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In Vitro Propagation of *Arbutus andrachne* L. and the Detection of Catechin

By

Zahra Yasser Al-jabary

In Partial Fulfillment of the Requirements for the Degree

Master of Science

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The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

***In Vitro* Propagation of *Arbutus andrachne* L and the Detection of Catechin**

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Zahra Yasser Al-jabary

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in biotechnology

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ABSTRACT

In Vitro Propagation of _Arbutus andrachne_ L. and the Detection of Catechin

By

Zahra Yasser Al-Jabary

Arbutus andrachne L., Arabic name "Qyqab", commonly known as Grecian Strawberry tree, is an evergreen small tree naturally distributed from East Mediterranean to the northern Black Sea area. In Palestine, very limited recovery of new plants has been observed possibly because of difficult seed germination under natural conditions and slow plant growth. In the past few decades, populations of Grecian strawberry tree in Palestine are facing extinction threat due to habitat fragmentation, over-exploitation of land, and damages resulted from extensive agricultural and human activities. Plant tissue culture offers a good alternative to propagate the endangered plant species. In the present work, the major objectives were to establish an effective micropropagation protocol for the purpose of conservation and to evaluate the presence of catechin, a flavonoid secondary metabolite, from different tissue sources. Micropropagation of *A. andrachne* was initiated from seeds. Seeds were pretreated by cold stratification at 4°C for 24 hours and soaking in 5.0 mg/l GA_3 followed by culture on WP media gave the highest germination percentage about 84% when compared with MS, B5 or water agar media 0.0%. *In vitro* shoot proliferation was tested with different 2i-P and zeatin levels (0.0 – 8.0 mg/l). Culture of microshoots on WP media supplemented with 6.0 mg/l zeatin gave the highest proliferation results (27.1 shoots/explant). For rooting, WP media supplemented with 15 g/l sucrose and 1.5 mg/l IBA was found to be the best medium in terms of rooting response 100% and number of roots (21.1). Rooted plantlets gave the highest survival percentage 83%. Compact callus was best initiated from seeds 70% on B5 media supplemented with 2,4-D at 1.0 mg/l, 30 g/l sucrose, and 1.0 g/l PVP. Callus was also induced from roots and cotyledons. Callus maintenance was achieved on WP media supplemented with 2.0 mg/l TDZ and 0.5 mg/l NAA. TLC analysis detected the presence of catechin in EtOAc extracts in *in vitro* and *ex vitro* vegetative parts. Qualitative and quantitative HPLC analysis revealed catechin in *ex vitro, in vitro* leaves, and callus tissue.

ملخص بالعربية

التكثير الدقيق داخل الأنابيب والكشف عن الكاتشين من***Arbutus andrachne* L**

زهرة ياسر الجعبري

شجرة القيقب المعروفة باسم شجرة الفراولة الإغريقية، هي شجرة صغيرة دائمة الخضرة، تنتشر على نطاق واسع من منطقة شرق البحر الأبيض المتوسط إلى منطقة شمال البحر الأسود. استخدمت قديما في الطب الشعبي لعلاج العديد من الأمراض، وذلك بسب احتوائها على العديد من المركبات الهامة. في العقود القليلة الماضية اعتبر القيقب واحدا من النباتات البرية المهددة بالانقراض في فلسطين بسبب الظروف القاسية التي يتعرض لها بسبب الرعي الجائر وبعض الممارسات الزراعية والبشرية الخاطئة بالإضافة إلى صعوبة الإنبات تحت الظروف البيئية الطبيعية وبطء نمو النبات. تم اللجوء إلى تقنيات تكثير النبات داخل المختبر بواسطة التكثير الدقيق حيث تعتبر بديل جيد لتكاثر النباتات المهددة بالانقراض. بالإضافية إلى إمكانية إنتاج المركبات الثانوية والمركبات الكيميائية الجديدة.
تهدف الدراسة الحالية إلى وضع بروتوكول لزراعة وتكثير نبات القيقب في المختبر ومن ثم نقله إلى البيئة الخارجية، بالإضافة إلى تأسيس بروتوكول لإنتاج الكالوس وتنميته من أجزاء مختلفة من النبتة، أيضا إجراء تحاليل نوعية وكمية لاستخلاص مركب الكاتشين و الكشف عنه وتحديد كميته في أجزاء مختلفة من النبات. تم بنجاح تأسيس نبات القيقب داخل الأنابيب من البذور و قد كانت نسبة الإنبات 84% بعد معالجتها بالتبريد لمدة 24 ساعة على درجة 4 سيليسيوس وغمسها في محلول الجبرلين بتركيز 5.0 ملغم\لتر، ثم زراعتها على بيئة WP. كما و أعطى نبات القيقب اعلى نسبة تفرع (27.1 تفرع /نبتة) عند استخدم بيئة WP مدعمه بمنظم النمو zeatin عند تركيز 6.0ملغم\لتر. حققت نسبة تجذير100% باستخدام بيئة WP بالإضافة إلى سكروز بتركيز 15 غ\لتر وهرمون IBA بتركيز 1.5 غ\لتر،تم بنجاح أقلمة النباتات المجذرة. أما الكالوس فقد تم استحثاثه بنجاح من البذور، الجذور، والبادرة على بيئة B 5 مع 1.0 ملغم\لتر من منظم النمو2,4- D وقد سجل الكالوس من البادرة أعلى وزن. على بيئة WP بإضافة TDZ على مستوى 2.0 ملغم\لتر + NAA على مستوى 0.5 ملغم\لتر تم الحصول على كمية كبيرة من النوع القاسي باللون الأحمر والأخضر. تم بواسطة TLC الكشف عن الكاتشين من مستخلص الإيثيل إستيت للأوراق التي نمت في الأنابيب والتي جمعت من الطبيعة، و لكن بواسطة HPLC تم الكشف عن الكاتشين من الأوراق التي جمعت من الطبيعة والتي نمت بالأنابيب بالإضافة إلى الكالوس وقد كانت كمية الكاتشين أعلى ما يكون في الأوراق التي جمعت من الأنابيب.

DECLARATION

I declare that the Master Thesis entitled "***In Vitro* Propagation of *Arbutus andrachne* L. and the Detection of Catechin "** is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Zahra Yasser Al-jabary

Date: 8th August 2011

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Date: 8th August 2011

DEDICATION

I would like to dedicate this thesis to my children. Without their patience, understanding, support, and most of love, the completion of this work would not have been possible. Thank you Zahwa and Yahya very much.

I would also like to dedicate this thesis to my parents, sisters, and brothers. Thank you all for your support and encouragement all the time.

This thesis especially dedicated to the memory of my husband who has been a major influence in my life, who has been the first person encouraged me to become at this scientific level.

Finally, I dedicate this work to all those I love them.

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ABBREVIATIONS

Word or sentence	Abbreviation
-And others	*et al*.,
-Analysis of variance	ANOVA
-6-Benzylaminopurine	BAP/BA
-Degree centigrade celcius	℃
-Completely Randomized Design	CRD
-2,4-Dichlorophenoxyacetic acid	2,4-D
- ethyle-acetate	EtOAc
- Gamborg *et al*., salt mixture	B5
- Gibberellic acid	GA_3
-High Performance Liquid Chromatography	HPLC
-Indole-3-butyric acid	IBA
-Indole-3-acetic acid	IAA
-6-Furfurylaminopurine	kinetin
-Least significant difference	LSD
-Liter	l
-Lloyd & McCown, salt mixture	WP
-Milligram	mg
-Milliliter	ml
-Micro mole per square meter per second	µmoles/m^2/sec
-Molar	M
- Murashige and Skoog salt mixture	MS
- Parts Per Million (1mg/l)	ppm
-Percent	%
-Photosynthetic photon flux density	PPFD
-Plant growth regulator	PGR
-PVP	polyvinylpyrrolidone
- Sterilized Distilled Water	SDW
-Thidiazuron	TDZ
-Thin layer chromatography	TLC
- Ultra Violet	UV
- 6-(4-Hydroxy-3-methylbut-2-enylamino) purine	zeatin

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS

CHAPTER

INTRODUCTION

1

Nature stands as a golden landmark characterized by natural products from plants and animals. Thousands of years ago, herbs and their products were used in traditional medicine for treating diseases. Until now, about 80% of people in developing countries still relay on herbal medicine based largely on natural products from medicinal plants for primary health care (Sheetal and Singh, 2008). Floristic analysis showed that there are about 500,000 plant species on our planet, out of these about 120,000 plant species are used to produce biologically active products, some of which can be used in disease treatment (Tivy, 1993).

The interest in studying the biological effects of traditional medicinal plants or isolating their active components for treatment of illness has been increasing all over the world and comprehensive screening programs have been established. New systematic methods for separation, identification, and determination of chemical constituents are applied in addition to different biological assays carried out on plant extracts and their chemical constituents. Most of the recent phytochemical studies are following similar

methodologies in plant collection, plant extraction, bioassay, chemical analysis, and statistical analysis (Buchbauer, 2000). However, a sustained supply of the source material often becomes difficult due to some factors like environmental changes, cultural practices, diverse geographical distribution, labor cost, selection of superior plant stock, and over exploitation by pharmaceutical industry (Joy *et al*., 1998).

1.1. Importance and uses of medicinal plants

Many people believe that medicinal plants are more natural and more accessible than manufactured drugs as they were used in treating a wide spectrum of diseases (Jennifer, 2000). According to Huang *et al*. (2008), medicinal plants have been screened for their potential uses as alternative remedies. Plants are used in traditional medicine for their antibacterial, antifungal, and anticancer activities (Schinella *et al*., 2002; VanderJagt *et al.,* 2002). Recently some products of higher plant origin have been shown to be effective sources of chemotherapeutic agents without undesirable side effects and strong biological activity (Huang *et al*., 2008). Medicinal plants are considered as a source of biologically active biochemicals like secondary metabolites, used for various applications in food, medicines, and industry (Huang *et al*., 2008).

1.2. Medicinal plants in Palestine

Palestine is described as a rich diversity land of flora; this richness is due to the diversity of the soil and climatic conditions (Shtayeh *et al*., 1998). Within a small Mediterranean area, about 2600 plant species are covering Palestine and Golan Heights including 700 for their uses as medicinal herbs (Said *et al*., 2002).

Many of plant populations with edible and medicinal significant in Palestine are becoming endangered and subjected to genetic erosion (e.g. *Iris* spp, *Crocus* spp, *Origanum syriacum*, *Arbutus andrachne*, *Pistacia atlantica* and *P. palaestina*; (ROTEM, 2002). *Arbutus andrachne* L. is one of these plants that is used in the Arab Tradition Medicine (Said *et al*., 2002), it will be investigated in this study.

1.3. *Arbutus andrachne* L.

1.3.1. Botanical description

Arbutus andrachne L. or "Grecian Strawberry tree", known in Arabic Qyqab, is a member of the Ericaceae family. It is an evergreen small tree, wide spread from the East Mediterranean to Northern Black Sea area (Davis, 1978). In Palestine, the plant grows on rocky hills with high clay content and low aeration, it flowers from March to April, and the seeds ripen from September to October. The flowers are hermaphrodite and pollinated by Bees. The plant is self-fertile (Danin, 2005). The reddish stems and evergreen foliage make the tree very attractive with high value in landscape (Fig.1.1). Additionally, fruits become sweet with good taste when ripe and can be eaten fresh, dried or as jam (Hedrick, 1972; Facciola, 1990).

Figure 1.1. *Arbutus andrachne* L. tree (a) flower (b) bark (c) leaves (d) fruits

1.3.2. Current status of *A. andarchne* in Palestine

In the past few decades, populations of *A. andrachne* as many other wild plants in Palestine are facing severe habitat fragmentation, over-exploitation, extensive agricultural and human activities, overgrazing, and premature harvest by local people, all these factors lead to the rarity of many wild plants and accelerated their extinction in many areas (ROTEM, 2002). Very limited recovery of *A. andrachne* tree in natural habitats has been observed possibly due to difficult seed germination in natural conditions and slow plant growth (Karam and Al-Salem, 2001). Because of difficult germination conditions of *A. andrachne,* there are strong challenges to maintain this species by applying alternative techniques used in multiplying endangered plant species.

1.3.3. Uses of *A. andrachne* in folk medicine

According to Said *et al.* (2002) and Sakar *et al.* (1991), *A. andrachne* is used traditionally as astringent and urinary antiseptic and for the treatment of urinary system, blood tonic, and cancer, aching joints and treating wounds. These uses caused increase demand for this species as well as other species.

1.3.4. Chemical constituents of *A. andrachne*

Tawaha *et al.* (2007) listed *A. andrachne* as the highest among 51 other medicinal plant species from Jordan that have antioxidant content. According to the available literature, the plant has triterpenoids and steroids in the fruits (Grishkovets *et al*., 1979).

Triterpenoides, sterols, and lipids are isolated from bark, leaves, and fruits, Arbutin, monotropeins, unedoside, and catechin were also isolated from bark and leaves (Saker *et*

al., 1991). The present study focused on detection of catechin from different explants of *A. andrachne*.

1.4. *In vitro* Micropropagation

Traditional ordinary methods of cloning plants most widely use cuts from vegetative parts, grafting, and budding. However, these methods are sometimes difficult, expensive, and unsuccessful. *In vitro* plant culture offers a good alternative means of plant propagation. Clonal propagation through tissue culture popularly called micropropagation, which is achievable in a shorter time. Use of plant tissue culture for micropropagation was initiated by Morrel (1960), who founded commercially viable approach for orchid propagation.

Micropropagation offers a good alternative to multiply novel and endangered plant species. Moreover, it is also used to provide sufficient number of plantlets for planting from few stock plants, which does not produce seeds or does not respond well to vegetative propagation (Srivastava *et al.,* 2005). In *vitro* propagation in many cases is more rapid than traditional methods and can offer virus and disease- free plants. Additional advantages of culturing medicinal plants via *in vitro* micropropagation is the possibility of producing secondary metabolites irrespective of seasonal and climatic conditions and for more efficient recovery due to the rapid growth of cell culture (Pierik, 1997). However, the greatest limitation of micropropagation is the cost and mechanization of the process would eliminate most of the labor cost associated.

There are four basic stages for successful micropropagation of plantlets. The first stage, the preparative stage, or stated as phase zero. It involves the correct pretreatment of the

starting plant material to ensure they are disease free as far as possible. The second phase is the establishment of clean starting tissue for aseptic culture and growth. It involves sterilizing protocol to produce aseptic tissues. These aseptic tissues will be used for the next stage of shoot multiplication, which can be carried out in a number of ways. Generally, plant growth regulators are used for shoot multiplication. The shoots obtained in phase two will be used for root induction at the third phase either *in vitro* or *in vivo*. Finally, at phase four, the *in vitro* planlets are acclimatized for better survival when transferred to greenhouse conditions or to the soil (Pierik, 1997).

1.4.1. Establishment of Aseptic Explants

The plant tissues or explants collected from *ex vitro* growing plant are usually contaminated with microorganisms. These microorganisms include bacteria, fungi, and viruses that must be removed during the preparation of aseptic explants otherwise; they would kill the explants either due to their overgrowth or due to the release of toxic substances into the medium. The potential sources of contamination in the cultures are the plant tissue, instruments, culture medium, and environment of the transfer area, technicians, and incubation room (Dodds and Roberts, 1995). To overcome contamination problem, commonly sterilizing agents must be used for obtaining aseptic tissues such as sodium hypochlorite and calcium hypochlorite. Sodium hypochlorite is available as commercial bleach, Clorox® with 5% (v⁄v) active ingredient. It is used at 20 % (v/v) concentration to sterilize plant material (Al-Wasel, 2000).

1.4.2. *In vitro* shoot proliferation

Growth medium composition is very important for induction and growth of shoots. The amount of Plant Growth Regulators PGRs in the culture medium is critical in controlling

the growth and morphogenesis of the plant tissues (Skoog and Miller, 1967). Generally low auxin and high cytokinin concentration in the medium results in the induction of shoot morphogenesis (Pierik, 1997).

Mostafa *et al*. (2010) and Bertsouklis and Papafotiou (2009) successfully propagated and proliferated shoots of *A. andrachne*. Another woody species is *A. unedo* was also successfully micropropagated by El-Mahrouk *et al*. (2010) and Mereti *et al*. (2002).

1.4.3. *In vitro* rooting

Auxin alone or with the presence of a very low concentration of cytokinin is an important role in root induction (Hopkins, 1995). In medicinal plants rooting of microshoots has been obtained in growth medium containing IAA, IBA, NAA individually or in combinations or sometimes in hormone free medium (Conger, 1982; Hopkins, 1995).

1.4.4. Acclimatization

Plantlets grown in small closed vessels at high air humidity and have low growth potential due to limited photosynthetic performance (Jeon *et al*., 2005). After multiplication, *in vitro* plantlets are transferred to the greenhouse in very different environmental conditions. A high percentage of micropropagated plants often do not survive during this acclimatization period. Often plantlets must produce new leaves to adjust to new conditions (Preece and Sutter, 1991). Failure to rapidly optimize their anatomy and physiology is likely to be responsible for poor performance during acclimatization. High survival rates of *ex vitro* acclimatized plants have been reported for different plant species like *Pyrus syriaca* Boiss. reached 95% survival (Shibli *et al*., 1997), and reported for *A. andrachne* 80% survival rate by Mostafa *et al*. (2010).

1.5. Callus Culture

Theoretically, all living plant cells are capable of giving rise to a full plant. This phenomenon is called “cellular totipotency”. In cultures, isolated plant cells/tissues may be induced to form an actively growing mass of cells called callus, which can be multiplied for an indefinite period by routine subculturing. It is an actively dividing and more or less undifferentiated tissue. Callus can be obtained from isolating tissues, organs, and embryos *in vitro*. First, undergo tissue should dedifferentiate before all division starts (Collin and Edwards, 1998). The greatest callus formation takes place under the influence of exogenously supplied growth regulators present in the nutrient medium. The type of growth regulator requirement and its concentration in the medium depend strongly on the genotype and endogenous hormone content of explants (Chawla, 2008). Generally, a high concentration of auxin and a low concentration of cytokinin supplemented into the medium could promote cell proliferation with the formation of callus (Pierik, 1997).

In many instances, addition of any one of auxins to a basal medium may be enough to initiate and maintain callus growth. The age and physiological state of the mother plant can affect the formation of callus, and the explants material should be healthy with vigorous growth.

Allan (1991) reported that tissues from plants that were about to enter dormancy were best for callus induction. The importance of plant age was obviously observed from tree species, where callus usually could only be initiated from juvenile tissue, and not explants from mature trees. However, Sahoo *et al*. (1997) reported that callus could successfully be induced from the internodes segments of five years old *Morus indica* L.

In general, large pieces of tissues were favored because of the large numbers of cells present increase the chance of obtaining a viable culture. Therefore, a high surface area/volume ratio was desirable for a maximum growth (Yeoman, 1973).

Callus could be established from many explant types. Establishment of callus growth had been obtained from many *in vitro* plant species. According to Yeoman (1973), most viable plant cells could be induced to undergo mitosis from shoot tips or isolated meristems, which contained mitotically active cells for callus initiation.

Allan (1991) reported that PGRs are important and the balance between auxin and cytokinin concentration was crucial in establishing callus cultures and maintaining them. Mostly callus formed from the same explants can normally be grown on the same medium.

1.6. Methods of isolation and quantitative determination of secondary metabolites

1.6.1. Extraction

Extraction is the second step in the criteria of studying medicinal plants after plant collection. Different methods of extraction can be used to extract secondary metabolite compounds from either wild plant or plant tissue culture. Many factors may affect the extract such as, pH for the extracting medium, stability of the constituents, and the biological activity of the chemical constituents of the plants. Type of solvents used in those methods may be organic or aqueous depending on study needs (Shatyeh and Jamous, 2008).

Extracting secondary metabolites using plant tissue culture has some advantages over extracting using *ex vitro* plant including: (1) specific metabolites can be produced in

cultures all through the year even in places where these crops are not grown. (2) Producing secondary metabolites without exhausted considering the future needs. (3) If not in all, at least in remarkable number of cases cells under culture tend to produce greater amounts of these metabolites than that is accumulated in nature (Narula *et al*., 2004).

Yoshimatsu (2008) reported that alkaloid contents of *Cephaelis ipecacuanha* was relatively high in plants propagated through tissue culture compared to the plants grown from seedling.

1.6.2. Chromatographic types and techniques

Chromatography was discovered and named in 1906 by Michael Tswett, a Russian botanist while he was attempting to separate colored leaf pigments (Skoog *et al*., 1998).

Chromatography is an analytical method consists of collective techniques that are widely used for the separation, isolation, identification, and quantification of components in a mixture. Chromatography is defined as a process for separating compounds in a mixture that contains a large number of organic compounds; separations are based on differences in migration rates among the sample components. Components of the mixture are carried through the stationary phase by the flow of a mobile phase (Fried and Sherma, 1994).

1.6.2.1. Thin layer chromatography

Thin layer chromatography TLC is a qualitative analysis of extracts that serves as one of the many methods in providing a chromatographic plant extract fingerprint (Wagner and Bladt, 1996). TLC was chosen over other chromatography methods because it is a simple,

quick, and inexpensive procedure that can be used for the analysis of plant extracts (Robards, and Antolovich, 1997).

TLC is a tool of liquid chromatography in which the sample is applied as a small spot of concentrated aqueous plant extract to the origin of a thin sorbent layer such as silica gel, alumina, or cellulose powder, supported on a glass, or metal plate. This layer consists of finely divided particles and constitutes the stationary phase. The mobile phase is a solvent or a mixture of organic and/or aqueous solvents in which the spotted plate is placed. The mobile phase moves through the stationary phase by capillary action (Fried and Sherma, 1994). Plates can be visualized, depending on the chemical structure of the compounds at visible light, UV-254 nm and 365 nm or by using spray reagents, phenols can detect at UV-254 (Wagner and Bladt, 2009). The term retention factor R_f, is commonly used to describe the chromatographic behavior of sample solutes. Usually, the center of each spot is the point taken for measurement (Robards and Antolovich, 1997).

1.6.2.2. High Performance Liquid Chromatography

High performance liquid chromatography HPLC is a method, which separates compounds that are dissolved in the sample. The separation of these components depends on different migration rate. It migrates in a system of two phases; stationary phase and mobile phase. The mobile phase is going through the stationary phase and the sample is drifted by the mobile phase. The differences in interaction with the column can help to separate different sample components from each other (Klouda, 2003). The advantages of this method are (1) no need of derivatization, (2) nondestructive operation, (3) greater separation, (4) and detection selectivity. The nondestructive nature of HPLC allows it to

be used as a preparative purification method as well as a quantitative technique (Nollet, 2000).

HPLC has been the most widely employed chromatographic technique in flavonoid analysis during the past 20 years (Merken and Beecher, 2000). Particular advantages are the improved resolution of flavonoid mixtures compared to other chromatographic techniques, the ability to obtain both qualitative and accurate quantitative data in one operation and the great speed of analysis (Markham and Bloor, 1998).

1.6.3. Catechin

1.6.3.1. Catechin description

Catechin is the basic structural unit subclass of flavonoids and subgroup of flavan-3-ols, which represent the largest class of naturally occurring C6-C3-C6 monomeric flavonoids, resulted of secondary plant metabolism (Li *et al*., 2002). Flavonoids are naturally occurring polyphenolic compounds containing two benzene rings linked together with a pyrone ring or with dihydropyrone ring flavonoids are usually glycosylated (Giuseppe *et al*., 2007). Catechin possesses two benzene rings called the A and B rings and a dihydropyran heterocycle the C ring with a hydroxyl group on carbon 3. There are two chiral centers on the molecule on carbons 2 and 3 (Rinaldo *et al*., 2010) (Fig.1.2). Catechin has four diastereoisomers, two isomers are in trans configuration are called catechin and the other two are in cis configuration are called epicatechin (Van Rensburg *et al*., 1997). The most common catechin isomer is the (+)- catechin and the common one of epicatechin is (-)- epicatechin, the different epimers can be distinguished using chiral column chromatography (Rinaldo *et al*., 2010). Catechin oxygenase, a key enzyme in the degradation of catechin, is present in fungi and bacteria (M Arunachalam *et al*., 2003).

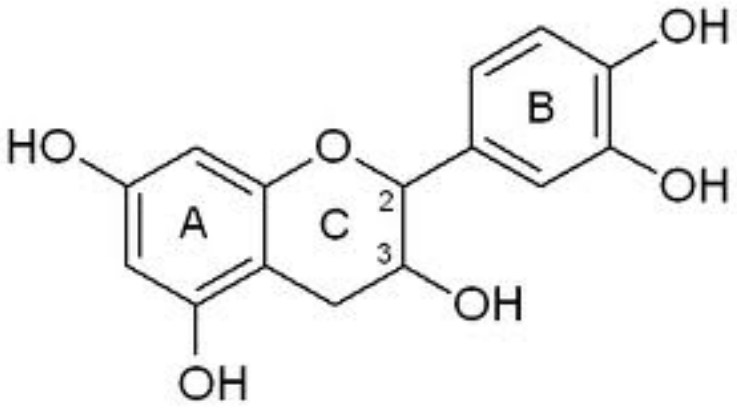


Figure 1.2. Molecular structure of catechin

In the strawberry fruits, catechin was identified as the main flavan-3-ols that can synthesis by malonate pathway according to Wulf *et al*. (2008). Figure 1.3 illustrates malonate pathway in strawberry fruits leading to catechin biosynthesis (Puhl and Treutter, 2008).

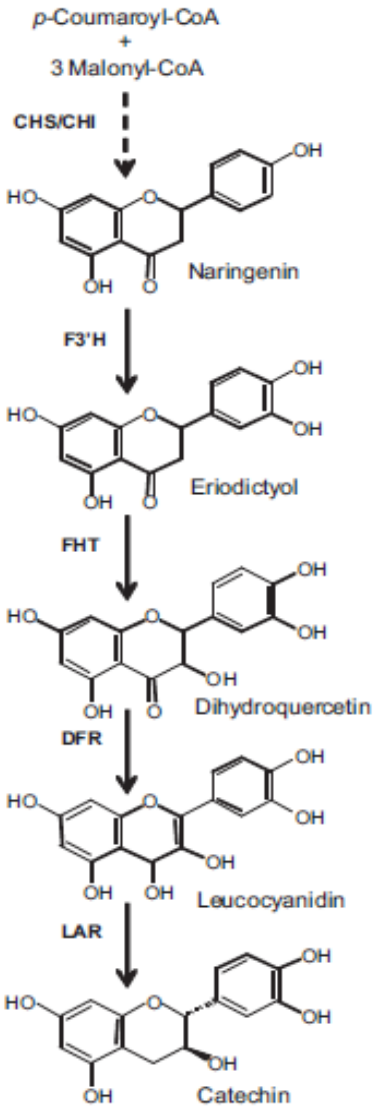


Figure 1.3 Simplified scheme of the flavonoid pathway in strawberry fruits showing the biosynthesis of Catechin. Enzymes involved CHS, chalcone synthase; CHI, chalcone/flavanone isomerase; F3'H, flavonoid 3'-hydroxylase; FHT, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; LAR, leucoanthocyanidin reductase

1.6.3.2. Catechin importance

Catechin has high pharmacological activities as free radical scavenging agents, and the most important property of this compound is the antioxidant activity (Nakao, 1998). (+)-catechin is the most powerful scavenger able to quench singlet oxygen due to, the presence of the catechol moiety on ring B and the presence of a hydroxyl group activating the double bond on ring C (Tournaire *et al*., 1993). Various pharmacological functions of catechin have been proved, as antibacterial, antiviral, anti-tumour, antioxidant and radical scavengers (Jha *et al*., 1998; Chattopadhyay *et al*., 2003). According to Hou *et al*. (2005) (+)-Catechin and (-)-epicatechin are also selective monoamine oxidase inhibitors of type B. They can be used as part of the treatment of Parkinson's and Alzheimer's patients Michael *et al*. (2001) proved that intestinal tumor formation can inhibits by eating fruits and vegetables contain a number of constituents associated with colorectal cancer prevention such as antioxidant compound as catechin. Catechin found in a wide variety of plant sources such as vegetables, herbs, and teas (Wollenweber and Dietz, 1981). It was isolated from bark and leaves of *A. andrachne* (Saker *et al*., 1991), and (K.Pallauf *et al*., 2008) was recorded the presence of catechin in the fruits of *Arbutus unedo*.

Few reports were studied catechin from *in vitro* plant, some of them by Farzami and Ghorbanli (2005), they were extracted (+)-catechin from the callus of *Rheum ribes* L. and (+)-catechin was extracted from the callus of *Taxus cuspidata* and *T. baccata* by Bulgakov *et al*. (2011).

CHAPTER 2

PROBLEM STATEMENT AND OBJECTIVES

2.1. Problem statement

As many other wild plants in Palestine, *A. andrachne* is considered an endangered species. Very limited recovery of plants has been observed possibly due to difficult germination under natural conditions and slow plant growth (Sakar *et al*., 1999). *A. andrachne* is a medicinal plant species that has a high antioxidant contents, possibly because of the presence of catechin compound.

Due to the above-mentioned reason, there is a tremendous need for alternative method to conserve this valuable endangered plant, which is *in vitro* propagation technique of *A. andrachne. In vitro* propagation of *A. andrachne* can enable conservation of endangered plants and enhance the production of valuable secondary metabolites such as catechin in a high quantity via *in vitro* plant.

2.2. Objectives

The main objectives of the present study are.

- To standardize micropropagation protocol of *A. andrachne* by:

  1- Establishing a protocol to break *A. andrachne* seed dormancy

  2- Optimizing a suitable medium and optimal concentration of growth regulators for aseptic culture initiation, shooting, and rooting

  3- Acclimatizing of *in vitro* grown *A. andrachne* plantlets

- To optimize growth medium for callus induction and maintenance from different tissues

- To detect catechin in, *ex vitro* leaves, *in vitro* leaves, and callus

  1- Qualitative identification of catechin using TLC

  2- Qualitative characterization of catechin using HPLC

CHAPTER 3

MATERIALS AND METHODS

This part of the experimental work was conducted in the Plant Tissue Culture Laboratory at the Biotechnology Training and Research Center, Palestine Polytechnic University, Hebron- Palestine.

3.1. Chemicals and reagents for *in vitro* culture

All basal salts for plant growth, PGRs, and other reagents were purchased from Duchefa Biochemie and Sigma-Aldrich chemical companies.

Murahige and Skoog (MS) medium (Murahige and Skoog, 1962) including all vitamins (Duchefa Prod. No: M0222.0050), (Gamborg *et al.*, 1968) B5 (Duchefa Prod.No: F0801.1000), and (Lloyd and McCown., 1980) WP Woody Plant (Sigma Cat. No: 047k2329) were used in this study. The chemical composition of these media is listed in Table 3.1. Plant growth regulators and other additives with providing company and Cat# are listed in the appendix Table 25.

Table 3.1 Basic composition of basal growth media used in this study; Lloyd & McCown (1980), Murashige and Skoog (1962), and Gamborg *et al*. (1968).

All components expressed in mg/L	Lloyd & McCown WoodyMedium	Murashige and Skoog MS	Gamborg B5
Ammonium Nitrate (NH_4NO_3)	400	1.650	-
Boric Acid(H_2BO_3)	6.2	6.2	3.0
Calcium Chloride ($CaCl_2.2H_2O$)	96	440	-
Calcium Nitrate ($Ca(NO_3)_2.4H_2O$)	556	-	-
Cobalt Chloride ($CoCl_2 \cdot 6H_2O$)	-	0.025	0.05
Cupric Sulfate ($CuSO_4 \cdot 5H_2O$)	0.025	0.025	0.025
Magnesium Sulfate ($MgSO_4$)	370	370	250
Manganese Sulfate ($MnSO_4 \cdot H_2O$)	22.3	16.9	3.0
Potassium Nitrate (KNO_3)	-	1.900	2.500
Potassium Phosphate (KH_2PO_4)	170	170	-
Sodium Molybdate ($Na_2MoO_4 \cdot 2H_2O$)	0.025	0.25	0.25
Zinc Sulfate ($ZnSO_4 \cdot 7H_2O$)	8.6	8.6	2.0
Ferrous Sulfate ($FeSO_4 \cdot 7H_2O$)	27.8	27.8	27.8
Na_2-EDTA	37.3	37.3	37.3
Inositol	100	100	100
Nicotinic Acid	0.3	0.3	1.0
Pyridoxine-HCl	0.3	0.3	1.0
Thiamine-HCl	1.0	0.4	10
2i-P	1.0	-	-
IAA	1.3	1.3	1.0
Kinetin	-	0.04-10	-
Sucrose	30.000	20.000	30.000
Agar	6.000	8.000	-

3.2. Media Preparation

According to each experiment's purpose, MS, B5 or WP salts was used as a basal media for *in vitro* plant growth. The assigned weight for each media was dissolved together with sucrose at 30g/l in deionized water. Growth regulators (in mg bases) were added according to the required amount. Finally, pH was adjusted to 5.8 with 1.0M NaOH or 1.0M HCl, and media was solidified with agar at 8.0 g/l. The media was poured into clean 250 ml Erlenmeyer flasks (50.0 ml each) or 15 × 150 mm test tubes (8.0 ml each). Flasks were closed with aluminum foil closures and test tubes were closed with autoclavable snap caps. Media was sterilized by autoclaving at 121°C and 121 pa for 20 min, then transferred to the media storage room where they kept till their further use.

3.3. Collection of plant material

Seeds of *A. andrachne* L. were collected from wild grown plants near Taffoh Town located 8 km to the west of Hebron in November 2007. The ripe fruits were soaked in tape water for 72 hours before they were separated by hand and washed from the fruit pulp.

3.4. Breaking seed dormancy and germination

According to Karam and Al-Salem (2001), *ex vitro* seed stratification and GA_3 pretreatment resulted in breaking seed dormancy and enhanced germination rate of *A. andrachne*. Here, four *in vitro* experiments have been conducted to adjust the effects of pretreatment with GA_3 and cold stratification on breaking seed dormancy and seed germination, respectively.

1) Control treatment where seeds were untreated and planted directly on the media,

2) Cold pretreatment at 4°C for 24 h,

3) Seeds soaked in 5.0 mg/l GA_3 solution for 24 h at room temperature

4) Seeds treated by stratification and immersed in GA_3.

Treated seeds were surface sterilized and cultured on four types of basal growth media; control (water agar), MS, B5, and WP. Media were prepared without using sucrose. Under the laminar air-flow, sterilized seeds of *A. andrachne* were inoculated on the surface of media filled in 15 × 150 mm test tubes (8 ml each). Treatments were arranged in a Completely Randomized Design CRD with fifty replicates (test tubes) per treatment and one seed /replicate. Seeds were kept in the growth room at light region of 16-h light/8 dark and photoperiod (photosynthetic photon flux density (PPFD) = 40-45 µmoles/m^2/sec), at (24 ± 1°C) for germination. Data was reported after six weeks for the percentage of germination in each treatment.

3.5. Surface Sterilization

Seeds were surface sterilized by washing thoroughly under running tap water for 2.0 min, then soaking with SDW and few drops of detergent for 30 min. Antiseptic solution of 20% (v/v) sodium hypochlorite (Clorox®) was prepared. Under the Laminar air-flow cabinet, seeds were immersed in the antiseptic solution for 5.0 min with continuous shaking. Seeds were then rinsed with SDW for three times (2 min. each). Finally, 70% ethanol solution was added to the seeds for 30 seconds then washed with SDW three times (2 min. each).

3.6. Initiation of Cultures

As described in section (3.4), sterilized seeds were cultured aseptically under the laminar flow hood on different basal growth media; MS, B5, or WP to overcome seed dormancy and obtain a sterile seedlings.

3.7. Micropropagation

3.7.1. *In vitro* shoot proliferation:

Cytokinins are generally used in plant tissue culture at a concentration range from 0.1-10.0 mg/l, when added in appropriate concentrations they may regulate cell division, stimulate auxiliary and adventitious shoot proliferation, regulate differentiation, and inhibit root formation (Pierik, 1997). After six weeks of inoculating seeds on the ideal germination media, primary microshoots appeared and 1.0 cm long of these microshoots were cultured in 250 ml Erlenmeyer flasks filled with 50 ml solid media. WP medium supplemented with zeatin and 2i-P at 0, 2.0, 4.0, 6.0, or 8.0 mg/l. Cultures were maintained in the growth room at light region of 16-h light/8 dark and photoperiod (photosynthetic photon flux density (PPFD) = 40-45 µmoles/m^2/sec), at (24 ± 1°C). Data were recorded after 6 weeks for the percentage of shoot proliferation, shoot height, number of proliferated leaves/ shoots, and plant performance was monitored. Treatments were arranged in a CRD with ten replicates (flask) per treatment and one microshoot /replicate.

3.7.2. *In vitro* rooting

Auxins are generally used in plant cell culture at a concentration range of (0.01-10.0 mg/l). When added in appropriate concentrations they may regulate cell elongation, tissue swelling, cell division, formation of adventitious roots, and inhibition of adventitious and

axillary shoot formation. *In vitro* rooting experiment was conducted using microshoots 1.0 cm long formed on seedlings in PGR-free WP medium. Each microshoot was cultured in test tube filled with 8.0 ml of WP medium supplemented with 15 g/l sucrose and 0, 0.5, 1.0, or 1.5 mg/l IAA or IBA. Cultures were maintained in the growth room. Data were reported after 6 weeks for the percentage of rooting, root number and length, shoot height, number of leaves, and also plant performance was monitored. Treatments were arranged in a CRD with ten replicates (test tube) per treatment and one microshoot /replicate.

3.7.3. Acclimatization to *ex vitro* conditions

Ex vitro acclimatization was performed as cited in Shibli (1997). *In vitro* young rooted plantlets were taken out of the test tubes, washed thoroughly with distilled water to remove any remainings of medium. Plantlets were transferred to plastic pots containing peatmoss and perlite at 1:1 (v/v), potted plantlets were covered with transparent polythene bags to ensure high humidity and watered when needed for 2 weeks under 16 h light (PPFD = 40-45 μmoles/m2/sec) /8h dark at (24 ± 1°C). Polythene bags were removed after 2 weeks in order to acclimatize plants to field conditions. Success of hardening protocol was determined by calculating survival percentage.

3.8. Callus culture

3.8.1. Callus Induction

Callus of treated seeds was initiated and induced on the surface of solid B5 growth medium supplemented with different concentrations of 2,4-D 0.0,1.0,or 2.0 mg/l, sucrose was added at 30 g/l and polyvinylpyrrolidone PVP at (1.0 g/l) (Abenavoli and Pennisi, 1998) were added. Inoculated seeds were placed in Petri dishes measuring 7.0 cm in

diameter, with the scutellum side up. The dishes were placed in a growth chamber for six weeks under conditions of total darkness and (24 ± 1℃) temperature. To induce callus from other explants like *in vitro* cotyledons, hypocotyls and roots, they were cultured on the media that showed best callus induction results from seeds. Treatments were arranged in a CRD with ten replicates (Petri dishes) per treatment and three explants/replicate. Data for callusing percentage and degree of callusing were recorded after 6 weeks of culture seeds.

3.8.2 Callus subculture

Based on the results obtained from callus induction carried out in the previous experiment, callus was subcultured on the induction media. Seeds, cotyledons, and roots in 5.0 mg were cultured to test growth performance and to estimate growth curve. Callus growth was monitored by fresh weight and dry weight bases. Fresh weight was reported weekly for four weeks to generate growth curve.

3.8.3. Callus maintenance and production

Induced callus from different explants were transferred to another culture medium for further growth. WP media supplemented with 2.0 mg/l TDZ and 0.5 mg/l NAA, and 1.0 g/l PVP was used for callus production. Medium (25.0 ml) was poured in 10.0 cm diameter plates. Five calli pieces (around 0.5 cm diameter) were placed on the surface of the media and kept in the growth room under 16h as described before.

3.9. Detection and determination of catechin content in *A. andrachne* by using TLC and HPLC methods

3.9.1. Collection of plant material

Three samples from *A. andrachne* explants were used; *Ex vitro* leaves, *in vitro* leaves, and callus. *Ex vitro* leaves were collected from Taffoh Town at the end of December 2010, *in vitro* leaves were grown for 4 months on WP medium supplemented with 6.0 mg/l zeatin from the flasks, and callus that grown and maintained for 4 months on WP medium supplemented with 2.0 mg/l TDZ + 0.5 mg/l NAA. Tissues were cleaned by SDW to remove remaining media and to be used for catechin extraction.

3.9.2. Chemicals and reagents for chromatographic analysis

All solvents, chemicals, and reagents were purchased from Sigma-Aldrich. Chloroform, acetone, acetic acid, H_3PO_4, and acetonitrile were analytical grade quality for TLC and HPLC analysis.

EtOAc, and methanol were used as extraction solvents, methanol (HPLC grade), Iron (III) chloride ($FeCl_3$) for TLC, and Standard solution (+)-catechin hydrate (Sigma, Cat# 22110-1G).

3.9.3. Extraction procedure

A number of experiments were carried out in order to find the optimal conditions for the extraction of catechin. Batch solvent extraction method was used based on mixing the sample and the solvent. EtOAc, and 5% methanol extraction solvents are the most commonly used solvents in catechin extraction, natural apple vinegar solvent was used to compare yield from chemical versus natural solvent. 1.0 g of cleaned tissue samples were weighted, and cut to small parts then soaked in 100.0 ml of three solvents, EtOAc,

5% methanol, and natural apple vinegar, stirred for 48 h at room temperature, supernatants were removed by centrifugation for 15 min at 5000 rpm . Solvents were air dried under the fume hood, the percentage yield of dry extracts was calculated in each treatment.

3.9.4. Sample preparation

Samples for catechin detection were prepared as follows, 100.0 mg of dried extracts from three tissue sources were dissolved in 1.0 ml of three types of solvents separately (EtOAc, 5% methanol, and natural apple vinegar), and were shaken for 24 h, centrifuged for 2 min at 5000 rpm, and the supernatant was taken for further analysis.

3.9.5. Reference solution

A solution of 10.0 mg of (+)-catechin hydrate in 1.0 ml of EtOAc (10.0 mg/ml) was prepared for TLC optimization and comparison. Another solution of 10.0 mg of (+)-catechin hydrate in 500 ml methanol (HPLC grade) was prepared for HPLC detection and comparison. TLC and HPLC chromatogram of standard catechin is shown in (Fig.3.1 a, b).

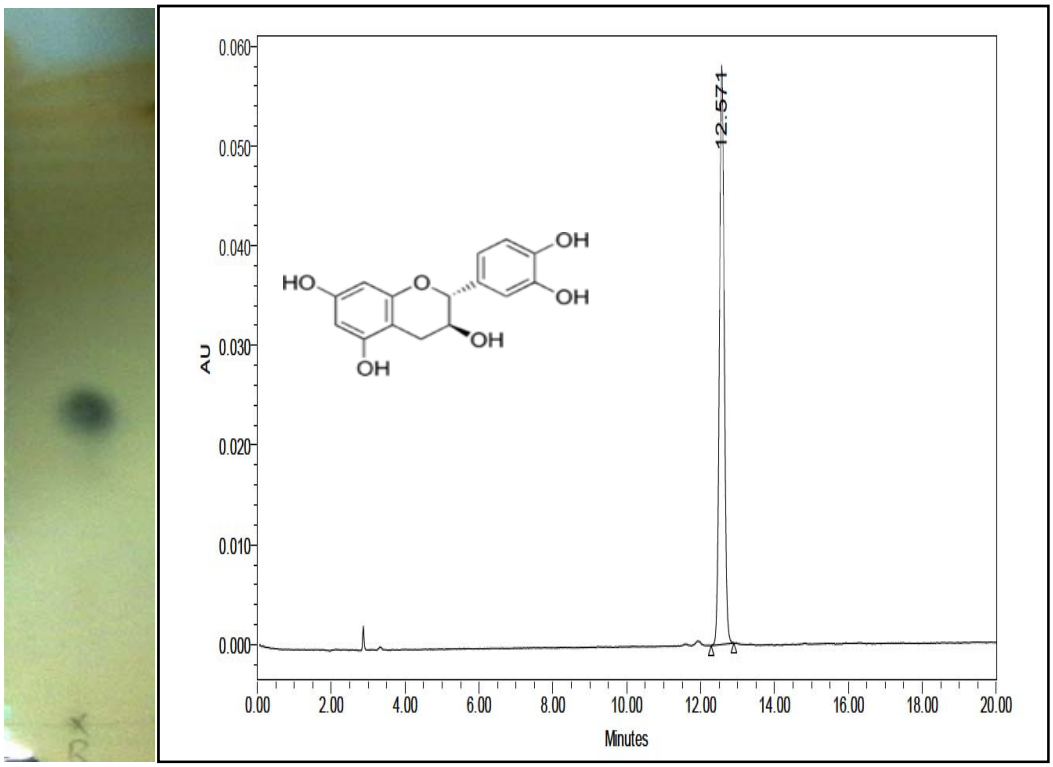


Figure 3.1. Typical a.TLC and b. HPLC chromatogram of standard (+)-catechin hydrate

3.9.6. TLC analysis of catechin

For qualitative detection of catechin, TLC analysis was performed using precoated TLC plates (Merck, Germany Silica gel 60 F_{254}, 0.25 mm) as stationary phase, 50 ml of chloroform:acetone:acetic acid was used as a mobile phase based on Wanger and Bladet (2009) but after different experiments chloroform:acetone:acetic acid was used at (65:21.5:13.5 v/v/v) . Plates were run three times in the mobile phase, after each run, the plates were air-dried. Aromatic substances were detected using UV light detector at 254 nm. Plates were sprayed with $FeCl_3$ solution (1.0 g of $FeCl_3$ dissolved in 100.0 ml water: methanol (50:50)) for visualization of phenolic substances, and heated until spots were

brown, grey or black. R_f value of catechin was measured by using the ratio referred to $R_f = \frac{distance\ spot\ moves}{distance\ solvent\ moves}$.

3.9.7. HPLC analysis of catechin

The HPLC analysis was conducted in the Center for Chemical and Biological Analysis at Alquds University. The quantitative analysis of catechin was performed on High Performance Liquid Chromatography HPLC (Alliance, Water 2692 separation module), using analytical HPLC column RP18 Water Symmetry Shield ^{TM}, (5.0µm, 4.6×250 nm). Samples for the analysis were prepared by dissolving 100.0 mg of dried EtOAc extract obtained from *in vitro*, *ex vitro* leaves, and callus in 0.7 ml HPLC-grade 8% methanol, total run time was adjusted at 21 min using the following gradient elution, 98% A, 2% B (0-19) min, 80%A, 20%B at 20 min, then back to 98% A, 2% B at 21 min, A: buffered water 1% H_3PO_4, B: acetonitrile. Flow rate was adjusted at 1.0 ml/min, UV detection was at 280 nm, and injection volume was 40 µl. Serial reference standard solutions of catechin (0, 1, 20, 40, 60, 80, and 100 ppm) were prepared using catechin dissolved in HPLC-grade 99.9% methanol to construct the calibration curve of catechin. Percentage yield of catechin from different explants was calculated at mg basis catechin per 100 mg of plant dry material against external catechin standard using the following equation: W/W% = (C× FV×D×100%)/W, where C is catechin concentration in the sample (mg/ml) extrapolated from the calibration curve linear regression, FV is the final volumn of the sample in milliliters, D is the dilution factor, and W is the sample weight in milligrams.

3.10. Statistical analysis

Each experiment was set up at Completely Randomized Design (CRD). Data in each experiment was analyzed with the analysis of variance (ANOVA) using SigmaPlot version 11.0 (Inc. SigmaPlot for Windows). Means were separated according to Fisher's least significant difference (LSD) test at the p=0.01 level of probability. Sample numbers for each measurement are provided in the captions of related illustrations.

CHAPTER 4

RESULT AND DISSCUSSION

The main objectives of this study are: (1) to conserve and propagate *Arbutus andrachne* tree through micropropagation technique. (2) to investigate the production of secondary metabolites in *Arbutus andrachne*, particularly the phenolic compound catechin by TLC, and HPLC analysis.

4.1. Breaking seed dormancy and germination:

Tissue growth and the quality of morphogenetic responses are strongly influenced by the type of growth medium and concentrations of PGRs present in the culture media (Niedz and Evens, 2007). Gibberelic acid plays an important role in seed germination and overcome seed dormancy (Takahashi *et al*., 1986). Many studies on *in vitro* tissue culture used GA_3 to overcome seeds dormancy in woody species (Kose, 1998; Tilki, 2004; Karam and Al-Salem, 2001; Harrington and Kraft, 2004).

The aim of this experiment was to investigate possible ways to overcome seed dormancy and germination. The experiment carried out according to the conditions described in section 3.4. Successful seed germination of *A. andrachne* was achieved in this study (Table 4.1). Our control treatment indicated that *A. andrachne* seeds could not germinate. Stratified and GA_3 treated seeds showed the highest germination percentage 84% compared to the control treatment 0.0%. The results confirmed that both stratification at

4ºC for 24 h and treatment with 5.0 mg/l GA_3 are directly involved together to breaking seed dormancy. Germination was not also observed in all treatments when seeds inoculated on MS and B5 media. Both WP and water- agar media showed germination at different percentages according to the treatment. Stratified seeds gave 4% in water- agar and 16% on WP media. Seeds pretreated with 5.0 mg/l GA_3 gave higher germination percentage 8% and 30% in water- agar and WP media respectively. The highest germination percentage 84% (Fig.4.1a) was obtained when both treatments (stratification and GA_3 pretreatment) were applied at WP media. Treatment with GA_3 + stratification was found to be successful for germination and breaking seed dormancy, the stratification process appears to enhance the production of some types of growth-promoting substances such as GA (Powell, 1987). Giba *et al*. (1993) reported that the inhibitory effect of retardants was overcome by gibberellic acid.

Table 4.1. Influence of different treatments and basal growth media on seed germination percentage.

Treatment	Basal growth media			
	Water-agar	**WP**	**B5**	**MS**
a) Control (untreated)	0.0^{b}	0.0^{d}	0.0	0.0
b) Stratified at 4º C for 24h	0.04^{b}	0.16^{c}	0.0	0.0
c) Soaked in 5mg/l GA3	0.08^{b}	0.30^{b}	0.0	0.0
d) Both(b+c)	0.44^{a}	0.84^{a}	0.0	0.0
P value	0.0	0.0	-	-
LSD	0.119	0.138	-	-

Fifty replicates (tubes) per treatment and one seed /replicate. Means were separated by Fisher's LSD at *p*=0.01.

Factors affecting seed dormancy and germination are well documented in literature. Karam and Al-Salem (2001) have tested various methods to break dormancy of *A. andrachne* seeds and found that nontreated seeds did not germinate. They also reported that either stratification of seeds at 4ºC for three to four months or treatment of seeds with 250 ppm GA_3 gave 86% of seed germinated. Compared to my study, the germination percentage was 84% that obtained by reducing the stratification time to 24 h and doubled the concentration of GA_3 to 500 ppm. Some researchers suggested that GA_3 may substitute for cold stratification and reported that GA_3 increased the germination percentage of *A. unedo* (Kose, 1998; Tilki, 2004).

WP media gave the highest germination result compared with B5 and MS media, the possible explanation of our result is that the major nutrient composition in WP medium corresponds to the mineral composition of the *A. andrachne* seeds, however, detailed mineral analysis for the seeds should be done before confirming this conclusion. It is well documented in literature that WP medium is being commonly used as a basic medium for propagation of woody plant species (Macarenhas *et al*., 1982).

4.2. Micropropagation

4.2.1. *In vitro* shoot proliferation

For obtaining desired responses in tissue culture, the role of growth regulators and their concentration will have to be carefully chosen. In shoot proliferation, high levels of cytokinins are used to overcome the apical dominance of shoots and to enhance the branching of lateral buds from leaf axils. Proliferation of shoot culture was carried out by culturing 1.0 cm of microshoots obtained from germinated seeds. Microoshoots were cultured on WP media with zeatin and 2i-P at 0.0, 2.0, 4.0, 6.0, or 8.0 mg/l.

The results of microshoots cultured on WP medium with zeatin are shown in Table 4.2. Culture of microshoots on 2i-P supplemented media resulted with no shoot proliferation (data not shown). Exhibited shoot proliferation varied depending on concentration of zeatin. Percentage of shooting on WP medium was 50% and 90% at (0.0, 2.0 mg/l) of zeatin respectively, but increased to 100% at (4.0, 6.0, and 8.0 mg/l) of zeatin. Zeatin increased the number of shoots (3.7-27.1) and leaves (13-31.2) compared with the control and resulted in the greatest shoot height (4.4 cm) at 6.0 mg/l (Table 4.2, Fig.4.1b). Zeatin at 8.0 mg/l decreased all growth parameters, likely due to its inhibitory effect at this high concentration.

Table 4.2. Influence of zeatin levels on the percentage of shooting, shoot height, number of shoots, and number of *in vitro* leaves microshoots of *A. andrachne*. Percentage of 100% were set to 1.0 for simplicity.

Concentration (mg/l)	Shooting (%)	No. of shoots	No. of leaves	Shoot height (cm)	Callusing
Zeatin					
0.0	0.5^{b}	3.7^{d}	13.0^{b}	3.0^{c}	-
2.0	0.9^{a}	6.8^{c}	20.0^{b}	3.85^{b}	-
4.0	1.0^{a}	15.7^{b}	20.4^{b}	3.38^{c}	-
6.0	1.0^{a}	27.1^{a}	31.2^{a}	4.4^{a}	-
8.0	1.0^{a}	3.8^{d}	18.2^{b}	3.08^{c}	-
P value	0.0005	0.0	0.0	0.008	
LSD	0.248	1.635	5.806	0.431	

Ten replicates (flask) per treatment and one microshoot /replicate. Different letters within columns showed significant difference at $P = 0.01$ as determined by Fisher's LSD.

Success of shoot proliferation on WP medium with zeatin indicates that zeatin increases the rate of shoot proliferation and results in production of a better quality shoot. Bertsouklis and Papafotiou (2009), were obtained only an average of 3.8–4.8 shoots/explants and 1.3–1.9 cm shoot height using zeatin-containing WP medium, which is much less than 27.1 shoots/explants and 4.4 cm shoot height that was obtained at 6.0 mg/l zeatin-containing WP medium in the present study.

According to Mostafa *et al*. (2010), they found that the proliferation was successful on MS medium supplemented with zeatin as a cytokinin type. Zeatin has increased shoot proliferation to 90–100% and gave average number of shoots 13.4–22.8 which is lower than 27.1 in this study. Zeatin also resulted in the highest shoot height 3.0 cm at 6.0 mg/l, while resulted in 4.4 cm when using zeatin with WP medium at 6.0 mg/l. Accordingly, it is apparent that zeatin was the most effective cytokinin for shoot proliferation of *A. andrachne* with both MS and WP media. Mereti *et al.* (2002) have studied the *in vitro* culture of *A. unedo*, a very close species to *A. andrachne* in Europe. They have reported that optimal shoot proliferation was achieved on WP media with MS vitamins, and 5.0 mg/l BA. In general, shoot proliferation was more successful on WP, which supports our finding by using WP media for woody plants in the *in vitro* micropropagation

4.2.2. *In vitro* rooting

To obtain viable plants, *in vitro* growing shoots must have roots before being transferred to *ex vitro* conditions. Thus, growth media was supplemented with different auxins to promote root initiation.

In the present study, both IAA and IBA have been used for rooting. By placing microshoots 1.0 cm long formed on plants in a PGR-free WP medium, they were cultured at WP medium supplemented with 15 g/l sucrose and 0.0, 0.5, 1.0, or 1.5 mg/l IAA, or IBA. Rooting rate and characteristics of the roots and explants were influenced by the type and concentration of auxin. Microshoots were successfully gave roots by adding IBA at 1.5 mg/l. There were high significant variations among and within rooting parameters of experiment (Table 4.3). No rooting was observed on PGR-free medium. Rooting rate was increased when the concentration of auxin was increased especially when IBA was used at 1.5 mg/l. IBA induced successfully rooting 100% at 1.5 mg/l whereas it was 50% at 1.5 mg/l IAA, the highest number of root/explants 21.1, and rooting length 5.2 cm were recorded for IBA at 1.5 mg/l (Fig.4.1c).

The result of this experiment showed that IBA and presence of sucrose were critical factors to initiate root formation. The results show that during root formation, sucrose is used as a source of energy and building blocks. Al-Khateeb (2001) finds that the root initiation and growth were high energy requiring processes that could only occur at the expense of available metabolic substrates, which were mainly sucrose.

Absence of rooting on PGR-free medium indicates the significance of auxin in enhancing rooting *in vitro*. Rooting inhibited with IAA in the current study, which is not in agreement with findings of Mereti *et al*. (2002), who reported that *A. unedo* microshoots rooted successfully on WP medium supplemented with 2.0 mg/l IAA or IBA. The maximum number of roots in the present work was observed in media with 1.5 mg/l IBA, this result was in accordance with the findings of Bertsouklis and Papafotiou (2009).

They were reported that rooting rate was 90% for *A. andrachne* on WP medium supplemented with 1.0 mg/l IBA.

Table 4.3. Influence of IBA levels on number of root, root length, shoot height, and number of leaves and shoots of *in vitro* microshoots *A. andrachne*.

Concentration (mg/l)	Rooting (%)	No. root/ explant	Root length (cm)	Shoot height (cm)	No. of leaves/explant
IBA					
0	0.2^{b}	0.3^{bc}	0.6^{bc}	2.1^{b}	1.6
0.5	0.9^{a}	8.3^{b}	2.2^{b}	2.24^{b}	2.1
1.0	0.8^{a}	5^{b}	1.42^{b}	2.35^{b}	3.7
1.5	1.0^{a}	21.1^{a}	5.2^{a}	3.4^{a}	2.3
P value	NS	0	0	0.0008	NS
LSD	0.306	5.511	1.384	0.645	-
IAA					
0	0.2	0.3b	0.6	2.1	1.6
0.5	0.4	4.1a	1.1	2.8	1.0
1.0	0.2	0.7b	0.25	2.25	1.4
1.5	0.5	1.4ab	0.5	2.3	1.7
P value	NS	0.034	NS	NS	NS
LSD	-	2.736	-	-	-

Ten replicates (test tube) per treatment and one microshoot /replicate. Different letters within columns showed significant differences at $P = 0.01$ as determined by Fisher's LSD.

4.2.3. Acclimatization to *ex vitro* conditions

One of the major obstacles in the application of tissue culture methods for plant propagation is the difficulty in successful transfer of plantlets from the laboratory to the field; the reasons for such a difficulty appear to be related to the dramatic change in the environmental conditions. Several workers have developed protocols to overcome some of these constraints. The acclimatization protocol used by Shibli *et al*. (1997) in micropropagation of wild pear successfully resulted in good number of healthy acclimatized *A. andrachne* plants. After sufficient rooting, *A. andrachne* plantlets were transferred to plastic pots containing peatmoss and perlite mixture. Agar was washed away from rooted plantlets. *Ex vitro* acclimatization successfully done (Fig.4.1d) from *in vitro* rooted plantlets of *A. andrachne* plant. Rooted plantlets showed different percentages according to the PGR used for rooting (Table 4.4). Plantlets rooted with IBA exhibited 83% survival, while with IAA 65%. This is in agreement with findings of Mereti *et al*. (2002) who reported that *A. unedo* rooted plantlets at IAA showed low survival rate during acclimatization. Survival rate obtained in the current study was relatively high 83% and similar to that reported for *A. andrachne* by Bertsouklis and Papafotiou (2009), and Mostafa *et al*. (2010).

Figure 4.1. (a) *Arbutus andrachne* plant germinated seeds on WP Woody Plant medium after stratification, and treatment with $GA_{3,}$ (b) shoot proliferation on WP at 6.0 mg/l zeatin (c) rooted plantlet on WP at 1.5 mg/l IBA (d) acclimatized rooted plant. A, b, c and d after six weeks of the beginning of each experiment

Table 4.4. Total survival and survival percentages, after 8 weeks of transferring rooted plantlet to *ex vitro* acclimatizing conditions according to the type of auxin used in rooting.

PGR	Survival Number	Survival Percentage
IBA	33/40	83%
IAA	26/40	65%

4.3. Callus culture:

4.3.1. Callus Induction and Maintenance:

Callus is an abnormal growth produces an unorganized mass of undifferentiated cells. Callus growth can be established from many *in vitro* or *ex vitro* explants. It is essential for callus induction to supply nutrient medium with plant hormones, as an auxin, cytokinin or gibberellins. Some plants require light and some produce callus in dark conditions. After getting a suitable size of callus, it was transferred to a fresh medium by sub culturing process.

The experiment was conducted to initiate and produce callus under dark condition on B5 supplemented with 2,4-D at 0.0, 1.0, or 2.0 mg/l, sucrose at 30 g/l and PVP 1.0 g/l. Different tissues were used to initiate callus tissue including seeds, cotyledons, hypocotyls, and roots of *A. andrachne*. The highest percentage of callus induction was obtained from seeds 70% with 2, 4-D at 1.0 mg/l (Table 4.5). Percentage of callus induction was decreased to 46% when 2, 4-D was used at 2.0 mg/l which was in accordance with Fu *et al.* (2006) reported that low levels of GA_3 facilitated induction of

embryonic callus from immature embryos. No callus induction was obtained from hypocotyls. However, successful high fresh weight and healthy friable callus was obtained from cotyledons, seeds, and roots. Pale brown colored callus was observed from seeds, roots, and white colored from cotyledonary tissue. Callus was successfully subcultured on the B5 media with 2,4-D at 1.0 mg/l, sucrose at 30 g/l and PVP 1.0 g/l. (Fig.4.2a,b,c).

Table 4.5. Influence of different levels of 2,4-D on callus induction from *A. andrachne* seeds cultured on B5 media

2,4-D (mg/l)	Induced Number	Induced Percentage
0	9/30	30%
1.0	21/30	70%
2.0	14/30	46%

Ten replicates (Petri dishes) per treatment and three explants/replicate.

Table 4.6 illustrate the differences in callus fresh weight during weekly inoculation. The mean of callus induction was significantly affected by different explants used and hormonal concentrations. Table 4.6 showed high significant variations among different explants, and within weekly callus fresh weight. Highest fresh weight was obtained from cotyledon, and the lowest from roots. For confirmation, Yeoman (1973) reported that for a maximum growth of callus, large pieces of tissues were favoured, so the larger number of cells in tissue increased the chance of obtaining a viable culture. Strong callus growth of *A. andrachne* was observed under light condition at solid WP media supplemented

with 2.0 mg/l TDZ + 0.5 mg/l NAA, callus grown were red green color (Fig.4.2d), and compacted in texture.

Table 4.6. Callus weight (mg) measured at weekly basis from different tissues

Time (week)	Cotyledon	Seeds	Root
1st Week	52.140[b]	39.520[c]	10.540[c]
2nd Week	60.060[ab]	45.140[bc]	13.120[bc]
3rd Week	63.680[a]	64.160[ab]	17.740[ab]
4th Week	70.600[a]	71.140[a]	21.680[a]
P value	0.019	0.017	0.0006
LSD	10.956	21.171	4.713

Ten replicates (Petri dishes) per treatment and one explants/replicate. Different letters within columns showed significant difference at $P = 0.01$ as determined by Fisher's LSD

To the best of our knowledge, there is no available data on callus induction of *A. andrachne*. In other species of *Arbutus* like *A. unedo*, callus was induced from internodal segments excised from *in vitro* axillary shoots and cultured on MS medium supplemented with various concentrations and combinations of PGRs (El-Mahrouk *et al*., 2010).

Figure 4.2. Callus induction and maintenance after six weeks (a),(b), and (c) from cotyledons, seeds, and roots respectively, on B5 media supplemented with 1.0 mg/l 2,4-D under dark condition, (d) maintained callus on WP media with 2.0 mg/l TDZ + 0.5 mg/l NAA under light condition.

4.4. Extraction and detection of catechin by Thin Layer Chromatography TLC and High Performance Liquid Chromatography HPLC

4.4.1. Extraction procedure

The main goal of this experiment was to optimize extraction methods in order to get the highest extraction rate for crude plant extracts of *A. andrachne.* Dried 1.0 g of *A. andrachne in vitro, ex vitro,* leaves , and callus were soaked separately in EtOAc, 5% methanol, and natural apple vinegar, mixtures were stirred for 48 h at room temperature and supernatant were removed by centrifugation, solvents were air dried in the fume hood. The percentages of extraction yield were calculated as a result of dividing the weight of the extracted powder by the weight of the extracted plant material (Table 4.7).

Table 4.7. Percentage extract yield (%) of 1.0 g of *A. andrachne* (*in vitro, ex vitro*, leaves, and callus) extracted by EtOAc, 5% methanol, and natural apple vinegar

Type of explants	EtOAc	5% methanol	Natural apple vinegar
In vitro leaves	43.3	17.7	18
Ex vitro leaves	28.3	13.4	9.2
Callus	20	10	23.5

The extraction solvents influenced the extraction yields. EtOAc extract gave best yield for *in vitro* and *ex vitro* leaves whereas highest yield for callus was obtained when natural apple vinegar was used. This result indicates the high solubility of catechin in organic solvent like EtOAc compared to aqueous solution, this is mainly due to the hydrophobic nature of catechin.

4.4.2. TLC analysis of catechin

Thin Layer Chromatography TLC is simple, cheap, and useful technique for follow up chemical changes during chemical processes. Although TLC cannot be used for accurate quantitative analysis, but still can offer information about the availability of known compounds by carefully comparing their R_f values with reference.

Catechin is our target molecules and its availability in different sources like *in vitro*, *ex vitro* leaves, and callus was initially determined by TLC.

Extracts derived from three tissues were investigated by TLC method with three solvent systems; EtOAc, 5% methanol, and natural apple vinegar, chloroform:acetone:acetic acid (65:21.5:13.5) was used as mobile phase. Catechin in 5% methanol and natural apple vinegar extracts was not detected whereas significant amount of catechin was noticed in EtoAC extract particularly in both *in vitro* and *ex vitro* leaves. Manki *et al*. (2009) confirmed detection of catechin compound in EtOAc extract. As catechin could exist in different isomers, the one we spotted is the (+)-catechin because its R*f* is identical to the standard (+)-catechin used as a reference in this study. The presented results of TLC analysis on silica gel with proper mobile phase (Figs.4.3, 4.4) have shown higher sensitivity in terms of standards. The identification of catechin was first detected under UV, and then stained with $FeCl_3$ solution, light dark spot was obtained after slow heating. $FeCl_3$ provided an excellent mean of selectively visualizing phenols.

The R*f* values of the major spot in *ex vitro in vitro* and leaves were dominantly observed in accordance to the reference *values* of catechin (R*f* = 0.4) (Fig.4.3), slightly more polar compound was also observed at lower R*f* values. According to TLC results, callus of *A.*

andrachne did not show any presence of catechin. For further confirm the presence of catechin, TLC sample from of *in vitro* leaves was repeated by running the plate three times in the mobile phase (Fig.4.4a, b), each time TLC was dried and re-run. After three runs, mix spot was not separated during the three runs that clearly indicate catechin from *in vitro* leaves match well with reference catechin (Fig.4.4 a1, a2, a3). Although the same extraction ratio (1:100, w/v) was used for all explants, according to TLC, catechin`s band from *in vitro* leaves was more concentrated than the pale one in *ex vitro* leaves (Fig.4.3), assuming that the concentration of catechin with *in vitro* leaves is higher compare to *ex vitro* leaves.

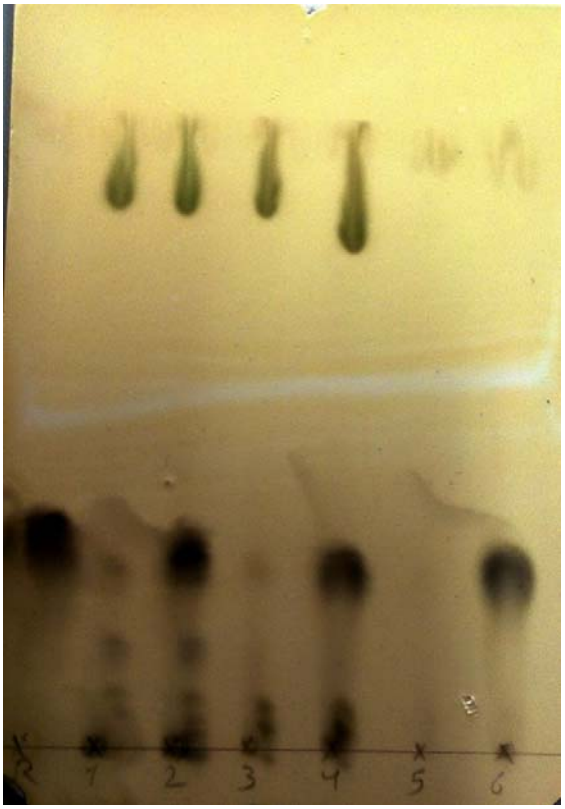


Figure 4.3. TLC plate spotted with EtOAc extract from different tissues of *A. andrachne* after being sprayed with $FeCl_3$. 1.0 µl of (R) Catechin standard, (1) *In vitro* grown *A. andrachne*, (2) mix 1 & R, (3) *Ex vitro* grown *A. andrachne*, (4) mix 3&R, (5) callus of *A. andrachne* and (6) mix 5&R were loaded.

a)

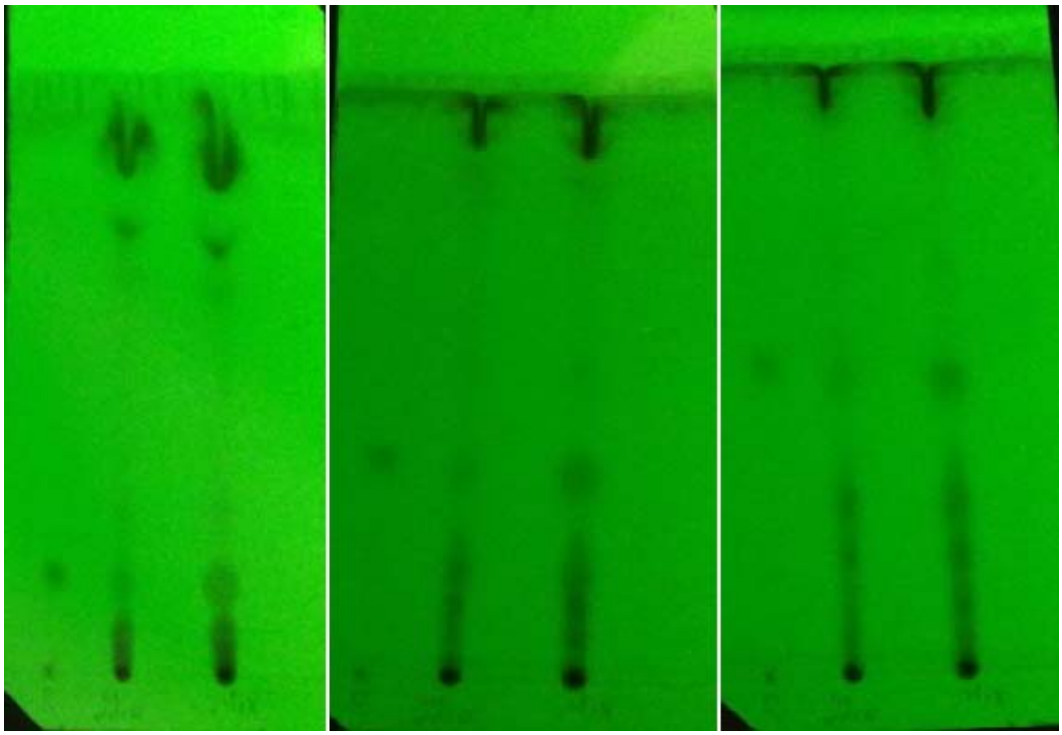


Figure 4.4. a) TLC plate under UV detector, spotted with EtOAc extract from *in vitro* leaves of *A. andrachne*. (R) Catechin standard. (1) *In vitro* grown *A. andrachne*.(2) mix 1 & R. (a1) run one, (a2) run two, (a3) run three.

b)

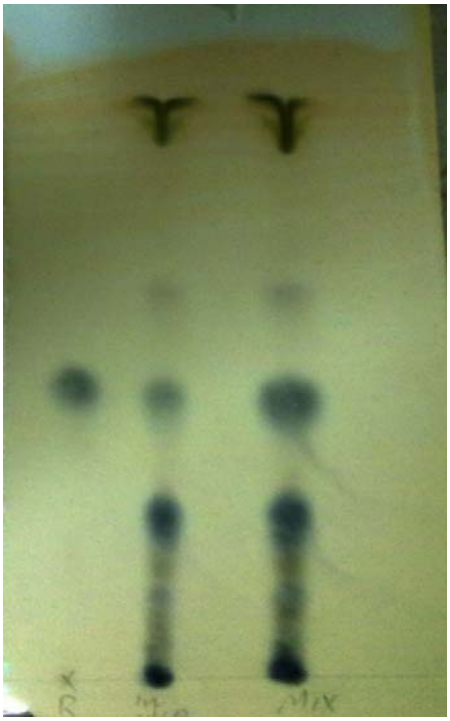


Figure 4.4. b) TLC plate after spraying with $FeCl_3$, spotted with EtOAc extract from *in vitro* leaves of *A. andrachne*. (R) Catechin standard. (1) *In vitro* grown *A. andrachne*. (2)mix 1 & R.

4.4.3. HPLC analysis of catechin

HPLC analysis involves qualitative and quantitative determination of active constituents present in plant extracts. HPLC analysis was carried out by using catechin as standard substance and EtOAc extract of *in vitro*, *ex vitro* leaves and callus of *A. andrachne*.

Under the chromatographic conditions described in methodology, the retention time of catechin was about 12.563 to 12.615 min. The chromatogram of standard catechin and that of catechin in *ex vitro in vitro*, leaves, and callus of *A. andrachne* is shown in (Fig.4.6-4.10) that proved the presence of catechin in three plant samples of *A. andrachne* especially of callus tissues, which could not be detected by TLC analysis. Catechin was quantified from a calibration curve using regression equation for catechin (Fig.4.5) resulted from serial reference standard solutions (0, 1, 20, 40, 60, 80, and 100 ppm).

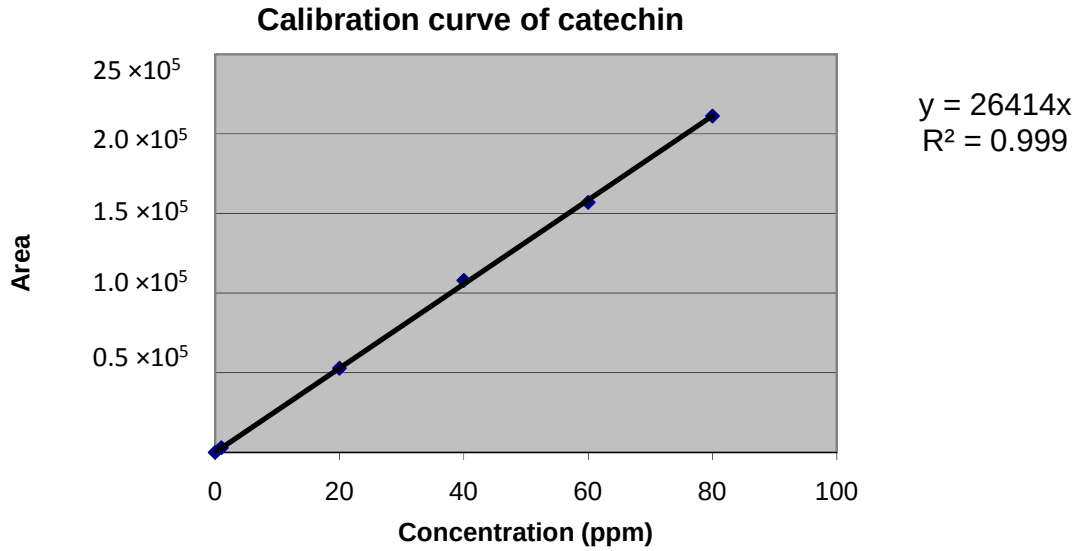


Figure 4.5. The calibration curve with the regeression equation for catechin determination

It was established the regeression equation with correlation coefficient of 0.999

Percentage yield of catechin from different explants was calculated as weight (mg) of catechin per 100 mg of plant dry material against external catechin standerd using the following equation: (W/W) % = (C× FV×D×100%)/W. Concentration of different explants was calculated, and the result as follows (*in vitro* leaves = 2.5%, *ex vitro* leaves = 0.5%, and callus = 0.063%). The highest yield of catechin was with *in vitro* leaves (Fig.4.6), and the lowest with callus tissues (Fig.4.8). HPLC result proved the accuracy of TLC result, which was presented in our study. The appearance of clear band in the *in vitro* leaves refers to the highest concentration of catechin, and start peak in callus tissue refers to low catechin concentration, which explain the absence of callus in TLC analysis. This result is in accordance with Collin and Edward (1998), they reported that in general dedifferentiated plant cells such as callus are known to reduce or stop production of secondary metabolites, which parent plant produces *in vivo.*

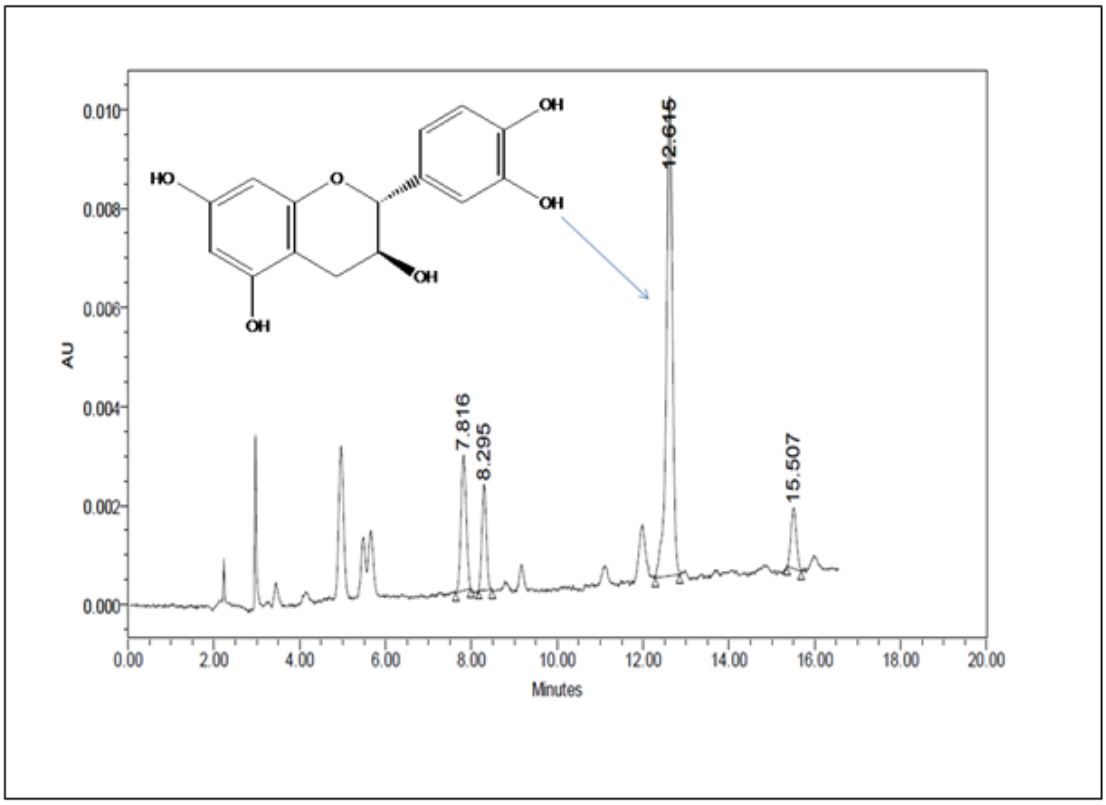
The highest catechin concentration was obtain from ethyl acetate extract of *in vitro* leaves this result confirm the idea mentioned before that by *in vitro* propagated plant higher quantity of catechin can be produced comparing with *ex vitro* plant. The present result is in accordance with Narula *et al*. (2004) they reported that extracting secondary metabolites using plant tissue culture tend to produce greater amounts of these metabolites than that is accumulated in nature.

Catechin as one of the flavonoid compounds is affected by physical environment. Light is the most imperative factor among all the physical factors. Flavonoids and phenolic biosynthesis requires light, and flavonoid formation is absolutely light-dependent, and its biosynthetic rate is related to light intensity and density (Graham, 1998). Other researchers concentrated their studies on the affects of different nutritional factors such as

sucrose concentration, ammonium nitrate level, and vitamins on the production of flavonoids specifically catechin (Farzami and Ghorbanli, 2005; Moumou *et al*., 1992, and Chattopadhyay *et al*., 2003)

The presence of catechin in *A. andrachne* grown under *in vitro* conditions indicates that this plant is capable to produce catechin under controlled environment (nutrient media, light, pH, and temperature). Thus improving catechin production can be achieved if further manipulation and optimization in the *in vitro* conditions are developed (pH, nutrients, light…etc) to simulate the preferable environmental conditions of certain spp.

Complete isolation and spectroscopic characterization of *A. andrachne* components has not been well documented in literature. By using HPLC, triterpenoids and steroids were detected (Grishkovets *et al*., 1979), Triterpenoides, sterols, arbutin, monotropeins, unedoside, and catechin were isolated (Saker *et al*., 1991), and highest yield of arbutin *in vivo,* was reported in leaves collected in August (Mostafa *et al*., 2010).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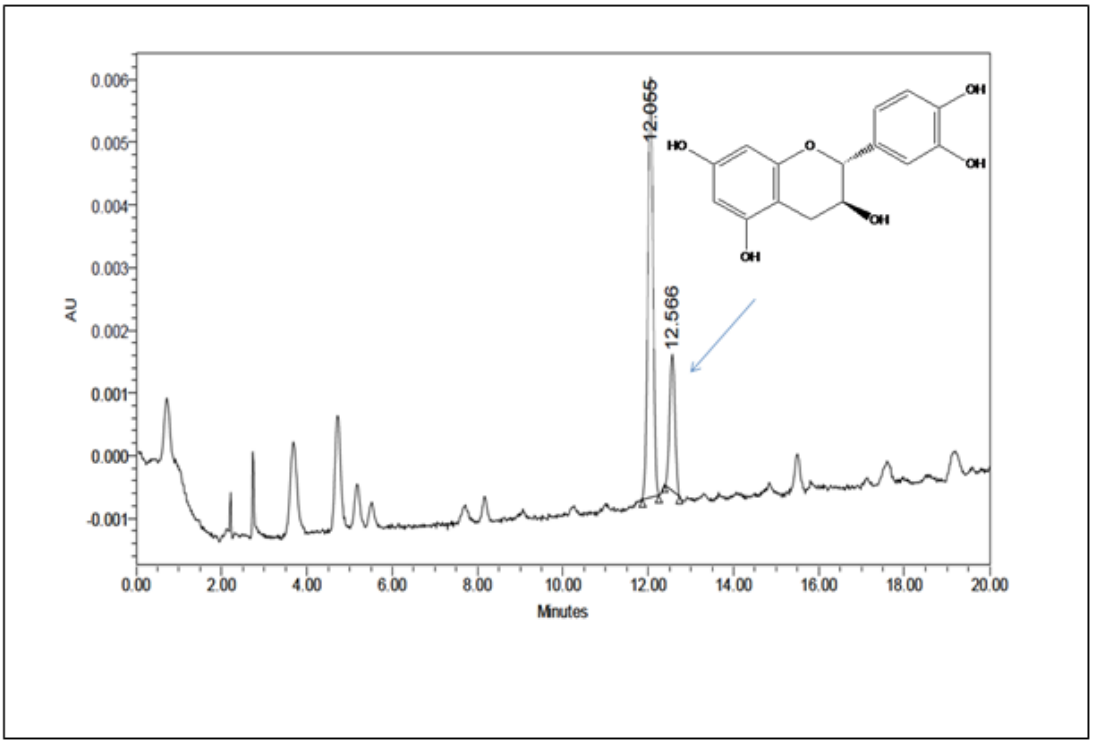
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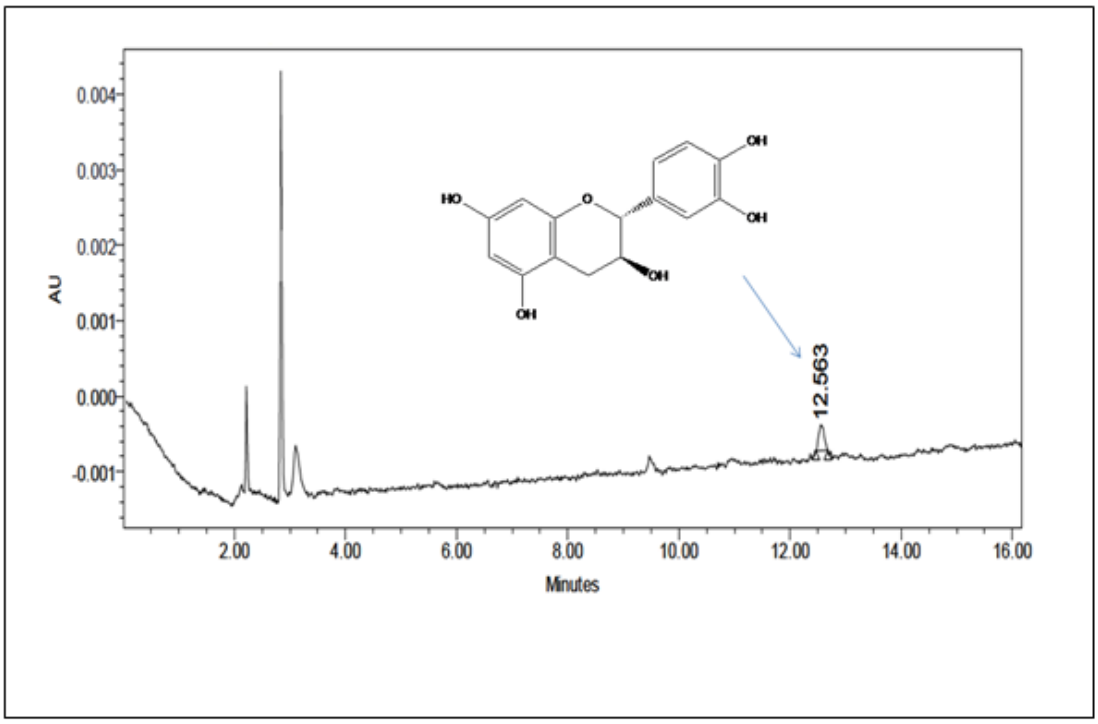

To the best of our knowledge, there is no study on this particular compound in *A. andrachne* via *in vitro* plant to support our findings; this study is the first that reported the presence of catechin contained by *in vitro A. andrachne*

(Fig.4.9, 4.10) involves overlaying catechin peaks of (catechin reference, *in vitro*, *ex vitro* leaves, and callus) to compare peak profiles.

According to overlay figures, emergence of peaks from three explants and reference were identical, that clearly confirm the presence of catechin in *A. andrachne in vitro* explants with variation of concentration

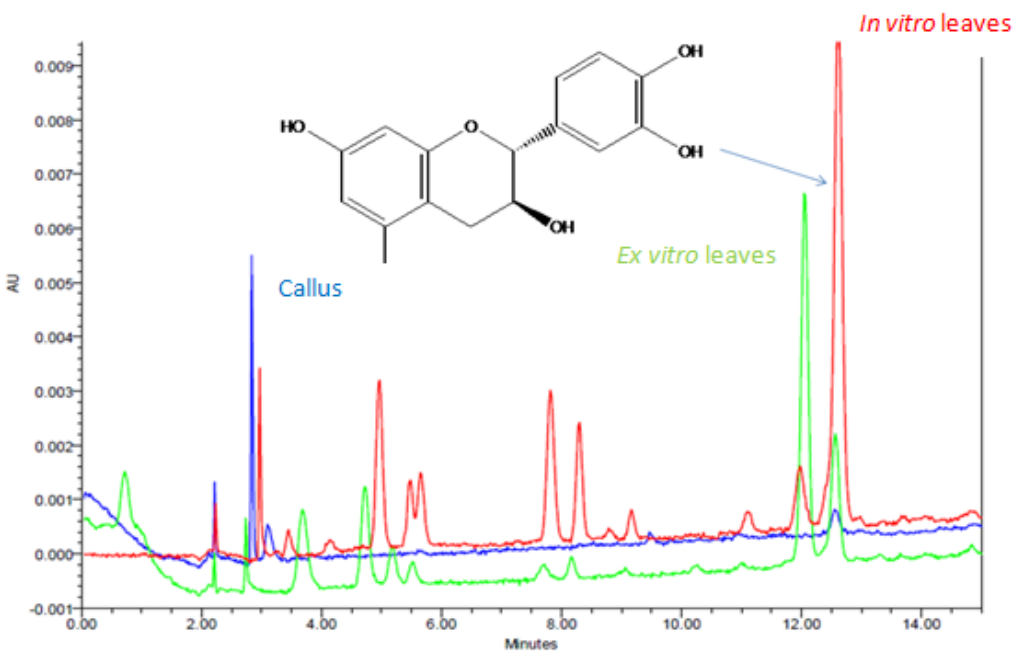

Figure 4.9. HPLC chromatogram for overlay catechin detection from *A. andrachne*

In vitro leaves *Ex vitro* leaves Callus

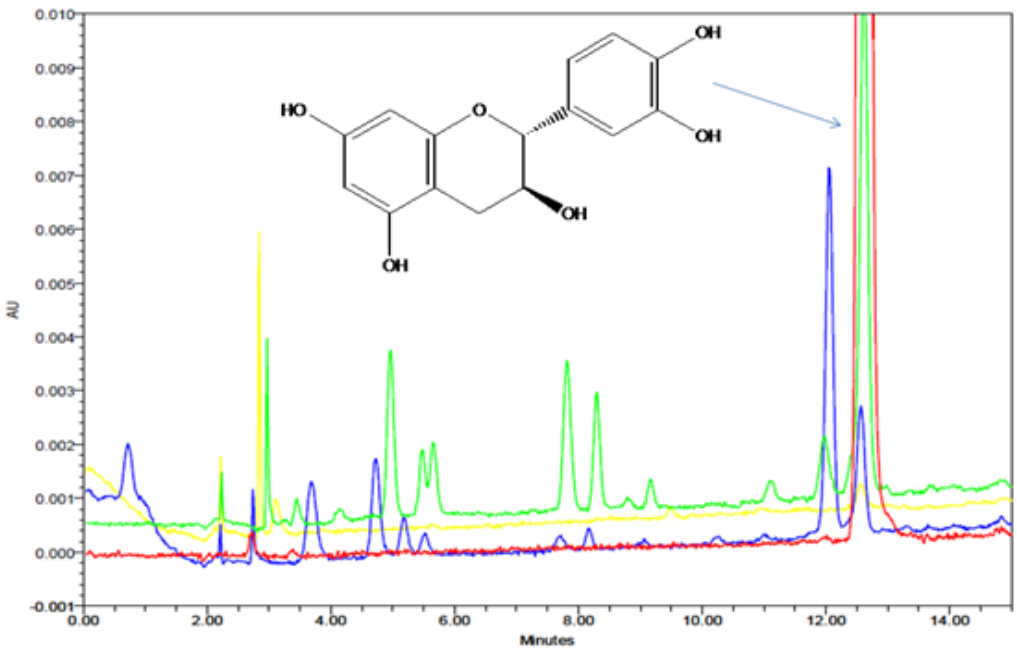

Figure 4.10. HPLC chromatogram for overlay catechin detection from *A. andrachne*

Catechin standerd *In vitro* leaves *Ex vitro* leaves Callus

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

5.1. Conclusions

Observations and conclusions in the present work:

- Tissue culture technique can be used to achieve multiplication of endangered plant species.
- Establishment of culture medium by adjusting their PGRs concentration and maintenance of aseptic conditions are the keys to success for *in vitro* multiplication of *A. andrachne*
- Seeds are considered a good starting material for *in vitro* establishment of *A. andrachne*.
- Callus could be induced and maintained from many explants type.
- Many medicinal plants that were reported to have the potential for medicinal purpose were investigated for useful bioactive compounds.
- It can be concluded that HPLC analysis used during catechin detection is much more rapid, sensitive, and accurate in comparing the quantity obtained from different explants than TLC analysis.

In the present work, we have attempted to develop suitable micropropagation protocol, establish a protocol for callus induction and maintenance from different plant explants, and qualitative and quantitative characterization of catechin using different explants of *A. andrachne.* All objectives were achieved as following:

1. Germination of seeds was 84% especially if they are pretreated with 5.0 mg/l GA3 at 4℃ for 24h, then cultured on WP medium containing 1.0 mg/l GA3.
2. Culturing microshoots on WP medium supplemented with 6.0 mg/l zeatin is recommended to achieve 100% shoot proliferation and the greatest shoot height, shoot, and leaf number.
3. Rooting is most successful on WP medium supplemented with 1.5 mg/l IBA.
4. Good rate of survival of plantlets was achieved, 83% when acclimatization was done with sterile peatmoss, and perlite.
5. Sucrose work as an inhibitor of shoot proliferation, but inducer of rooting at low percentage
6. Establishment of callus growth had been obtained from pretreated seeds and *in vitro* explants cultured on B5 medium supplemented with 1.0 mg/l 2,4-D, 30g/l sucrose, and 1.0 g/l PVP
7. Induced callus maintained on WP medium with 2.0 mg/l TDZ, and 0.5 mg/l NAA
8. According to growth curve the highest induced callus weight was obtained from cotyledon followed by seeds and roots
9. The crud EtOAc extract gave the best percentage yield from *in vitro*, *ex vitro* leaves, and callus of *A. andrachne*

10. Chloroform: acetone: acetic acid (65:21.5:13.5) was a good TLC solvent system to detect catechin in *ex vitro*, and *in vitro* leaves
11. Catechin was detected in *ex vitro, in vitro* leaves, and callus using HPLC
12. Catechin quantity was calculated depending on calibration curve and the highest result was obtained from *in vitro* leaves, followed by *ex vitro* leaves and callus.

5.2. Future Work

- Further studies are needed to increase yield of catechin *in vitro*.
- More subculturing should be done to examine the subculture stages at which maximum shoot multiplication takes place and the stage when the multiplication rate starts to decline
- The produced powder extracts will be subjected to bioassay analysis concerning anti-cancer activity
- In the future, purification of the solvent extracts based on the different polarities of the fractions/compounds will be necessary to produce more purified powders.

REFERANCES

1- Abenavoli, M.R. and Pennisi, A.M. 1998. The Effect of PVP on Chestnut Callus Formation. Acta Hort. 457:17-20. http://www.actahort.org/books/457/457_1.htm497.

2- Ali-Shtayeh, M. S., Yaghmour, R. M., Faidi Y. R., Salem Kh., Al-Nuri M. A. 1998. Antimicrobial activity of 20 plants used in Folkloric Medicine in Palestinian Area. Journal of Ethnopharmacol. 60: 265-271.

3- Al-Khateeb AA. 2001. Influence of different carbon sources on in-vitro root formation of date palm (Phoenix dactylifera L.) cv khanezi. 559-566.

4- Al-Wasel, A.S. 2000. Micropropagation of Acacia seyal (Del.) *in vitro*. J Arid Envi, 46: 425-431

5- Bertsouklis, K.F., Papafotiou, M. 2009. *In vitro* propagation of *Arbutus andrachne* L. Acta Hortic. 813:477–480

6- Buchbauer, G. 2000. The detailed analysis of essential oils leads to the understanding of their properties. Perfumer & Flavorist. **25**: 64-67.

7- Bulgakov Victor, P. G. K., Tchernoded, M. V., Veselova, S. A., Fedoreyev, T. I., Muzarok, Y. N., Zhuravlev. 2011. Catechin production in cultured cells of Taxus cuspidate and Taxus baccata. Biotechnol Lett. DOI 10.1007/s10529-011-0632-6

8- Chattopadhyay, S. K., Agarwal, S., Banerjee, S., Kulshrestha, M., Kumar, S., Mehta, V.K., Sharma, R.P. 2003. Process for the Production of a Biologically

Active Phenolic Compound(+) Catechin, Patent Genius**,** U.S Patent No: 6620599 B1

9- Chawla, H.S. 2008. Types of culture. Introduction to plant biotechnology. 2nd ed. New Delhi: Oxford and IBH publishing. 27-36.

10- Collin, Hamish A., Edwards, Sue. 1998. Plant cell culture; plant micropropagation. Springer (New York). 10-15.

11- Conger, B.V. 1982. Cloning Agricultural Plants Via *In Vitro* Techniques. CRC Press, Inc. Boca Raton. Florida. 2-3.

12- Danin, A. 2005. Flora and Vegetation of Israel and Adjacent areas. The Hebrew University of Jerusalem. http://www.botanic.co.il/a/articls/WebsiteVegIsr.htm

13- Davis, P. H. 1978. Flora of Turkey and the East Aegean Islands. 6. Edinburgh, Edinburgh University Press.

14- Dodds John, H., Roberts Lorin, W. 1995. Experiments in Plant Tissue Culture. 3rd edtion. Cambridge University Press, New York.

15- Eckhard Wollenweber and Volker H. Dietz. 1981. Occurrence and distribution of free flavonoid aglycones in plants. Phytochemistry. 20:869-932

16- El-Mahrouk, M., Dewir, Y.H., and Omar, A.M. 2010. I*n Vitro* Propagation Of Adult Strawberry Tree (*Arbutus Unedo* L.) Through Adventitious Shoots And Somatic Embryogenesis. Propagation of Ornamental Plants. 10(2): 93-98.

17- Facciola, S. 1990. Cornucopia - A Source Book of Edible Plants. Kampong Publications

18- Fang Mingyuan., Fang Ruizheng., He Mingyo., Hu Linzhen., Hu Ling-cheng., Yang Hanb., Qin Haining., Min Tianlu., David F., Chamberlain, Peter F., Steven Gary D., Wallace. Arne Anderberg .2005. Flora of China. 14: 242-517

19- Farzami, M., and Ghorbanli, M. 2005. Formation of Catechin in Callus Cultures and Micropropagation of *Rheum ribes* L. Bakistan Journal of Biological Sciences. 8 (10): 1346-1350.

20- Fried, B. and Sherma, J. 1994. Thin-layer chromatography: techniques and applications.Third edition, revised and expanded. Chromatographic science series 66. Marcel Dekker, Inc. New York, USA. 451 p.

21- Fu FL, Feng ZL, Qu BY, Li WC. 2006. Relationship between induction rate of embryonic callus and endogenous hormones content maize. Acta Agr Nucl Sinica. 20: 10-14

22- Gamborg, OL., Miller, RA. and Ojima, K. 1968. Nutrient Requirements of Suspension Cultures of Soybean Root Cells. Experimental Cell Research. 50:151-158

23- Gamborg, O.L. and Phillips, G.C. 1995. Media Preparation and Handling. In O.L. Gamborg and G.C. Phillips (Eds.). Plant Cell, Tissue and Organ Culture: Fundamental Methods, Springer, Germany, 21-34.

24- Giba Z, Grubišic´D, and Konjevic´R. 1993. The effect of white light, growth regulators and temperature on the germination of blueberry (Vaccinium myrtillus L.) seeds. Seed Science and Technology. 21: 521–529.

25- Giuseppe, G., Barreca, D., Gargiulli, C., Leuzzi, U., Caristi, C. 2007. Flavonoids composition of citrus juice. Molecules. 12: 1641-1673.

26- Graham, TL. 1998. Flavonoid and flavonol glycoside metabolism in Arabidopsis. Plant Physiol. Biochem. 36: 135-144

27- Grishkovets, VI., Sergienko, TV., Chirva, VY. 1979. Triterpenoids and steroids from the fruit of Arbutus andrachne. Chem Nat Compounds 6:866–867

28- Harrington, C.A., Kraft, J.M. 2004. Cold Stratification of Pacific Madrone Seeds. Native-Plant-Journal, 1: 66–74.

29- Hdider, C., and Desjardins, Y. 1994. Effect of Sucrose on Photosynthesis and Phosphoenolpyruvate Carboxylase Activity of *In vitro* Cultured Strawberry Plantlets. Plant Cell Tiss. Org. Cult. 36: 27-33.

30- Hedrick, U.P. 1972. Sturtevant's Edible Plants of the World. Dover Publications.

31- Hopkins, W.C. 1995. Introduction to plant physiology. John Wiely and Sons. I.N.C. New York. 445-453

32- Hou WC, Lin RD, Chen CT, Lee MH. 2005. Monoamine oxidase B (MAO-B) inhibition by active principles from Uncaria rhynchophylla. *J Ethnopharmacol* 100 (1-2): 216–20.

33- Huang, G., Jiang, J., Dai, D. 2008. Antioxidative and antibacterial activity of the methanol extract of artemisia anomala S. Moore, African Biotech**.** 7: 1335-1338.

34- Jennifer, K. 2000. Medicinal plants for livestock beneficial or toxic http://WWW.ansci.cornell.edu/plants/inedicmal/index.html

35- Jeon, MW., Ali, MB., Hahn, EJ., Paek, KY. 2005. Effects of photon flux density on the morphology, photosynthesis and growth of a CAM orchid, Doritaenopsis during post micropropagation acclimatization. Plant Growth Regul 45:139–147

36- Jha, S., Sanyal, D., Ghosh, B., Jha, TB. 1998. Improved Taxol Yield in Cell Suspension Culture of Taxus wallichiana (Himalayan Yew). Plant Medica. 64:270-272.

37- Joy, PP., Thomas, J., Samuel, M., Baby, PS. 1998. Medical Plants. Agricultural University Aromatic and Medicinal Plants Station. 3-5.

38- Karam, N. S., and Al-Salem. M. M. 2001. Breaking dormancy in *Arbutus andrachna* L. seeds by stratification and gibberellic acid. Seed Science and Technology. 29: 51–56.

39- Klouda, P. 2003. Moderní analytické metody, 2nd ed. 132 p. ISBN 80-86369-07-2

40- Kose, H. 1998. Studies on the Germination of some Woody Ornemental plants Existing in Turkish Flora. 1. *Arbutus unedo* L. and *Arbutus andrachne* L. Anadolu. Journal of Aegean Agricultural Research Istitute. 8: 55–65.

41- Kozai, T., and Kubota, C. 1988. The Growth of Carnation Plantlets *in vitro* cultured Photoauto- and Photomixotrophically on Different media. Environ. Control Biol. 28: 21-27.

42- Langford, P.J., and H, Wainwright. 1987. Effects of Sucrose Concentration on the Photosynthetic Ability of Rose Shoots *in vitro*. Ann. Bot. 60: 633-640.

43- Li, W. K.; Fong, H. H. S.; Singletary, K. W.; Fitzloff, J. F. 2002. Determination of catechins in commercial grape seed extract. J. Liq. Chromatogr. R. T. 25: 397–407.

44- Lloyd G & McCown B. 1981. Woody Plant Medium (WPM) a Mineral Nutrient Formulation for Microculture for Woody Plant Species. Hort. Sci. 16: 453.

45- M Arunachalam, M Mohan Raj, N Mohan and A Mahadevan. 2003. Biodegradation of Catechin. Proc. Indian natn Sci Acad. 4: 353-370

46- Macarenhas, A.F., Hazara, S., Potdar, U., Kulkarni, D.K., Gupta, P.K. 1982. Rapid Clonal Multiplication of Mature Forest Trees through Tissue Culture. Fujiwara (ed) Proceedings of the Fifth International Congress of Plant Culture, Tokyo, Japan. 719–720

47- Manki, S. Maoela., Omotayo, A. Arotiba., Priscilla, G.L. Baker., Wilfred, T. Mabusela., Nazeem Jahed, Everlyne, A. Songa, Emmanuel, I. Iwuoha. 2009. Electroanalytical Determination of Catechin Flavonoid in Ethyl Acetate Extracts of Medicinal Plants. Electrochem. Sci., 4:1497 – 1510

48- Markham, K. R., Bloor, S. J. 1998. Analysis and identification of flavonoids in practice. In: Rice-Evans CA, Packer L, eds. Flavonoids in Health and Disease. New York, USA: Marcel Dekker Inc., 1– 33

49- Mereti, M., Grigoradou, K., Nanos, GD. 2002. Micropropagation of the strawberry tree, *Arbutus unedo* L. Sci Hortic 93:143–148

50- Merken, H. M., Beecher, G. R. 2000. Measurement of food flavonoids by high-performance liquid chromatography: A review, J. Agric. Food Chem., 48: 577–599.

51- Michael, J. Weyant, Adelaide, M. Carothers, Andrew, J. Dannenberg, and Monica, M. Bertagnolli. 2001. Catechin Inhibits Intestinal Tumor Formation and Suppresses Focal Adhesion Kinase Activation in the Min/1 Mouse Cancer research. 61:118–125

52- Morel, G. 1960. Producing virus free Cymbidiums. Amer. Orchid Soc. Bull., 19: 495-497.

53- Mostafa, S.E., Karam, N.S., Shibli, R. A., Alali, F. Q. 2010. Micropropagation and production of arbutin in oriental strawberry tree (*Arbutus* andrachne L.). Plant Cell Tiss Organ Cult. 103:111–121

54- Moumou, J Vasseur, F Trotin, and J Dubois. 1992. Catechin production by callus cultures of *Fagopyrum esculentum.* Phytochemistry. 31: 1239-1241

55- Murashige T & Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473.

56- Mustafa Yildiz, Sertac Önde, and Murat Özgen. 2007. Sucrose Effects On Phenolic Concentration and Plant Regeneration From Sugarbeet Leaf and Petiole Explants. Journal of Sugar Beet Research. 44: 1- 2.

57- Nakao, M., Takio, S., and Ono, K. 1998. Alkyl peroxy radical-scavenging activity of catechins. Phytochemistry. 49: 2379–2382.

58- Narula, A., Kumar, S., Bansal, K.C., and Srivastava, P. S. 2004. Biotechnological approaches towards improvement of medicinal plants. *In*: Srivastava, P. S., Narula, A. and Srivastava, S. eds. Plant Biotechnology and Molecular Markers. 78-116. Anamaya Publishers. New Delhi

59- Ncube, B., Ngunge, V.N.P., Finnie, J.F., Van Staden, J. 2011. A comparative study of the antimicrobial and phytochemical properties between outdoor grown and micropropagated Tulbaghia violacea Harv. Plants. Ethnopharmacology 134: 775–780.

60- Niedz, RP., Evens, TJ. 2007. Regulation of plant tissue growth by mineral nutrition. *In vitro* Cell Dev. Biol. Plant 43: 370-381.

61- Nollet, M.L.L. 2000. Food Analysis by HPLC. 2nd ed. USA: Marcel Dekker. ISBN 08247-8460-X.

62- Pallauf, K., Rivas-Gonzalo, J.C., del Castillo, M.D., Cano, M.P., S. de Pascual-Teresa. 2008. Characterization of the antioxidant composition of strawberry tree (*Arbutus unedo* L.) fruits. Journal of Food Composition and Analysis 21: 273–281

63- PEnA. 1999. National Biodiversity Strategy and Action Plan for Palestine, Palestinian Environmental Authority (PEnA)

64- Pierik, R.L.M. 1987. *In vitro* Culture of Higher Plants. Martinus Nijhoff Publishers, Dordrecht, Netherlands. 213-218.

65- Pierik, R.L.M. 1997. *In vitro* Culture of Higher Plants, 4th edn. Martinus Nijhoff , Netherlands

66- Powell Le. 1987. Hormonal aspects of bud and seed dormancy in temperate-zone woody plants. Hortscience. 22: 845–850.

67- Preece, JE., Sutter, EG. 1991. Acclimatization of micropropagated plants to the greenhouse and field. In: Debergh PC, Zimmerman RM. eds. Micropropagation: technology and applications. Kluwer Academic Publisher, Dordrecht, The Netherlands. 71–93

68- Puhl, I. and Treutter, D. 2008. Ontogenetic variation of catechin biosynthesis as basis for infection and quiescence of *Botrytis cinerea* in developing strawberry fruits. Plant Diseases and Protection. 115 (6): 247–251

69- Rinaldo D, Batista JM Jr, Rodrigues J, Benfatti AC, Rodrigues CM, dos Santos LC, Furlan M, Vilegas W. 2010. Determination of catechin diastereomers from the leaves of Byrsonima species using chiral HPLC-PAD-CD. Chirality. 22(8):726-33.

70- Robards, K., Antolovich, M. 1997. Analytical chemistry of fruit bioflavonoid: a review, Analys., 122**,** 11R– 34R.

71- ROTEM. 2002. Constructing Red Number for endangered plant species. Israel flora as a test case. http://rotem.huji.ac.il/red_number.htm

72- Said, O., Kha lil, K. Fulder S., and Azaizeh, H. 2002. Ethnopharmacological survey of medicinal herbs in Israel, the Golan Heights and the West Bank region. Journal of Ethnopharmacology. 83:251-265.

73- Sahoo, Y., Pattnaik, S. K. and Chand, P. K. 1997. Plant regeneration from callus cultures of *Morus indica* L. derived from seedlings and mature plants. Scientia Hortic. 69(1/2): 85-89.

74- Sakar, M. K. Berkman, M. Z. Calis, I and Ruedi, P. 1991. Constituents of *Arbutus andrachne*. Fitoterapia. 62: 176-177.

75- Schinella, G. R., Tournier, H. A., Prieto, J. M., Mordujovich de Buschiazzo, P., Ríos, J. L. 2002. Antioxidant activity of anti-inflammatory plant extracts, Life Sci., 70: 1023-1033.

76- Shang-lian Hu, Jian-ying Zhou, Ying Cao, Xue-qin Lu, Ning Duan, Peng Ren and Ke. 2011. *In vitro* callus induction and plant regeneration from mature seed embryo and young shoots in a giant sympodial bamboo, Dendrocalamus farinosus

(Keng et Keng f.) Chia et H.L. Fung. African Journal of Biotechnology Vol. 10(16). 3210-3215.

77- Shatyeh, M. S., Jamous, R. M. 2008. Traditional Arabic Palestinian Herbal Medicine. (1st ed.), Nablus: Biodiversity and Environmental Research Center.

78- Sheetal Verma, and Singh SP. 2008. current and future status of herbal medicine. Veterinary World. 1(11): 347-350.

79- Shibli, R.A., M. Ajlouni. A. Jaradat, S. Aljanabi, and M. Shatnawi. 1997. Micropropagation of wild pear (*Pyrus syriaca*). Sci. Hort.972:1-6.

80- Skoog, F. And Miller, C. C. 1967. Chemical regulation of growth and organ formation in plant tissue cultivated *in vitro*. In: Biological Action of Growth Substances, 11th Symposium Society of Experimental Biology, 11: 118-131.

81- Skoog, A., Hollar, F., Nieman, A. 1998. Principles of instrumental analysis. 5th ed. USA: Thomson learning Inc.

82- Srivastava, P. S., Narula, A. and Srivastava, S. 2005. Plant Biotechnology and Molecular Markers. Anamaya Publishers. New Delhi.

83- Takahashi, N., I. Yamaguchi, and H. Yamane. 1986. Gibberellins. Chemistry of plant hormones. CRC Press, Boca Raton, Fla.

84- Talal Aburjai, Mohammad Hudaib, Rabab Tayyem, Mohammed Yousef, Maher Qishawi. 2006. Ethnopharmacological survey of medicinal herbs in Jordan, the Ajloun Heights region. Ethnopharmacology. 110: 294–304

85- Tawaha, K., Alali, F., Gharaibeh, M., Mohammad, M., El-Elimat, T. 2007. Antioxidant activity and total phenolic content of selected Jordanian plant species, Food Chemistry. 104: 1372–1378.

86- Tilki, F. 2004. Improvement in seed germination of *Arbutus unedo* L. Pakistan Journal of Biological Sciences, 7: 1640–1642.

87- Tivy, J. 1993. Biogeography, a study of plants in the ecosphere. Essex, England: Longman House Burnt Mill, Halow. 12.

88- Tournaire C., Croux S., Maurette M.-T., Beck I., Hocquaux M., Braun A.M., Oliveros E. 1993. Antioxidant activity of flavonoids: Efficiency of singlet oxygen (1Δ(g)) quenching. *Journal of Photochemistry and Photobiology B: Biology*, 19 (3): 205-215.

89- Van Rensburg, H., Van Heerden, P. S., Bezuidenhoudt, B. C. B., and Ferreira, D., 1997b. Enantioselective synthesis of the four catechin diastereomer derivatives. Tetrahedron Lett 38: 3089-3092

90- VanderJagt, T. J., Ghattas R., VanderJagt, D. J., Crossey, M., Glew, R. H. 2002. Comparison of the total antioxidant content of 30 widely used medicinal plants of New Mexico, Life Sci. 70: 1035-1040.

91- Wagner, H. and Bladt, S. 2009. Plant Drug Analysis: A Thin Layer Chromatography Atlas. Second edition. Springer-Verlag Berlin Heidelberg New York Tokyo.

92- Watson, L., and Dallwitz, M.J. 1992. The families of flowering plants: descriptions, illustrations, identification, and information retrieval. Version: 4th March 2011. http://delta-intkey.com

93- Yeoman, M.M. 1973. Tissue Culture Techniques. In: Plant Tissue and Cell Culture (H.E. Street, ed.). Botanical Monograph. Vol. II. Black Well Scientific Publications. Osney Mead, Oxford.

94- Yoshimatsu, K. 2008. Tissue Culture of Medicinal Plants: Micropropagation, Transformation and Production of Useful Secondary Metabolites. Studies in Natural Products Chemistry. 34: 647-752.

APPENDICES

Appendix Table 1.

Analysis of variance (ANOVA) for break seed dormancy and seed germination on control media using different treatment.

Source of Variation	DF	SS	MS	F	P
Between Groups	3	6.160	2.053	22.458	0
Residual	196	17.920	0.0914		
Total	199	24.080			

Appendix Table 2.

Analysis of variance (ANOVA) for break seed dormancy and seed germination on WP media using different treatment.

Source of Variation	DF	SS	MS	F	P
Between Groups	3	19.935	6.645	54.404	0
Residual	196	23.940	0.122		
Total	199	43.875			

Appendix Table 3.

Analysis of variance (ANOVA) for percentage of shoots at different levels of zeatin after six weeks of *in vitro* microshoots of *A. andrachne*.

Source of Variation	DF	SS	MS	F	P
Between Groups	4	1.880	0.470	6.221	0.0005
Residual	45	3.400	0.0756		
Total	49	5.280			

Appendix Table 4.

Analysis of variance (ANOVA) for number of shoots at different levels of zeatin after six weeks of *in vitro* microshoots of *A. andrachne*.

Source of Variation	DF	SS	MS	F	P
Between Groups	4	4031.880	1007.970	305.857	0
Residual	45	148.300	3.296		
Total	49	4180.180			

Appendix Table 5.

Analysis of variance (ANOVA) for shoot height at different levels of zeatin after six weeks of *in vitro* microshoots of *A. andrachne*.

Source of Variation	DF	SS	MS	F	P
Between Groups	4	3.637	0.909	3.967	0.008
Residual	45	10.313	0.229		
Total	49	13.950			

Appendix Table 6.

Analysis of variance (ANOVA) for number of leaves at different levels of zeatin after six weeks of *in vitro* microshoots of *A. andrachne*.

Source of Variation	DF	SS	MS	F	P
Between Groups	4	1762.720	440.680	10.607	0
Residual	45	1869.600	41.547		
Total	49	3632.320			

Appendix Table 7.

Analysis of variance (ANOVA) for percentage of rooting at different levels of IBA after six weeks of *in vitro* microshoots of *A. andrachne*

Source of Variation	DF	SS	MS	F	P
Between Groups	3	3.875	1.292	11.341	0.2829
Residual	36	4.100	0.114		
Total	39	7.975			

Appendix Table 8.

Analysis of variance (ANOVA) for number of root/explants at different levels of IBA after six weeks of *in vitro* microshoots of *A. andrachne*

Source of Variation	DF	SS	MS	F	P
Between Groups	3	2381.675	793.892	21.503	0
Residual	36	1329.100	36.919		
Total	39	3710.775			

Appendix Table 10.

Analysis of variance (ANOVA) for root length at different levels of IBA after six weeks of *in vitro* microshoots of *A. andrachne*

Source of Variation	DF	SS	MS	F	P
Between Groups	3	120.169	40.056	17.203	0
Residual	36	83.825	2.328		
Total	39	203.994			

Appendix Table 11.

Analysis of variance (ANOVA) for shoot height at different levels of IBA after six weeks of *in vitro* microshoots of *A. andrachne*

Source of Variation	DF	SS	MS	F	P
Between Groups	3	10.581	3.527	6.973	0.0008
Residual	36	18.209	0.506		
Total	39	28.790			

Appendix Table 12.

Analysis of variance (ANOVA) for number of leaves/explant at different levels of IBA after six weeks of *in vitro* microshoots of *A. andrachne*

Source of Variation	DF	SS	MS	F	P
Between Groups	3	24.275	8.092	2.761	0.056
Residual	36	105.500	2.931		
Total	39	129.775			

Appendix Table 13.

Analysis of variance (ANOVA) for percentage of rooting at different levels of IAA after six weeks of *in vitro* microshoots of *A. andrachne*

Source of Variation	DF	SS	MS	F	P
Between Groups	3	0.675	0.225	1.000	0.404
Residual	36	8.100	0.225		
Total	39	8.775			

Appendix Table 14.

Analysis of variance (ANOVA) for number of root/explants at different levels of IAA after six weeks of *in vitro* microshoots of *A. andrachne*

Source of Variation	DF	SS	MS	F	P
Between Groups	3	87.875	29.292	3.220	0.034
Residual	36	327.500	9.097		
Total	39	415.375			

Appendix Table 15.

Analysis of variance (ANOVA) for root length at different levels of IAA after six weeks of *in vitro* microshoots of *A. andrachne*

Source of Variation	DF	SS	MS	F	P
Between Groups	3	5.938	1.979	1.118	0.347
Residual	36	134.550	1.770		
Total	39	140.488			

Appendix Table 16.

Analysis of variance (ANOVA) for shoot height at different levels of IAA after six weeks of *in vitro* microshoots of *A. andrachne*

Source of Variation	DF	SS	MS	F	P
Between Groups	3	2.769	0.923	1.372	0.267
Residual	36	24.225	0.673		
Total	39	26.994			

Appendix Table 17.

Analysis of variance (ANOVA) for number of leaves/explant at different levels of IAA after six weeks of *in vitro* microshoots of *A. andrachne*

Source of Variation	DF	SS	MS	F	P
Between Groups	3	2.875	0.958	0.487	0.694
Residual	36	70.900	1.969		
Total	39	73.775			

Appendix Table 18.

Analysis of variance (ANOVA) for callus fresh weight of *A. andrachne* cotyledon during 4 weeks of incubation.

Source of Variation	DF	SS	MS	F	P
Between Groups	3	885.940	295.313	4.422	0.019
Residual	36	1068.432	66.777		
Total	39	1954.372			

Appendix Table 19.

Analysis of variance (ANOVA) for callus fresh weight of *A. andrachne* seeds during 4 weeks of incubation.

Source of Variation	DF	SS	MS	F	P
Between Groups	3	3406.274	1135.425	4.554	0.017
Residual	36	3989.504	249.344		
Total	39	7395.778			

Appendix Table 20.

Analysis of variance (ANOVA) for callus fresh weight of *A. andrachne* roots during 4 weeks of incubation

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	3	365.922	121.974	9.869	0.0006
Residual	36	197.740	12.359		
Total	39	563.662			

Appendix Table 21.

HPLC analysis for retention time, area, % area, and height of peaks appeared in catechin standard sample

	RT	Area	% Area	Height
1	12.571	528322	100.0	58205

Appendix Table 22.

HPLC analysis for retention time, area, % area, and height of peaks appeared in *A. andrachne in vitro* sample

	RT	Area	% Area	Height
1	7.816	23160	16.02	2726
2	8.295	15174	10.50	2142
3	12.615	95384	65.99	9675
4	15.507	10836	7.50	1229

Appendix Table 23.

HPLC analysis for retention time, area, % area, and height of peaks appeared in *A. andrachne ex vitro* sample

	RT	Area	% Area	Height
1	12.055	56576	74.85	6744
2	12.566	19006	25.15	2162

Appendix Table 24.

HPLC analysis for retention time, area, % area, and height of peaks appeared in *A. andrachne* callus sample

	RT	Area	% Area	Height
1	12.563	2315	100.0	333

Appendix Table 25.

List for plant growth regulators and suppliers used in this study:

2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma Cat. No: 125k0703),
6-benzylaminopurine (BAP) (Duchefa Prod. No: B-0904.0005),
70% ethanol (Chatham Cat. No. 7408/10)
European Bacteriological Agar (Hy-labs Prod. No: 5080021/sB-080472)
Gibberellic acid (GA_3) (Duchefa Prod. No: 60907.0005)