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Induction, Elicitation and Determination of Total Anthocyanin Secondary Metabolites from *In vitro* Growing Cultures of *Arbutus andrachne L.*

By

Abla Ghassan Jaber

In Partial Fulfillment of the Requirements for the Degree

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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:

Induction, Elicitation and Determination of Total Anthocyanin Secondary Metabolites from *In vitro* Growing Cultures of *Arbutus andrachne* L.

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Abla Ghassan Jaber

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Graduate Advisory Committee:

CommitteeMember (Student's Supervisor)	Date
Dr.Rami Arafeh, Palestine Polytechnic University	
Committee Member (Internal Examine)	Date
Dr Hatim Jabreen Palestine Polytechnic University	
Committee Member (External Examine)	Date
Dr.Nasser Sholie , Agriculture research center	

Approved for the Faculties

Dean of Graduate Studies and Scientific Research	Dean of Faculty of Science
Palestine Polytechnic University	Bethlehem University
Date	Date

Induction, Elicitation and Determination of Total Anthocyanin Secondary Metabolites from *In vitro* Growing Cultures of *Arbutus andrachne* L.

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Anthocyanin pigments are important secondary metabolites that produced in many plant species. They have wide range of uses in food and pharmaceutical industries as antioxidant and food additives. Medically, they prevent cardiovascular disease and reduce cholesterol levels as well as show anticancer activity. This study aims at utilizing a rare medicinal tree, *A. andrachne* in the production of anthocyanin pigments via plant *in vitro* culture techniques. The effects of different physical and chemical factors on total anthocyanin content (TAC) in callus and cell suspension culture of *A. andrachne* have been investigated. Anthocyanins have been detected in the callus of *A. andrachne* with the presence of light. McCown Woody Plant medium (Mcc) supplemented with 0.5 mg/l NAA, 2.00 mg/l TDZ and 1.0 g/l PVP resulted in red colored callus occurred after 16 days of culture. The highest anthocyanin concentration was observed in MCc salt mixture, sucrose and a mix of starch and agar for gelling. Cell suspension growth and anthocyanin production was the highest in Gamborg's B5 media that supplemented with 0.2mg/l Kineitin, 0.1 mg/l NAA, 0.25 g/l casein hydrolysate and 0.3% w/v sucrose. The effect of osmotic stress by using sorbitol enhanced the production of anthocyanin pigments at earlier stage than control (sucrose alone). Elicitation of anthocyanins with salicylic acid at 1.0 mg/l for 48 hours showed significant increase in TAC from 2.6 to 3.8 mg/l. This study outlined a protocol for large scale anthocyanin production via plant *in vitro* culture technique.

Key words: Anthocyanin, *Arbutus andrachne* L., callus culture, cell suspension culture, elicitation.

ملخص باللغة العربية

تحفيز انتاج مركب صبغة الأنثوسيانين في نبات القيقب *Arbutus Andrachne* المكثر في المختبر على شكل نسيج الكاللوس و الخلايا معلقة في وسط سائل

تعتبر صبغة الأنثوسيانين من المركبات الثانوية الهامة المنتجة في النبات ، ولهذه الصبغة استخدامات عديدة في الصناعات الغذائية والدوائية. يعتبر مركب الأنثوسيانين مضاد أكسدة وله عدة استخدامات طبية في الوقاية من أمراض القلب كارتفاع مستوى الكولسترول في الدم وكذلك يستخدم كمضاد سرطان لبعض الأورام السرطانية. تهدف هذه الدراسة الى تحفيز انتاج مركب الأنثوسيانين من انسجة وخلايا نبات القيقب *Arbutus andrachne* المكثر داخل أنابيب الاختبار. تم دراسة تأثير عدد كبير من العوامل افيزيائية والكيميائية على امكانية انتاج الأنثوسيانين من الكالاس والمحلول المعلق. تم ملاحظة وجود صبغة الأنثوسيانين في نسيج الكاللوس وذلك بعد تعريضه للاضاءة في الوسط الغذائي من نوع MCcوالمضاف إليه 0.5 mg/l NAA، 2.0 mg/l TDZو 1.0 g/l PVP حيث تمت ملاحظة الصبغة بعد اليوم السادس عشر من بداية الزراعة. سجل أعلى تركيز لصبغة الأنثوسيانين في الوسط الغذائي المضاف إليه نشا الذرة بالاضافة الى الآجار. تم قياس صبغة الأنثوسيانين في الخلايا المعلقة في الوسط الغذائي السائل المحتوي على ميديا غامبورغ B5 والمضاف إليها 0.2 mg/l Kinetin، 0.1mg/l NAA و 0.25 mg/l casein hydrolysate بالاضافة الى 0.3% w/v سكروز. ان تأثير الضغط الأسموزي عبر استخدام السوربيتول ادى الى تحفيز انتاج صبغة الانثوسيانين أبكر بكثير من انتاجها في الوسط المحتوي على السكروز لوحده. ان زيادة تحفيز انتاج الأنثوسيانين عبر استخدام التحفيز الكيماوي باستخدام ال salicylic acid بتركيز 1.0mg/lلمدة 48 ساعة ادى الى زيادة تركيز الأنثوسيانين بصورة معنوية من 2.6الى 3.8 mg/l بالمقارنة مع الشاهدز نجحت الدراسة في وضع الخطوط العريضة لامكانية انتاج صبغة الأنثوسيانين بكميات كبيرة من نبات القيقب عن طريق تقنية زراعة الأنسجة والخلايا النباتية.

كلمات افتتاحية : زراعة الخلايا المعلقة والانسجة النباتية ، *Arbutus andrachne*، صبغة الأنثوسيانين ، تحفيز انتاج المواد الثانوية

DECLARATION

I declare that the Master Thesis entitled "**Induction, Elicitation and Determination of Total Anthocyanin Secondary Metabolites from *In vitro* Growing Cultures of *Arbutus andrachne* L."** 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.

Abla Ghassan Jaber

Date: 30.12.1014

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DEDICATION

الإهـــداء

بسم الله الرحمن الرحيم

(قل إن صلاتي ونسكي ومحياي ومماتي لله رب العالمين)

صدق الله العظيم

إلى الله ، إلى الليالي الطوال التي كان بها أنيسي الوحيد جل جلاله لا يغيب.

إلى من كان صوتها ودعائها وصلاتها سببا لاستمراري رغم كل الضعف والاعياء والغربة "أمي" لك الحياة

إلى رجل المستحيل والأساطير الممكنة "والدي" لك كل ما لا أستطيعه إلى الآن وأكثر أكثر بكثير

إلى من جعلت الحلم ممكنا من تحملت فوضاي ومزاجيتي الحادة "جدتي" ...لعينيك هذا التعب الجميل

إلى رفيق السهر من تحملني في أسوء أحوالي " خالد" لك البدايات لانها الأجمل دائما.

إلى من يشاكسني دائما ليلون حياتي بالفرح والدموع معا " مُحمّد" ....

إلى من حملت اسمها فكان نصيبي بهاءٌ على بهاء "جدتي" ...

إلى الفارع حد السماء ..من أرادني ان أكمل هذا الطريق حتى آخره "جدي"...

الى ذكراهم الباقية فينا للأبد جدي "عبد الوهاب" وعمي "عدنان" لروحهما السلام

إلى من ساندوني لحظةً بلحظة ، إلى الرفاق الغائبين الحاضرين دائما....إلى من كان وجودهم دافعا للاستمرار والحب والنهوض بعد كل سقطةٍ بأملٍ جديد..

و أخيرا إليَّ بطبيعة الحال إلى طفلةٍ كنتها ولا تزال ..

إلى كل أحلامي المؤجلة ..

إلى المستقبل الذي أريده أن يكون أجمل

إلى "بحر" الذي أنتظر

لابد من فرح لابد من انتصار

وبطبيعة الحال

"شامٌ" و"سليمان".

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ABBREVIATIONS

Term/phrase	Abbreviation
- **Absorbance at different wavelengths**	A
- **And others**	*et al*.
- **Analysis of variance**	ANOVA
- **Degree centigrade (Celsius)**	**°C**
- **Completely Randomized Design**	CRD
- **2,4-Dichlorophenoxyacetic acid**	2,4-D
- **Dilution factor**	DF
- **Figure**	Fig
- **Gamborg *et al*., salt mixture**	B5
- **Gram**	G
- **Kinetin**	KIN
- **Liter**	L
- **Lloyd & MCcown woody plant medium**	MCc
- **Microgram**	µg
- **Microliter**	µl
- **Micro mole per square meter per second**	μmol /m^2/sec
- **Milligram**	mg
- **Milliliter**	ml
- **Molar extinction coefficient**	Ɛ
- **Molecular weight**	MW
- **Murashige and Skoog Basal Medium**	MS
- **Napthalene Acetic Acid**	NAA
- **Percentage**	%
- **Photosynthetic Photon Flux Density**	PPFD
- **Plant growth regulator**	PGR
- **Polyvinylpyrrolidone**	PVP
- **Quoirin & Lepoivre Medium**	QLM
- **Retention Value**	R_f
- **Salicylic acid**	SA
- **Thin Layer Chromatography**	TLC
- **Thidiazuron**	TDZ
- **Total anthocyanin concentration**	TAC
- **Ultra Violet**	UV
- **Volume per volume**	v\v

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Chapter1

INTRODUCTION

A story is a starting point for every research. Many centuries ago there was an old myth that talked about Arbutus (Madrone) "Sacred trees". Redskin people were the first who had thought about this tree. The first enticed point, which had taken their attention, was the "red bark". First of all, they looked at this tree as a holy tree, they assumed it contains all knowledge and the secret of life, so they begun adoration and blessed with all parts of this tree specifically the bark. Then, they thought to use this tree to heal wounds. Moreover, they had used the red bark as a soup to treat diabetes as well to prevent hypertension and cardiovascular disease (Johnson 2010).

Arbutus is a genus that belongs to the Ericaeae family in which 14 species of *Arbutus* has been described. Most of them share main characteristics like overall shape, color and main chemical constituent. On the other hand, they differ from each other in flowering time, geographical distribution, fruit shape and total phenolic compounds as well as antioxidant capacity. From Hileman *et al*. (2001) *Arbutus* species were split into two groups based on the phylogenetic relationship among Arbutoideae between western North America (New World) and the Mediterranean Basin (Old World). These two main groups are listed in (Table 1.1).

Table 1.1: Geographical distribution of 10 *Arbutus* species in the New and Old World according to Hileman *et al.* (2001).

Old World species	Geographical Distribution	New world species	Geographical Distribution
Arbutus andrachne	Mediterranean region and southwestern Asia	*Arbutus arizonica*	New Mexico, Arizona and Eastern Mexico south to Jalisco
Arbutus pavarii	North Africa (Libya)	*Arbutus menziesii*	Western coast of North America
Arbutus unedo	Mediterranean region, Western France, and Western Ireland	*Arbutus glandulosa*	Central and southern Mexico
Arbutus canariensis	Canary Islands	*Arbutus peninsularis*	Baja California Peninsula of Mexico
		Arbutus xalapensis	Texas, New Mexico and northeastern Mexico
		Arbutus tessellata	Mexico

Arbutus andrachne L. is the core of this research however, as listed in (Table 1.1) *A. andrachne* is the species of *Arbutus* that can be found in the Eastern Mediterranean region.

A. andrachne is a medicinal tree which naturally distributed from Eastern Mediterranean to the Northern Black Sea region (Davis 1978). *Arbutus andrachne* L. or "Grecian Strawberry Tree ", Arabic name is (Qyqab or Qatlab) is a rare and endangered plant species in Palestine (Said *et al*. 2002). It is an evergreen small tree with white flowers, reddish stem and sweet red fruits.

Plant tissue culture is a technique which plays an important role in utilization of endangered plants and the production of many pharmaceutical substances like alkaloids, terpenoids, anthocyanins, phenolics, flavonoids, saponins, steroids and amino acids (Rahimi *et al*. 2012). Additionally, the technique of plant tissue and cell suspension culture would help in exploitation and preservation of *A. andrachne* as well as to produce different secondary metabolites in efficient and nondestructive manner.

Anthocyanin pigments are members of the flavonoid family responsible for red, pink and blue color of fruit, flowers and vegetables of many plants like strawberry and grape. Anthocyanins

have an important role in human health. They have a potent antioxidant properties as well as anti-diabetic and anti-carcinogenic effect (Shipp and Abdel-Aal 2010). Moreover, anthocyanins have wide commercial uses including food coloring substituting synthetic dyes. On the other hand, anthocyanins are unstable compound which produced from plant in a small amount (0.1%- 1%) of dry weight. For that, plant cell and tissue culture technique can be used as an alternative method to produce commercial amounts of anthocyanins for commercial industrial, cosmetics pharmaceutical and food uses.

1.1 Study species; *Arbutus andrachne* L.

There are two similar species of *Arbutus*, *Arbutus unedo* and *Arbutus andrachne*, which distributed along Mediterranean region. These two species differ in their flowering time, the total phenolic compound and their geographical distributed (Serçea *et al*. 2010). In Palestine, *A. andrachne* is the species that occur in some mountainous areas of the west bank (Figure 1.1).

Fig 1.1: *A. andrachne plant parts as appear in nature (A) a branch with leaves (B) stem bark (C) flowers (D) ripe fruits.*

Recently, *Arbutus andrachne* as many other wild plants in Palestine is facing an extinction threat due to many factors like sever habitat fragmentation, over-exploitation, extensive agricultural and human activities, overgrazing and premature harvest by local people (BERC 2002).

1.1.1 Importance of *Arbutus andrachne* L. in traditional medicine

A. adnrachne is used in traditional medicine as astringent and urinary tract antiseptic and for the treatment of urinary system, blood tonic, aching joints and healing wounds as well as anticancer agent (Said *et al*. 2002, Sakar *et al*. 1991). Additionally, *A. andrachne* is classified as homeopathic remedy for eczema associated with gouty and rheumatic symptoms (Rehman 2003). Moreover, the recent study that adapted from Issa *et al*. (2008) showed that *A. andrachne* has antityrosinase effect and can be used as skin whitening agent These uses have increased the demand on this species.

1.1.2 Chemical properties of *A. andrachne*

Arbutus andrachne is listed as the highest antioxidant content within 51 medicinal plant species in Jordan (Tawaha *et al*. 2007). Fruit and leaves contain several classes of naturally occurring antioxidants such as: phenolic compounds like anthocyanins; gallic acid derivatives, tannins and flavonoids, organic acids; vitamin C, vitamin E and carotenoids (Serçea *et al*. 2010). Moreover, triterpenoides, sterol and lipids are isolated from bark, leaves and fruits, while arbutin, monotropeins, unedoside, and catechin are isolated from bark and leaves (Sakar *et al*. 1991).

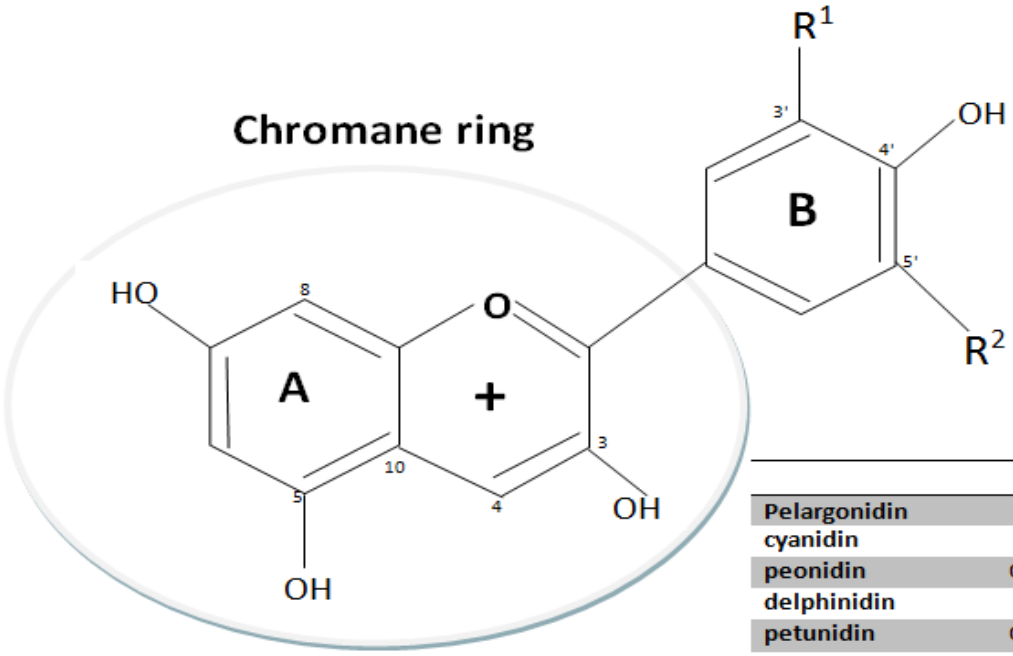
Anthocyanins are one group of natural phenolic compounds that found in *A. andrachne* and *A. unedo*. Anthocyanin compounds have been detected from *A. unedo* as three different structures: delphinidin 3-O galactoside, cyanidin 3-O glucoside and cyanidin 3-O arabinoside (Pawlowska *et al*. 2006).

1.2 Anthocyanin compounds

Anthocyanins are important pigments with antioxidant properties that commonly found in plants like strawberry and grape. These pigments are responsible for the red, purple and blue color of many flowers, fruits, vegetables and cereal grains. The root of the word anthocyanins was come from (Greek *anthos*, flower and Greek *Kyanos*, blue) (Strack and Wray 1994). They are phenolic compounds which are members of flavonoid family. The concentration of anthocyanins in most fruits and vegetables were ranges from 0.1% up to 1.0% of the dry weight (Delgado-Vargas and Paredes-López 2002).

1.2.1 Chemical structure of anthocyanins

Anthocyanins are one class of flavonoid compounds that derived from the basic structure of the flavylium cation, which is composed of two aromatic rings connected by three-carbon units and condensed by an oxygen atom (figure 1.2). The anthocyanin structure composes of an aglycon (anthocyanidin), sugars, and in many cases acyl groups. Their structure is based on C_{15} skeleton that arranged in the pattern C_6- C_3-C_6 skeleton (Andersen and Jordheim 2006). Anthocyanins are positively charged at acidic pH because of flavylium cation form (Jordheim 2007). There are about eighteen different aglycon occur naturally, six of them are common anthocyanins (figure 1.2). They differ from each other in the number of hydroxyl or methoxy groups, however cyanidin is the most frequent (50%), followed by delphinidin, malvidin and pelargonidin 12% for each, while the percentage of peonidin and petuinidin is about 7.0% of the aglycon present in nature (Cooke *et al*. 2005).



	R^1	R^2
Pelargonidin	H	H
cyanidin	OH	H
peonidin	OCH_3	H
delphinidin	OH	OH
petunidin	OCH_3	OH
malvidin	OCH_3	OCH_3

Fig 1.2 Basic structure of anthocyandin pigment (aglycon) and the most common anthocyndins occur in nature (Simoes *et al*. 2012).

1.2.2 Anthocyanin biosynthesis pathway

Anthocyanins are one of secondary metabolites that synthesized in the cytoplasm and transported into vacuole where they bind with a protein matrix and form anthocyanins vacuolar inclusion in plant (Conn *et al*. 2003). These compounds are synthesized via falvoniod synthesis pathway; however the biosynthesis began with pentosephosphates pathway, continuous with shikimate pathway and follows the pathway of flavonoid (Oancea and Oprean 2011). The flavonoid pathway can be divided into two sections; the basic flavonoid upstream pathway which includes early biosynthetic genes (EBGs) and the specific anthocyanin downstream pathway, which includes late biosynthetic genes (LBGs) (Ananga *et al*. 2013). Furthermore, phosphoenolpurivate which produce from pentosephosphate pathway is a precursor compounds of anthocyanins biosynthesis (Delgado-Vargas and Paredes-López 2002) (Fig 1.3). There are two main parts of anthocynin biosynthesis pathway: 1) general phenylpropanoid metabolism and 2) specific step of anthocyanin biosynthesis towards flavonoid pathway (Delgado-Vargas and Paredes-López 2002) (Fig 1.3). It is resolved that flavonoid pathway started with phenylalanin amino acid followed by the elimination of the amino group to produce trans-cinnamic acid, which will be hydroxylated by cinnamate-4-hydroxylase (C4H), then it converted to coumaryl-CoA by 4-coumaryl-CoA ligase (4CL) (Delgado-Vargas and Paredes-López 2002).

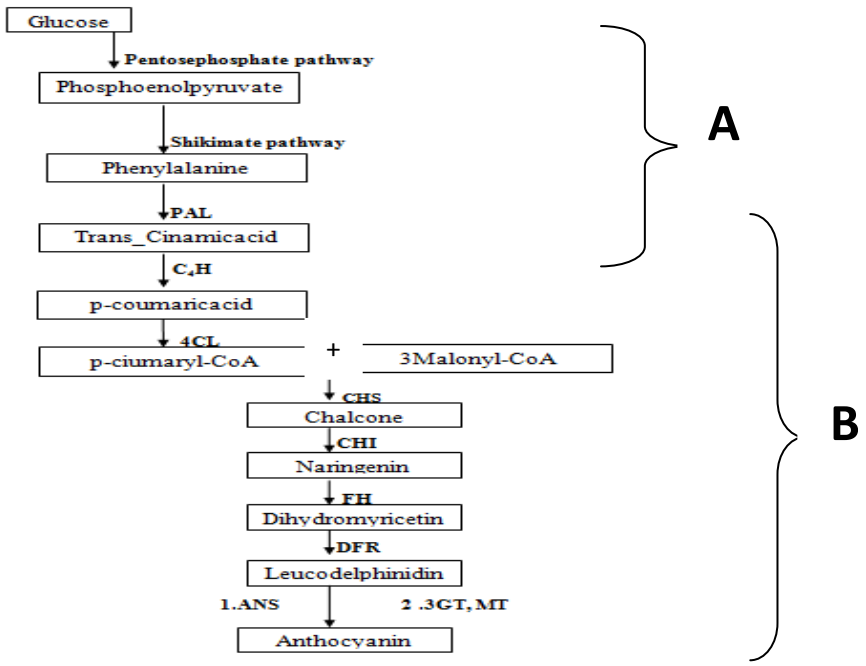


Fig 1.3 Anthocyanin biosynthesis pathway. A) General phenylpropanoid metabolism three enzymes were involved in this metabolism: PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase and 4CL, 4-coumaryl: CoA ligase. B) Specific step of anthocyanin biosynthesis six enzymes were involved in this step: CHS, chalcone synthase; CHI, chalcone isomerase; FH flavonol hydroxylase; DFR, dihydroflavonol-4-reductase; ANS; anthocyanin synthase; 3GT, glucosyltransferase; MT, methyl transferase.

Chalcone synthase (CHS) which considered as the key enzyme in flavonoide biosynthesis, it catalyzes the condensation reaction between p-ciumaryl-CoA and 3Malonyl-CoA to form the intermediate chalcone. Then, chalcone Isomerase enzyme (CHI) converted chalcone (yellow color) by isomeration to naringenin. Additionally, naringenin is a precursor of flavonoid and isoflavonide, however it was converted to dihydromyricitin by flavonol dihydroxylase (FH) (Delgado-Vargas and Paredes-López 2002). After that, the dihydroflavonol-4-reductase (DFR) catalyzes the conversation of dihydromyricitin into leucoanthocyanidin (Delgado-Vargas and Paredes-López 2002). Then, anthocyanidin synthase enzyme (ANS) was used to transformed leucoanthocyanidins to colored anthocyanidins (Delgado-Vargas and Paredes-López 2002). Finally, anthocyanidins were converted to anthocyanins by glycosylation reaction, which catalyze by glucosyl-transferase enzyme (GT) (Delgado-Vargas and Paredes-López 2002).

1.2.3 Anthocyanins role in plants

Anthocyanins are found in the form of glycoside in the vacuoles of different plant cells (Oancea and Oprean 2011). Furthermore, anthocyanins give the plants attractive colors and these pigments help in pollination. Additionally, they have a strong absorption of light so, they protect plant from photoinhibition (He and Giusti 2010). Moreover, anthocynins pigment in some species is connected with resistance to pathogens and herbivores (Simoes *et al*. 2012).

1.2.4 Anthocynanin medicinal uses

Recently, anthocyanins have beneficial effects on human health. In the United State, the estimated anthocyanin daily intake in human diet is around 12.5 mg / day (Wu *et al*. 2006). Additionally, there are a numerous studies on anthocyanin in *in vitro* tissue from animals and human, all these studies have shown that anthocyanins are not only useful for food industries also as therapeutic agents (Shipp and Abdel-Aal 2010).

Antioxidant activity and protection against DNA damage are the most important roles of anthocyanins (Oancea and Oprean 2011). Anthocyanins have an ability to capture dangerous free radical compounds like singlet oxygen as a result of oxidative stress. Basically, more than one hundred human disease conditions are associated with oxidative stress (Dalle-Donne *et al*. 2006). For that, the other biological effects of anthocyanins are related to their antioxidant activity, includes:

1) Prevention of cardiovascular disease

According to (Mazza 2007) anthocyanins display many effects on blood vessels and platelets, which reduce the risk of coronary heart disease. Furthermore, the role of anthocyanins in the protection of cardiovascular disease is strongly linked to oxidative stress protection (Lila 2004).

2) Anti-inflammatory activity

Anthocyanins pigment demonstrated inhibitory effect on cyclooxygenase enzyme COX-1 and COX-2 that means it show a strong anti-inflammatory activity (He and Giusti 2010).

3) Anti-carcinogenic activity

According to Wang and Stoner (2008) they showed that anthocyanins inhibit the development of cancer in carcinogen treated animals and in animals which have a hereditary predisposition to cancer. Furthermore, anthocyanins exhibit anti-carcinogenic activity against multiple cancer cell types *in vitro* and tumor types *in vivo* (Wang and Stoner 2008). Anthocyanins effect on animals model showed a chemopreventive effect against gastrointestinal tract GIT cancer, colon cancer and skin cancer (He and Giusti 2010; Wang and Stoner 2008).

4) Prevention of obesity

According to Tsuda (2008) consumption of anthocynanis improve the function of adipocytes that may prevent the metabolic syndrome and obesity. Furthermore, many studies investigated that intake of a fruits and vegetables that contain anthocyanins significantly inhibited weight gain (Lucioli 2012).

5) Control of diabetes

The consumption of anthocyanin was found to inhibit the elevation of blood glucose and improve insulin sensitivity accordingly , it lower the risk of diabetes (Lucioli 2012).

6) Improvement of eye vision

According to the results adapted by Canter and Ernst (2004) they showed that the consumption of anthocyanins improves normal night vision.

7) Antimicrobial activity

Anthocyanins have an antimicrobial activity, however, anthocyanins are active against different microbes, Gram-positive bacteria usually are more susceptible to the anthocyanin action than Gram-negative ones (Cisowska *et al*. 2011).

8) Preventive neurodegenerative disease

Anthocyanins consumption improve learning and memory performance in rats however, anthocyanins have a neuroprotective effect against neurodegenerative disease such as cerebral ischemia (Lucioli 2012).

1.3 Biotechnological methods used in secondary metabolites production

Some plant biotechnological techniques enhanced the production of pharmaceuticals and food additives qualitatively and quantitatively (Rao and Ravishankarb 2002). Table (1.2) lists some of the biotechnological aspects that are used in secondary metabolites production from medicinal plants.

Table 1.2. Different aspects of biotechnological approaches in medicinal plants that produced secondary metabolites (Rao and Ravishankarb 2002).

Aspects of biotechnological approaches to plant that derived secondary metabolite
1.Plant cell tissue and organ cultures
Cell culture
Shoot culture
Root culture
Scale-up of cultures
2.Transgenic plants/organisms
Metabolic engineering
Heterologous expression
Molecular forming
3.Micropropgation of medicinal plants
Endangered plants
High-yielding varieties
Metabolically engineered plants
4.New sources
Algae
Other photosynthetic marine aquatic plants

Moreover, there are several strategies that used to enhance the secondary metabolites production from plant cell and tissue culture listed in (Table 1.3).

Table 1.3 Different strategies that are used to enhance the production of secondary metabolites (Roa and Ravishankarb, 2002).

Strategies to enhance production of secondary metabolites in plant cell cultures
1. Obtaining efficient cell lines for growth.
2. Screening of high-growth cell line to produce metabolites of interest
a. Mutation of cells
b. Amenability to media alteration for higher yields
3. Immobilization of cells To enhance yields of extracellular metabolites and to facilitate biotransformation.
4. Use of elicitor to enhance productivity in a short period of time.
5. Permeation of metabolites to facilitate downstream processing.
6. Adsorption of the metabolites to partition the products from the medium and to overcome feedback inhibition.
7. Scale-up of cell culture in suitable bioreactors.

1.3.1 Plant cell and tissue culture techniques

Plant cell and tissue culture technique has an important role in the production of many pharmaceutical substances like alkaloids, terpenoids, anthocyanins, phenolics, flavonoids, saponins, steroids and aminoacids (Rahimi *et al*. 2012). Plant tissue culture is the *in vitro* aseptic culture technique of cells, tissues, organs and their component under aseptic conditions (Thorpe 2007). This technique was proposed by Gottlieb Haberlandat in 1902 (Haberlandt 1902). In the mid of 1960s and through the 1970s and 1980s these applications could be divided into 5 broad areas , 1) cell behavior , 2) plant modification and improvement, 3) pathogen-free plants and germplasm storage, 4) clonal propagation, and 5) product formation (Thorpe 1990).

Basically, cell culture system has major advantages over the traditional cultivation including: 1) production of useful compounds under controlled conditions, 2) cultured cells are free of contaminations and insects, 3) the cells of plants would be easily multiplied to yield their specific metabolites, 4) reduced labor costs and improve productivity of secondary metabolites, 5) many organic substances are extracted from callus tissue (Vijaya *et al*. 2010). Moreover, the main advantage of this technology is to provide a continuous, reliable source of plant biochemicals at a large scale (Pascal and Johan 2002).

there are many examples in the production of many pharmaceutical substances from plant cell and tissue culture such as Taxol, the anticancer agent which is produced from *Taxus* species cells, morphine and codeine the analgesics which produced from callus and suspension

cultures of *Papaver somniferum*, and diosegenin a steroidal drugs which could be produced from cell culture of *Dioscora deltoidea* (Pascal and Johan 2002).

1.3.2 Anthocyanin from plant cell and tissue culture

Oancea and Oprean (2011) listed some factors that influence the amounts of flavonoid as well as anthocyanins in plant tissue culture:

1- The genetic difference between different plant species.
2- The environmental factors that affect plant development like light, temperature and media pH.
3- The methods of cultivation.
4- The further processing of plant materials.

Anthocyanin pigments can be found in cell culture of many plant species like *Vitis vinifera*, *Euphorbia spp.*, *Daucus carota* and *Perilla frutescens*. Anthocyanins were also found in a high yields in *Pe. frutescens* cell culture than in intact plant (Roa and Ravishankarb 2002). Moreover, there is approximately 33 different plant species cell culture that was established to produce anthocyanins pigment (Deroles 2009).

Biotechnology applications could be used in different approaches (Table 1.2-3) to enhance the production of anthocynains for commercial applications.

1.3.3 Elicitation of anthocyanins in *in vitro* culture

Elicitation is a process that induces or enhances the synthesis of secondary metabolites in plant *in vitro* culture. The application of elicitors is considered as one of the most effective methods to improve the synthesis of secondary metabolites in medicinal plants in different pathways (Patel and Krishnamurthy 2013; Karl-Hermann *et al*. 2009).

Elicitors are molecules that capable to induce the production of phytoalexins or stimulate any type of plant defense like the production of ROS (Reactive Oxygen Species) and the hypertensive response (Rao and Ravishankarb 2002; Montesano *et al*. 2003).

In recent studies, wide varieties of elicitors have been employed in order to modify cell metabolism. These modifications were designed to increase the production of important secondary metabolites in plant tissue culture and cell suspensions (Rao and Ravishankarb

2002). However, the elicitations technique showed increasing yield of secondary metabolites and cutting production costs (Miao *et al*. 2000).

According to Ramadan *et al*. (2003) elicitors are classified into physical or chemical, biotic or abiotic. The physical elicitors include: temperature, humidity, and light as well pH of the growth media. The chemical elicitors include; nutrient concentrations, osmotic stress and trace amount of metal ions. On the other hand, biotic elicitors are origin from biological origin such as yeast extracts while, abiotic elicitors they do not belong to biological origin. Both elicitors had created a stress on plant biosynthesis pathway to increase the production of secondary metabolites.

The chemical compositions of nutrient media could elevate the production of secondary metabolites from cell and tissue culture (Briskin 2007). For example, increasing the sucrose level in growth media enhanced the production of anthocyanin in *Perilla frutesens* (Zhong and Zhu 1995). Carbohydrates chemicals like starch, cellulose and sugar types are also elicitors that results in the production of secondary metabolites in plant cell culture (Angelova *et al*. 2006).

It is noteworthy that manipulation of nutrients in cell suspension culture increases the accumulation of certain secondary metabolites like anthocyanins. For that, the expression of many secondary metabolites pathways could be altered by extraneous factors including nutrient levels, stress factors, light and growth regulators (Rao and Ravishakarb 2002).

1.4 Factors that enhancing the *in vitro* production of anthocyanins

Basically, anthocyanins are unstable compounds so there are many factors that may affect the stability of their chemical structure and production such as: pH, temperature, light, presence of oxygen and interaction with other molecules like metal ions (Jackman and Smith 1996).

1.4.1 Light effect on anthocyanins production

Light is one of the essential factors in anthocyanins biosynthesis in plant tissue culture (Chalker-Scott 1999). Moreover, light stimulate the signal transduction and gene expression that involves in anthocyanins biosynthesis (Mola *et al*. 1996). Additionally, it induces the expression of genes that responsible for the activation of the promoter for the flavonoid pathway genes (Azuma *et al*. 2012). However, UV- A light significantly stimulates genes that encoding the enzymes of shikimate pathway, also UV-B and -C irradiation light enhances the

production of anthocyanins in grape berries (Ananga *et al*. 2013). Some species of plant tissue culture have an ability to produce anthocyanin in dark conditions. There are a difference between cell suspension culture conditions and callus culture conditions on the same species to produce a large amount of anthocyanins pigment. Furthermore, the initiation of high concentration of anthocyanins from plant cell suspension culture independent of light will influence the production of anthocyanins at a large scale by using bioreactor technology (Ananga *et al*. 2013).

1.4.2 Temperature effect on anthocyanins production

Temperature is another important physical factor that influences the accumulation of anthocyanins in the cell vacuole. According to Mori *et al*. (2007) they showed that the elevated temperature decreases anthocyanin production from the skin of grape due to the inhibition of mRNA transcription as well as increasing the degradation of anthocyanins.

1.4.3 The effect of media pH on anthocyanins production

Media pH is also an important factor that affects the stability of anthocyanins and the activity of enzyme systems that drive anthocyanins biosynthesis pathway in plant cell (Iercan and Nedelea 2012). Moreover, the media pH is associated with the metabolism of nitrogen as well the solubility of salts, so it affects the nutrition level in the culture media of cell suspension growth and production of anthocyanins (Ananga *et al*. 2013).

1.4.4 The effect of nutrients on anthocyanins production

Generally, culture under stress like osmotic stress or starvation nutrition increases the production of anthocyanins (Deroles 2009). Moreover, the source of carbon and nitrogen as well as the concentration of phosphate are important factors to enhance or to inhibit the production of anthocyanins from *in vitro* growing materials. Hence, sugars are mainly used as source of carbon as well as osmotic agent at high concentrations (Simoes *et al*. 2012). Sucrose is the most frequently sugars that used in *in vitro* culture which influence the production of anthocyanins (Simoes *et al*. 2012). The additions of alcoholic sugars like sorbitol or mannitol create an osmotic stress and enhance the production of anthocyanins (Simoes *et al*. 2012). According to Rajendran *et al*. (1992) the supplementation of mannitol in addition to sucrose showed the significant increase in the anthocyanins in the *in vitro* callus of *Daucus carota*.

Basically, the nitrogen source came from either ammonium (NH_4^+) or nitrate (NO_3^-) salts and the ratio between these two salts significantly affects on the production of anthocyanins in callus or cell (Deroles 2009; Simoes *et al*. 2012). According to Do and Cormier (1991) the high concentration of ammonium salt in cell suspension decreases the production of anthocyanins from *in vitro* cell suspension of *Vitis vinifera*.

Additionally, the concentration of phosphate salt had markedly effect on the accumulation of anthocynains (Deroles 2009, Simoes *et al*. 2012). Furthermore, the low concentration of phosphate salts (42.5-85 mg/l) produces high concentration of anthocyanins from callus culture of *Daucus carota* (Rajendran *et al*. 1992) and from cell suspension culture of *Vitis vinifera* (Dedaldechamp *et al*. 1995; Dedaldechamp *et al*. 1999).

1.4.5 The effect of chemical elicitors on anthocyanins production

A trace amount of metal ions act as elicitors to enhance the production of anthocyanin in plant cells. Moreover, salicylic acid and methyljasmonate increased the production of anthocyanins by increasing cytoplasmic Ca^+ that increased the biosynthesis pathway of anthocynains (Sudha and Ravishakar 2003a 2003b). Additionally, the enhancement of the production by using elicitors is a useful tool to maximize the production at the commercial scale from different plant cell culture from different species (Deroles 2009). According to Mahi *et al*. (2010); Saw *et al*. (2010); Riedel *et al*. (2012) salicylic acid is one of the elicitors that showed an impact to enhance the production of anthocyanins and other phenolic acids from *in vitro* growing material of plants like cell suspension of *Vitis vinfera.*

1.5 Methods of isolation and quantitative determination of anthocyanin

1.5.1 Extraction of anthocyanin pigment

Anthocyanins are unstable water soluble compounds that give different colors under different media pH, also they can be extracted by using acidified solution (Hua *et al*. 2013). The extraction of anthocyanins is commonly carried out by acidified methanol or ethanol at 1.0% HCl (Abdel-Aal and Hucl 1999). However, acidified methanol is still the most efficient solution for anthocynins extraction (Hua *et al*. 2013).

1.5.2 UV-VIS spectrometry and anthocyanin detection

UV-VIS spectrophotometer can be used for the detection and characterization of anthocyanins. Anthocyanins spectral absorption depends on pH (Shipp and Abdel-Aal 2010).

There are two distinctive bands of absorption in the detection of anthocyanins , one in the UV region (260-280nm) and the second in the visible region (490 – 550 nm) (Giusti and Wrolstad 2001). All anthocyanins compounds had shown the two distinctive absorption in these two spectra; however the shape of each spectrum indicates the number and position of glycoside substitution and the number of cinamic acid acylation (Giusti and Wrolstad 2001). The solvent that used for anthocyanins extraction will affect the shape and the position of the spectrum (Giusti and Wrolstad 2001).

1.5.3 Determination the Total monomric anthocyanin concentration

Basically, anthocyanins undergo reversible structure transformation under different media pH (Fig 1.4), however the pH differential methods that used to calculate the total monmeric anthocyanins is based on the reaction that adapted from Giusti and Wrolstad (2001). The differential method is measuring the absorbance at two different pH(s) to determine total anthocyanin concentration. The absorbance is taken at pH 1.0 and 4.5 at 520nm and 700nm (Giusti and Wrolstad 2001). For that, the pH differential method has been described as a rapid and easy way to calculate the total anthocyanin concentration (Giusti and Wrolstad 2001).

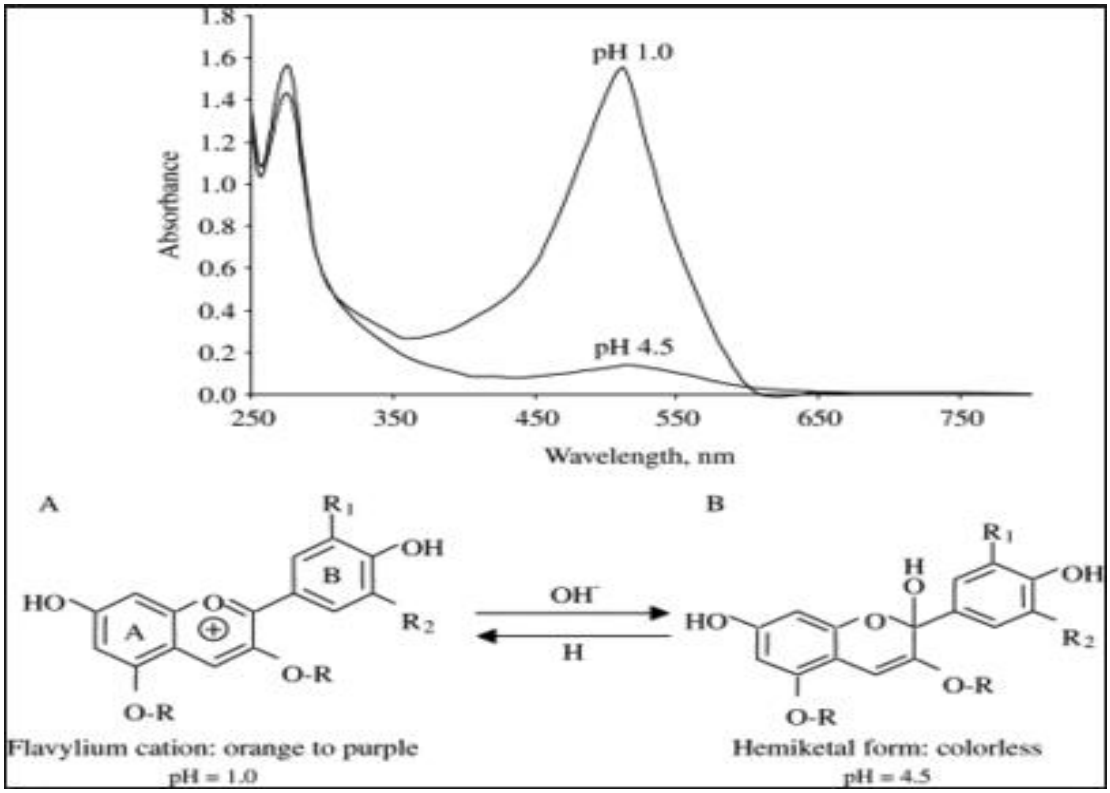


Fig 1.4: Spectrum and molecular structures of an anthocyanin at pH 1.00 and pH 4.5 (Giusti and Wrolstad 2001).

1.5.4 Thin layer chromatography TLC

Generally, TLC is used for the analysis, identification and separation of pigments like anthocyanins from other mixture compounds. Moreover, TLC is a qualitative analysis of plant extract (Wagner and Bladt 1996). It is also simple, rapid, and inexpensive method that can be used for analysis anthocyanins from plant extracts. In the study of Devi *et al*. (2012), TLC method was used to identify anthocyanins from different plant species like red sorghum. Although, the spots of anthocyanins pigment were detected under ultraviolet light 365nm. Moreover, the retention factor R_f was used to provide corroborative evidence to identify the compounds by comparing R_f from extracts with R_f for the reference compound under the same condition by using the same elution buffer (Wagner and Bladt 1996).

Chapter2

PROBLEM STATEMENT AND OBJECTIVES

2.1 problem statement:

Arbutus andrachne is one of medicinal plants that forcing extinction threat in Palestine (BERC 2002). Because of its importance many studies have focused on the propagation and utilization of this medicinal plant, which consequently would help in its conservation and the production of some important secondary metabolites from it (Mostafa *et al*. 2010; Aljabari *et al*. 2014). An important group of secondary metabolites exits in *A. andrachne* is anthocyanins. They are chemical compounds that responsible for the red pigment color of flower, fruit and stem. Anthocyanins are phenolic compounds that produced primarily from flavonoid synthetic pathway.

Anthocyanin pigments have wide range of uses in human health as well as commercial application in pharmaceutical, food and cosmetic products. This pigment is found in trace amounts in the plant (~0.1% to 1.0%), accordingly, this study aims at investigating some chemical and physical factors that influence the production of anthocyanins in the *in vitro* growing callus and cells of *A. andrachne*.

2.2 Objectives:

The main objective of this study is focused on the induction, detection and elicitation of anthocyanins from *in vitro* cell and tissue culture of *A. andrachne*.

The specific objectives in this study are:

- Qualitative and quantitative detection of total anthocyanins that extracted from different callus lines and cell suspension culture.
- Study the effect of different chemical factors like basal media components, sugars (source of carbon), gelling agent and physical factors like media pH on the production of anthocyanins pigment from different callus lines and cell suspension culture.
- Study the effect of elicitors on anthocynins production.

Chapter 3

MATERIALS AND METHODS

The experimental work in this study was conducted in plant tissue culture laboratory at the Biotechnology Research center (BRC) at the Palestine Polytechnic University in Hebron, Palestine.

3.1 Chemical and reagent for different maintenance callus

All basal media for plant growth, plant growth regulators (PGRs) and abbreviations, chemical reagents as well as buffers were obtained from Duchefa Biochemie and Sigma-Aldrich chemical companies. All chemicals and reagents are listed with abbreviations in Appendix Table 1.

3.2 Plant material

Seed of *Arbutus andrachne* were collected from wild trees in Taffouh near Hebron city, (LAT: 31°32´00´´), (LONG: 35°05´42´´) in November of 2012.

3.3 seed sterilization, germination and callus induction

This section was carried out by following similar protocol that was described in Aljabary (2011). According to Aljabary (2011) seeds were cold stratified for 24 hours then soaked in 1.0 mg/l Gibberellic acid then culture on MCc media. The callus tissue was established from cotyledonaray explants of *A. andrachne* on modified B5 media supplemented with 1.0 g/l 2,4-Dichlorophenoxyacetic acid (2,4-D), sucrose was added at 30 g/l and 1.0 g/l polyvinylpyrrolidone (PVP). Twelve milliliter media was poured in 5.0 cm sterile Petri dishes. Five cotyledonary explants were placed in growth room for six weeks under dark condition at (24±1 °C). After that, callus were transferred to another culture medium which is MCcown media (MCc) supplemented with 2.0 mg/l Thidiazuron (TDZ) and 0.5 mg/l NAA and 1.0 g/l PVP. Five calli pieces around (0.2 g) were cultured on (MCc) media and kept in the growth room under cool-white fluorescent illumination of 40-45 μmol /m^2/sec

Photosynthetic Photon Flux Density (PPFD) at (24±1 °C). Callus was transferred after 4 weeks to MCc media periodically to propagate calli and increase the starting materials.

3.4 Experimental media for callus growth and anthocyanins production

This study tested the influence of changing growth media type and components on callus maintenance and growth, in addition to anthocyanin pigments accumulation within *in vitro A. andrachne* callus at fixed operating temperature, humidity, light, media pH and growth regulator. This experiment was based on preparing 14 different treatments (Table 3.1). These treatments were prepared by using one of the basal salts of MS, B5. QLM and MCc. All media types were prepared according to the manufactures instruction and 30.0 g/l of different sugars: (sucrose, fructose and glucose) were added. Additionally, two treatments were based on adding alcoholic sugars (sorbitol and mannitol) at 4.0 g/l in addition to other carbon source (sucrose). These media were supplemented with 2.0 mg/l TDZ, 0.5 mg/l NAA and 1.0 g/l PVP. Then, the medium pH was adjusted to 5.8 by pH meter with1.0 M KOH or 1.0 M HCl. After that, media was solidified with different gelling agents according to the treatments: (agar, gelrite, starch). All components were heated in the microwave until they completely dissolved.

Table 3.1 The list of 14 treatments on solid media for callus culture, this media was supplemented with 2.0 mg/l TDZ, 0.5 mg /l NAA and 1.0 g/l PVP.

Media	Salt	Sugars	Gelling agent
Control media (1)	MCc basal media	30.0 g/l Sucrose	8.0 g/l Agar
Treatment (2)	MCc basal media	30.0 g/l Fructose	8.0 g/l Agar
Treatment (3)	MCc basal media	30.0 g/l Glucose	8.0 g/l Agar
Treatment (4)	MCc basal media	15.77 g/l Glucose + 15.77 g/l Fructose	8.0 g/l Agar
Treatment (5)	MCc basal media	30.0 g/l Sucrose + 4 g/l Mannitol	8.0 g/l Agar
Treatment (6)	MCc basal media	30.0 g/l Sucrose + 4 g/l Sorbitol	8.0 g/l Agar
Treatment (7)	MCc basal media	30.0 g/l Sucrose	30.0 g/l Starch + 4.0 g/l Agar
Treatment (8)	MCc basal media	30.0 g/l Sucrose	3.0 g/l Gel rite
Treatment (9)	MS basal media	30.0 g/l Sucrose	30.0 g/l Starch + 4.0 g/l Agar
Treatment (10)	B5 basal media	30.0 g/l Sucrose	30.0 g/l Starch + 4.0 g/l Agar
Treatment (11)	QLM basal media	30.0 g/l Sucrose	30.0 g/l Starch + 4.0 g/l Agar
Treatment (12)	MS basal media	30.0 g/l Sucrose	8.0 g/l Agar
Treatment (13)	B5 basal media	30.0 g/l Sucrose	8.0 g/l Agar
Treatment (14)	QLM basal media	30.0 g/l Sucrose	8.0 g/l Agar

3.5 cell suspension culture

3.5.1 Initiation and maintenance of cell suspension culture

In order to initiate cell suspension culture, a previously grown cotyledonary (1.0 gm) was placed in 500 ml Erlenmeyer flask containing 100 ml of liquid media.

This experiment was started with MCc liquid media that supplemented with 2.0 mg/l TDZ and 0.5 mg/l NAA, which is a similar solid media that was used for callus maintenance as described in section 3.3. Although, this media showed negative result there is no cell formed or growth in this media, for that, another liquid media has been tested.

B5 basal media was supplemented with (0.1 mg/l NAA, 0.2 mg/l kinetin and 0.25 g/l casein hydrolysate) and 30 g/l sucrose according to Min *et al*. (2010).

Moreover, this experiment studied the effect of alcoholic sugars (mannitol or sorbitol) in addition to sucrose there (Table3.2). The cell suspension was induced from callus that placed in the three different media that used in this experiment. The flasks were maintained on orbital shaker at 120 rpm under two different conditions; dark at 29±1 °C, and light at 23±1 °C to initiate the cell suspension cultures and to study the effect of these two conditions on cell growth. The cell suspension culture was maintained by transfer 5.0 ml of liquid media containing cells to other fresh media. The 1st subculture was performed after cells suspension fresh weight reached 70 mg/ml and this obtained after 32 days from inoculation.

Table 3.2 The 3 different media were used to induce cell suspension from callus of *A. andrachne*

Media	Alcoholic sugars
1 (control)	No alcoholic sugars (only sucrose)
2 (S+M)	Mannitol 2.0 g/l + sucrose
3 (S+S)	Sorbitol 2.0 g/l + sucrose

3.5.2 Determination of cell suspension growth curve

The growth curve for both induced and subcultured cells was determined within 32 days and 24 days respectively. One gram of callus from control solid media was placed into 12 flasks in which they were divided into three groups. Each flask was filled with 100 ml liquid media (1, 2 and 3) as listed in (Table 3.2) respectively. Figure 3.1 illustrates the experiment layout. At four days interval, samples of 1.0 ml from each cell suspension flask was taken and the cell fresh weight was recovered after centrifuging at 10000 rpm for 12 minutes and dried. The cell growth curves were determined within 24 days. From the cell suspension, aliquots of 5.0 ml were transferred into six flasks, each contained 50 ml of control media without alcoholic sugars. The evaluations were made in a similar way as in the induced cell suspension growth curve.

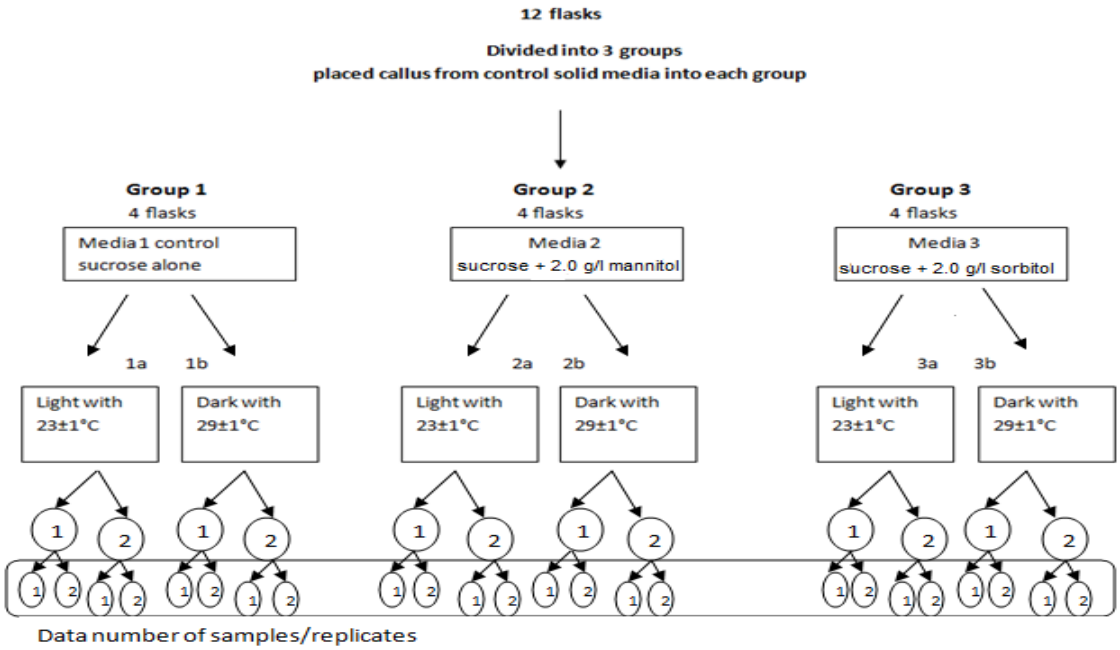


Fig 3.1 Experiment plan of cell suspension culture

3.5.3 Effect of different concentrations of sorbitol on cells growth and total anthocyanin concentration (TAC)

This part focused on the effect of different concentrations of sorbitol on cells growth and anthocyanins production. Since mannitol treatment showed inhibition of anthocyanin production, while sorbitol treatment increased the production of anthocyanins. Aliquots of 5.0 ml were transferred from group 3b to a new 6 flasks which divided into 3 groups as described in (Figure 3.2). The cell growth curves for these treatments were determined as described previously in section 3.4.2.

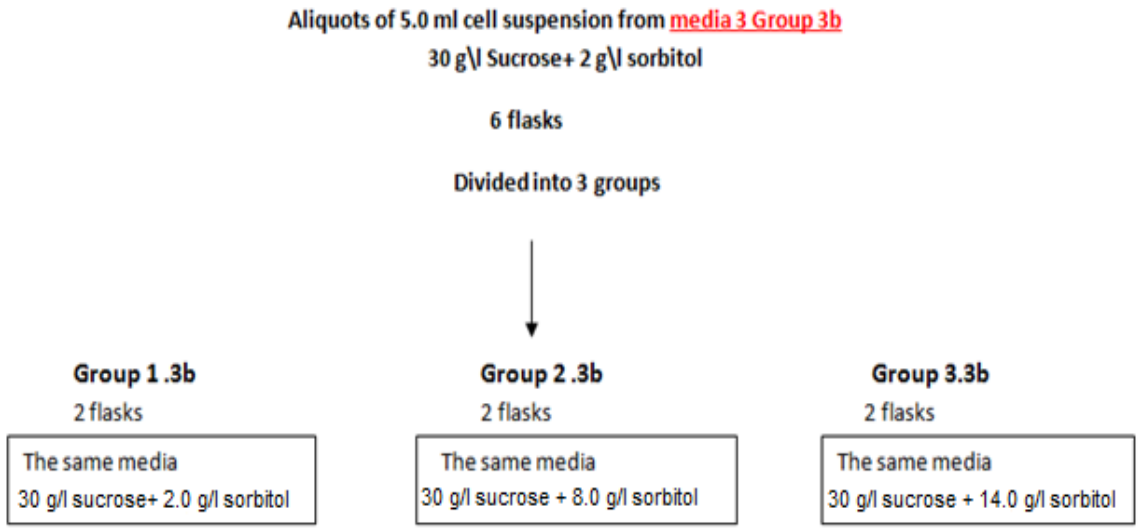


Fig 3.2 The experimental layout of three different concentrations of sorbitol that tested on subculture cell suspension of *A. andrachne*.

3.5.4 Effect of changing media pH on cell suspension growth and (TAC)

Five milliliters from the control treatment were transferred to 15 flasks each contained 50.0 ml of media at different pH levels as described in (Figure 3.3). The cell growth curves for these treatments were determined as illustrated in section 3.4.2.

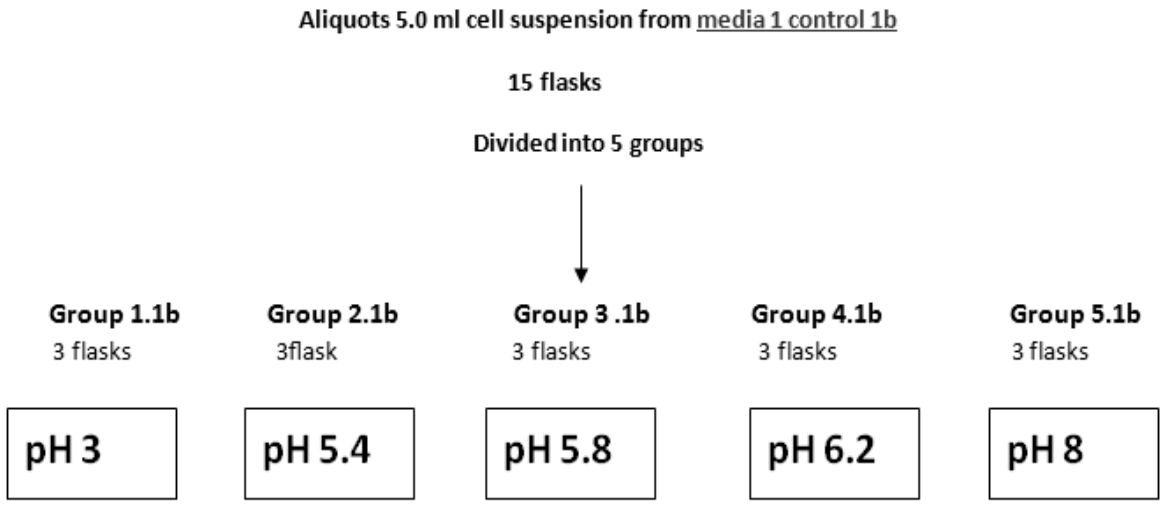


Fig 3.3 Description of different pH(s) that used to study the effect of different pH levels on cell growth and total anthocyanin concentration (TAC).

3.5.5 Extraction of anthocyanin pigment from cell suspension culture

Hundred milligrams of fresh weight cells were immersed in 3.0 ml acidified methanol (1% v/v HCl) in 50 ml falcon tube in dark at 4 °C for 24 hours. The extracted solution was centrifuged for 20 minutes at 2800 rpm .The supernatant was dried from media residues in the laminar-flow cabinet. The yield of extract was estimated from cell suspension at weight bases. Additionally, the clear supernatant was used to detect anthocyanin and determine the total anthocyanin concentration (TAC) by pH differential methods , which described at section 3.7 (Giusti and Wrolstad 2001).

3.6 Elicitation of anthocyanin pigments

As preliminary results shows that the highest cells growth point on the curve was recorded on the day 16^{th}, consequently the elicitor was added at its best time between the days 14^{th} and 18^{th} (Obinata et al. 2003).

3.6.1 Elicitation with salicylic acid (SA)

In order to examine the effect of salicylic acid on anthocyanin pigments accumulation in the cell suspension, aliquots 5.0ml of cell suspension were transferred to nine flasks which contained different media (Figure 3.4). The salicylic acid solution was filtered under aseptic condition by using 0.4µ syringe filter. The nine flasks were incubated on orbital shaker under dark condition at 29 ±1 °C for 14 days. On the day 14^{th}, the salicylic acid was added at two different concentrations as illustrated in figure (3.4). The detection of anthocyanin pigments and growth of cells suspension were determined within 48 hours and 96 hours from the SA addition to compare the effect of salicylic acid on the *in vitro* cells suspension culture.

3.7 Detection of anthocyanin pigment

Anthocyanin pigments were extracted by using acidified methanol 1% HCl (v/v). Additionally, the pigments were detected by using UV-VIS (SPECTROLINE MODEL ENF-240C/FE) from 700 nm to 200 nm.

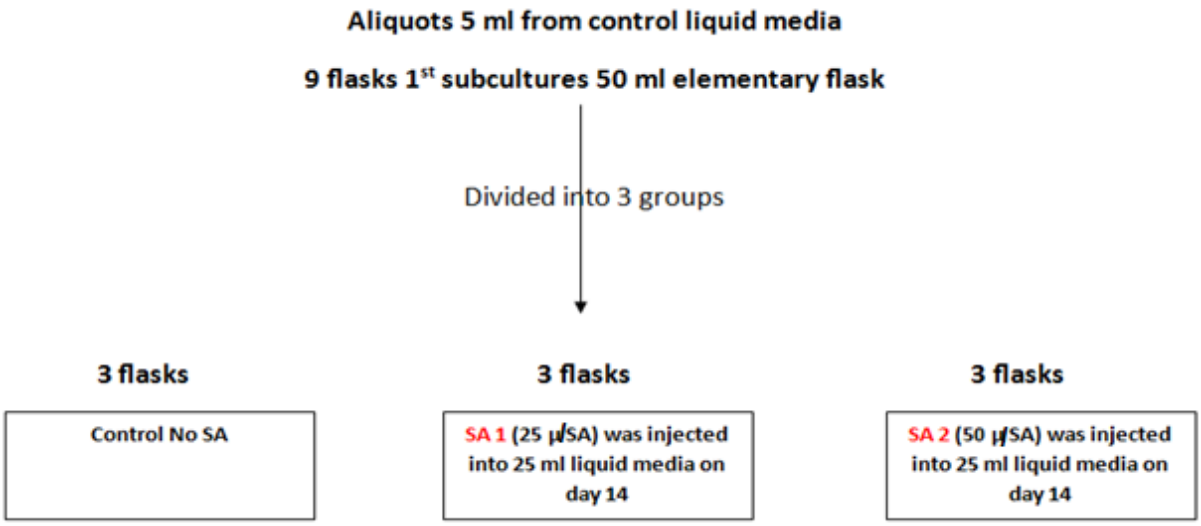


Fig 3.4 The effect of different concentrations of SA on anthocyanins production in cell suspension culture

3.7.1 Anthocyanin pigments extraction procedure

Five grams of callus fresh weight were used from 14 different treatments which described in (Table 3.1). Each 5.0 g callus was immersed in 25 ml of acidified methanol solution with 1% HCl in 50.0 ml falcon tube. The extraction was carried out on 24 hours in dark condition at 4°C. After that, the extracted solution was centrifuged for 20 minutes at 2800 rpm to separate the supernatant then the supernatant was dried under sterile condition. The yield was calculated by using the equation:

Yield = Pigment weight after extraction / weight of fresh callus * 100%

Equation 3.1.

3.7.2 UV spectrophotometric analysis

To confirm the presence of anthocyanin pigments a screening was carried out using UV- VIS spectrophotometer from 700-200nm then the curve was compared with the spectrum of a standard anthocyanin spectrum of strawberry fruit extract.

3.7.3 Thin Layer Chromatography (TLC)

The sample extracts were separated by Thin Layer Chromatography (TLC) on prepared silica gel plates of DC Fertigofoline ALUGRAM (REF# 818-133) with different elution buffers. 50.0µl of supernatant extracted samples were spotted on silica plates and air dried. Two different elution buffers were used; 1) butanol: acetic acid: water used at 25: 5: 10, and 2) ethyl acetate: acetic acid: formic acid: water used at 50: 5.5: 5.5: 13. The anthocyanin compounds were detected visually and by using UV light detector at 365 nm. R_f value of anthocyanins were measured by using the ratio referred to equation (3.2). The two bands were scraped and re-dissolved in methanol 1.0% HCl to measure the absorbance of each band at 520 nm and 700 nm to confirm the concentration.

R_f = distance of the spot moves / distance of the solvent moves

Equation (3.2)

3.7.4 Determination of total anthocyanin concentration

The pH differential method was used to determine the total anthocyanin concentration (TAC) as reported in Giusti and Wrolstad (2001). Each 2.0 ml supernatant were adjusted by (HCl or KOH) to reach pH 1.0. The absorbance was taken at the two different wavelengths (520 nm and 700). After that, 2.0 ml of supernatant were evaporated under sterile condition. The dried pigments were re-dissolved in acetate buffer (sodium monohydrate + distilled water + glacial acetic acid) that was adjusted at pH 4.5. The absorbance was measured at two wavelengths (520 nm and 700 nm). The (TAC) was calculated according to the equation (3.3) that adapted from Fuleki and Francis (1968).

$A = (A_{520} - A_{700})_{pH\ 1.0} - (A_{520} - A_{700})_{pH\ 4.5}$

Monomeric anthocyanin pigment (mg/l) = (A * MW * DF*1000)/ (ε *1)

Where, MW = 449.2 for unknown anthocyanin used the MW for cyanidin-3-glycoside
DF = 1, ε= 26900,

Equation (3.3)

3.8 Statistical analysis

For callus maintenance part, completely randomized design (CRD) was used to arrange all treatments in all experiments. Data in each experiment was analyzed with the analysis of variance (ANOVA) using SPSS version 20.0. On the other hand, elicitation experiment, standard error was calculated in each time the sample was taken. For more than one factors change the data was analyzed with t-test analysis using Microsoft Excel at p=0.05.

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Chapter 4

RESULTS AND DISCUSSION

In summary, the main objective of this study is to explore the *in vitro* culture of *A. andrachne* for secondary metabolites production, particularly anthocyanin pigments. The specific objectives are (1) studying the effect of different chemical constituents like sugars, basal salts, and gelling agent on callus maintenance, callus growth and total anthocyanin concentration (TAC). (2) Induction of cell suspension culture with best growth curve and highest anthocyanin pigments concentration.

4.1 Seed germination

The surface sterilized seeds gave 84% with 6% of contamination and 10% of abnormal seedlings. This result is quite similar to the result recorded by Aljabari (2011).

4.2 Callus induction and maintenance

After four weeks from germination on MCc media, cotyledonary segments were subcultured on B5 media supplemented with 1.0 mg/l 2,4-D and 0.1% w/v PVP. Explants were incubated in full dark conditions. After six weeks cotyledonary explants started to show compact callus clumps at their edges. Callus appeared with yellow color which is possibly be chalcone compound (fig 4.1 a). Callus was successfully transferred and maintained on MCc media supplemented with 2.0 mg/l TDZ, 0.05 mg/l NAA and 1.0 g/l PVP. These results agree with the results achieved by Aljabary (2011).

The callus have been grown in MCc media that supplemented with 2.0 mg/l TDZ, 0.05 mg/l NAA and 1.0 g/l PVP showed mixed color of green, yellow and red areas (fig 4.1.b). After few subcultures, the callus color tended to be pure dark red (fig 4.1 c).

Callus with reddish color started to appear after the day 16 from subculture. The callus color intensity became more reddish after the third subculture (fig 4.1 b, c). The red pigment was observed to be light dependent.

Regular callus subculture was performed with maximum of 25 days intervals to prevent tissue browning. By monitoring callus growth and total anthocyanin concentration (TAC) that accumulated within 32 days. The results showed that in the day 25th the highest TAC accumulation in callus was observed. For this reasons, a 25 days callus was taken to compare callus growth and TAC (fig 4.8).

According to (Figure 1.3) the specific step of anthocyanin biosynthesis was started with chalcone compound. Chalcone is an aromatic ketone with a yellow color; it is a mediate product before anthocyanin. Figure 4.1 emphasized that callus induction under dark condition has yellow color which related to chalcone compound, while callus maintained under light condition has red color which referred to anthocyanin pigments.

When induced callus are transferred to MCc media under light condition, light enhanced the expression of different genes that involves in anthocyanin biosynthesis pathway (Mola *et al.* 1996).

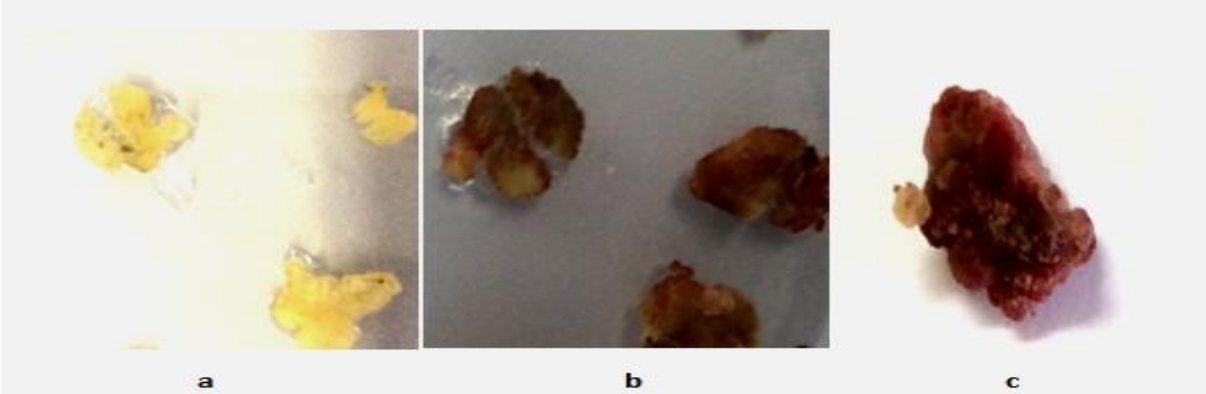


Fig 4.1: a) callus induction from cotyledonary explants on B5 media under dark condition after 6 weeks, b) callus maintained under light condition on MCc media on the day 16, and c) callus maintained after third subculture under light on MCc media on the day 25.

4.3 Testing callus growth and TAC with different treatments

These experiments investigated the influence of different chemical constituents of growth media on callus growth and TAC accumulation in callus after different treatments. These factors (treatments) were divided into three groups: testing the effect of different carbon sources, the effect of different basal media (salt), and the effect of different gelling agents. Each treatment was carried out at fixed conditions including: temperature, light, growth regulator as well as cultivation and incubation time. Figure (4.2) shows clearly the variation effects of changing the factors mentioned above. Changing the basal media influenced the

callus coloration, where using MS media gave greenish color, B5 gave mix of green and red, QLM gave dark green to brownish and MCc showed clear red color. Changing the gelling agent also affected callus coloration where using gelrite gave pale red color and the additive of starch to agar at 4.0 g/l resulted in deep red color. Changing the carbon source from the control (sucrose) to other sources (fructose and glucose) has a slight effect on callus coloration; however, changing the carbon source affected clearly the callus growth.

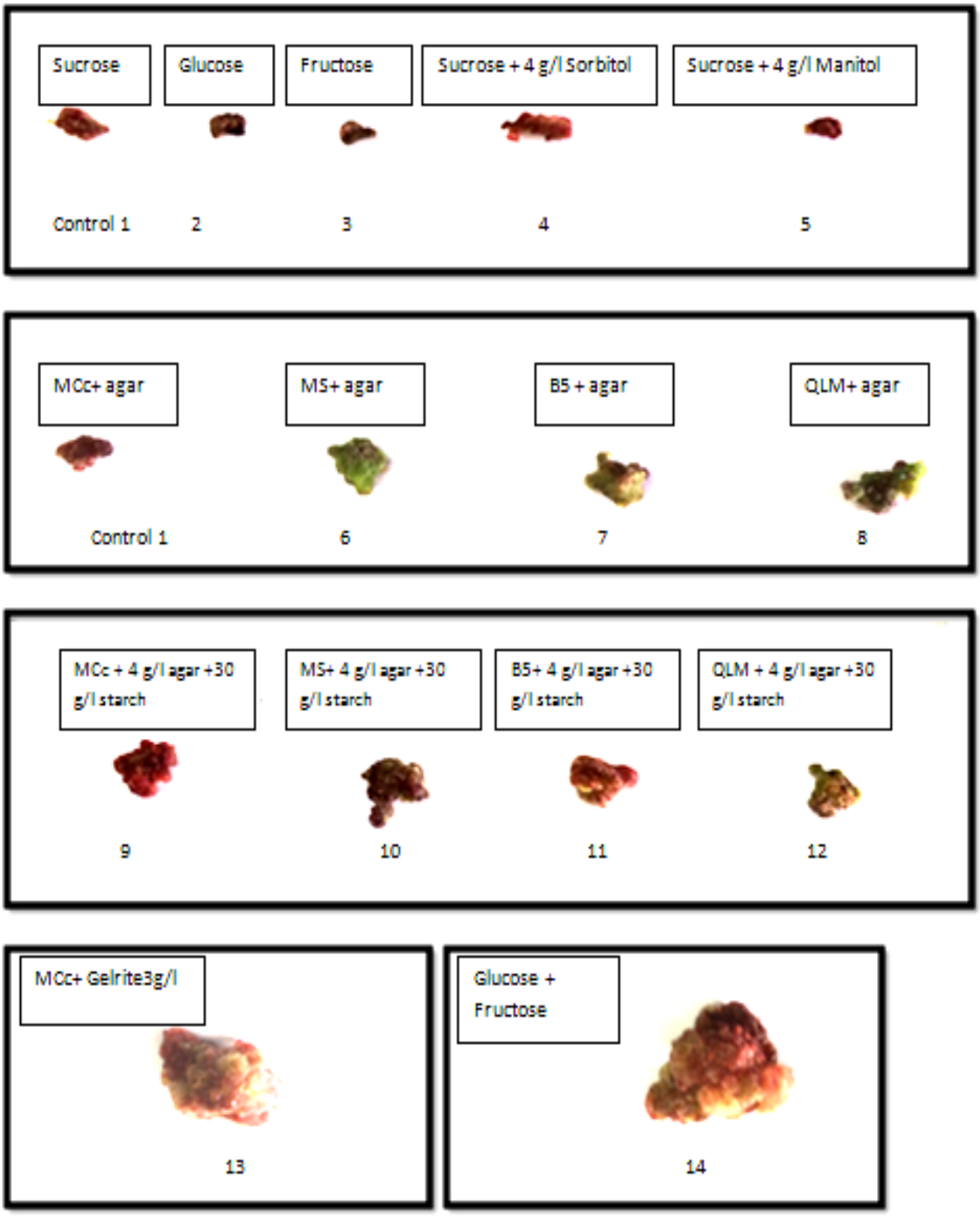


Fig 4.2: Callus maintenance after 25 days of on 14 different treatments.

The callus growth (fresh weight) on 14 different media combinations on the 25 day is presented in (fig 4.3). The callus fresh weight was detected from 3 replicates for each treatment at the same time. The highest significant effect that showed the best callus growth resulted from the synergistic effect between B5 basal media that was gelled with starch and agar. By comparing the compositions of 4 basal media listed in (Appendix Table 22), it was noticed that Gamborg B5 media contained the highest vitamin concentrations which include thiamin hydrochloride and pyridoxine hydrochloride then it is followed by MCc media. These two basal media showed the highest growth effect respectively, when these two media were gelled with starch and agar. On the other hand, QLM media showed the highest affect on growth without starch it contained the highest concentration of different salt without sodium chloride. On contrast, the highest significant effect that showed the lowest callus growth was observed when glucose was used as a source of carbon instead of sucrose.

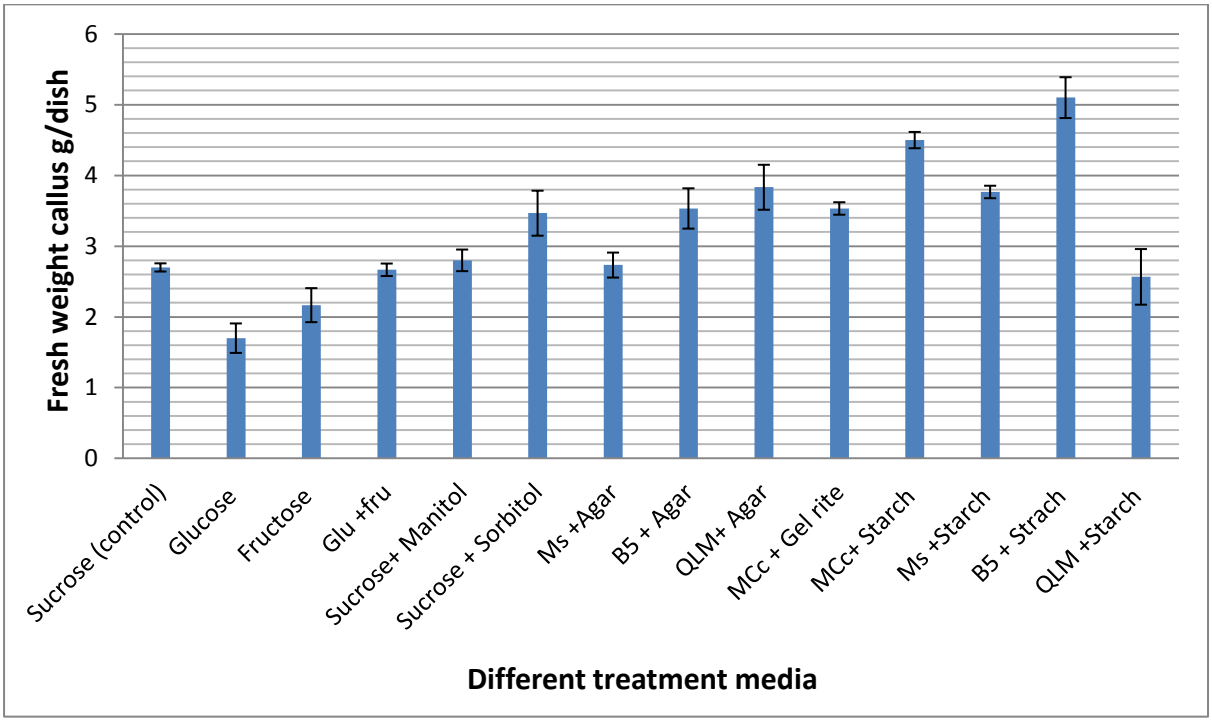


Fig 4.3: Callus fresh weight of *A. andrachne* on 14 different treatments where the sources of carbon, basal media salt, or the gelling agent were changed after 25 days of culture. Each value represents the mean of 3 replicates; vertical lines represent standard error of the mean.

The clear supernatant from callus has red color with different intensities accordingly with the treatments (Fig 4.4). The extraction yield percentage from different treatments was calculated by using Eq (3.1).

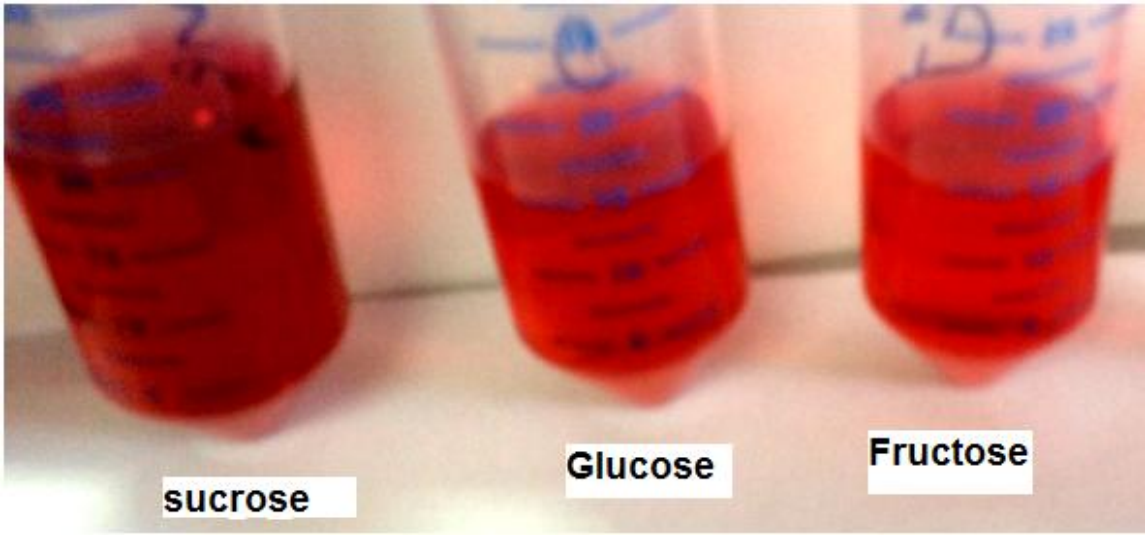


Fig 4.4: Anthocyanin pigment show different intensity of red color according to the media. Media with sucrose shows dark red color, while glucose and fructose resulted in the pale red color.

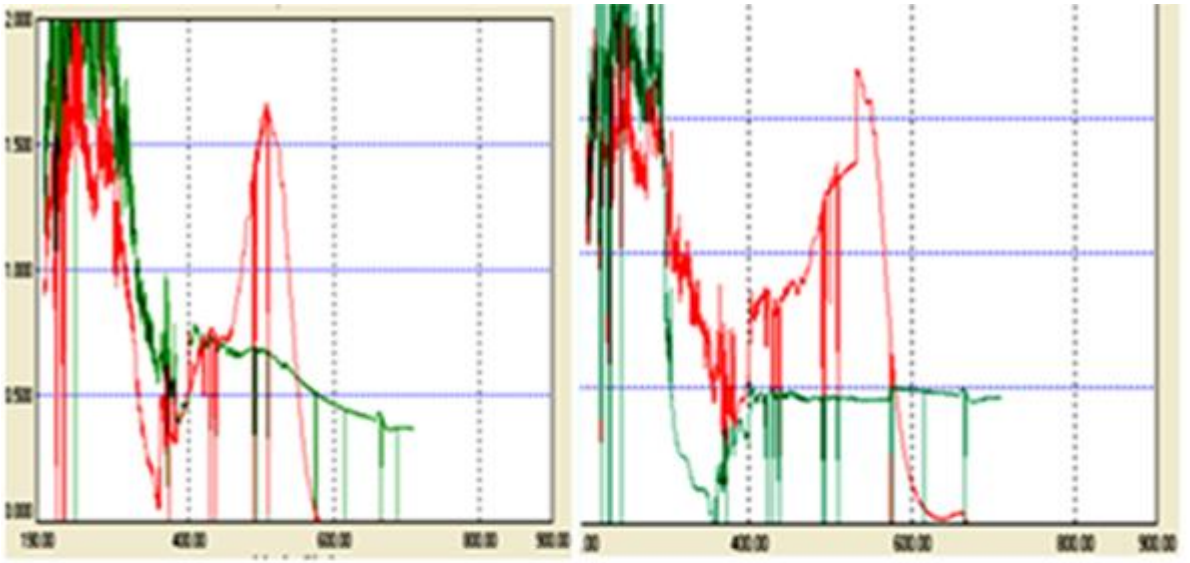
The extract yield was represented in (table 4.1). The highest extract yield does not mean highest anthocyanin concentration due to the presence of other flavonoids and carotenoids in callus extracts. It is known that anthocyanin is unstable compound which is affected with changing pH.

Table 4.1: Total extracts yield percentage from callus of *A. andrachne* from 14 different treatments after 25 day. Five grams fresh callus was dissolved in 25.0 ml acidified methanol 1% HCl (v/v).

Treatment	Yield%
Control	1.025%
Glucose	0.95%
Fructose	1.344%
Glu+Fru	0.785%
Sucrose+ 4.0 g/l sorbitol	1.201%
Sucrose + 4.0 g/l manitol	1.4%
MS + Agar	1.073%

B5 + Agar	1.009%
QLM + Agar	1.177%
MCc + gelrite	0.895%
MCc + Starch	1.111%
MS + Starch	1.063%
B 5 + Starch	0.843%
QLM + starch	1.1115%

The anthocyanin spectra showed two distinctive absorption areas, one exists in the UV region at 200 to 280 and the second one exists in the visible region between 500 - 550 nm. Here, in this study the shape of spectrum that resulted from callus was compared with the spectrum of strawberry as standard anthocyanin compound (Lopes-da-Silva *et al*. 2002) (Fig 4.5). A comparison between the spectrum of callus extract from control media with the reference spectrum that illustrated in (Fig 4.6) according to Devi *et al*. (2011) showed that the spectrum of *A. andrachne* callus extracts is parallel to apigeninidin spectrum (Fig 4.6). Apigeninidin is a 3-deoxyanthocyanidins that is a derivative from pelargonidin (Costantino *et al*. 1995). The detailed of chemical structure need further studies to confirm the final anthocyanin compound.

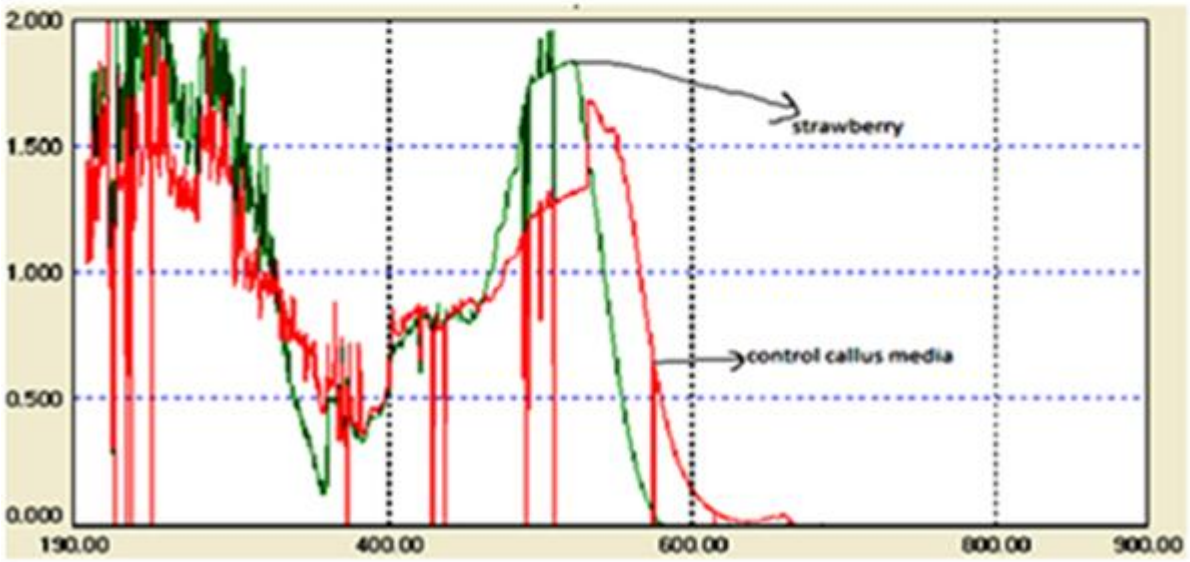


Strawberry anthocyanin spectrum

Red at pH1, Green at pH 4.5

MCc +3o g/l Sucrose +4 g/l agar (control media)

Red at pH 1, Green at pH 4.5



overlay of the two spectrum revealed from strwaberry (green), and callus of *A. andrachne* (red)

Fig 4.5: The spectrum of anthocyanin pigments were measured at 200-700nm. The highest peaks were found twice the first one at 500-550nm and the second one at 200-280nm. Comparison between the spectrum of callus of *A. andrachne* from control media and the spectrum of standard strawberry juice.

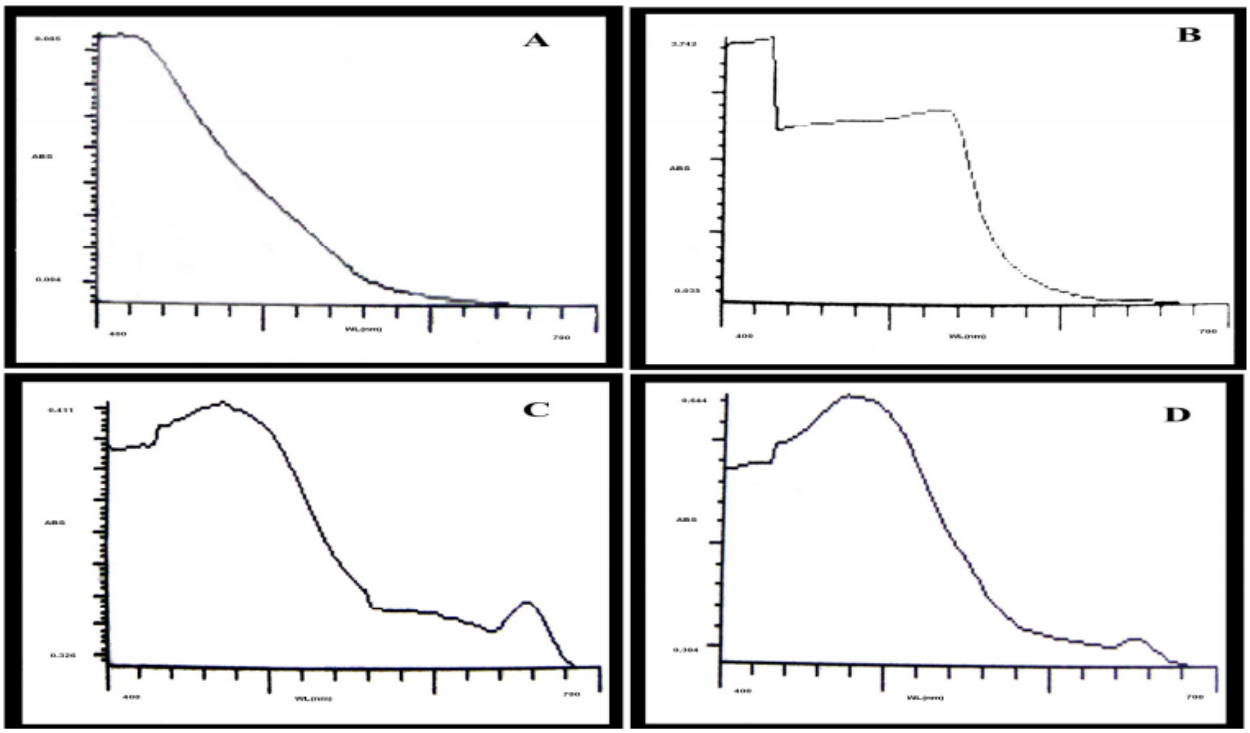


Fig 4.6 : Illustration of anthocynins stucture from red sorghum
A – Methanol extraction of Anthocyanin from husk of red sorghum
B – Acidified methanol extraction of Anthocyanin from husk of red sorghum
C - Luteolinidin
D – Apigeninidin
The figure adapted from Devi *et al*. (2011).

The total anthocyanin concentration (TAC) was calculated by using pH differential methods as described in (3.6.5). The concentration of anthocyanin pigment form control and different treatments is represented in (Fig 4.7).

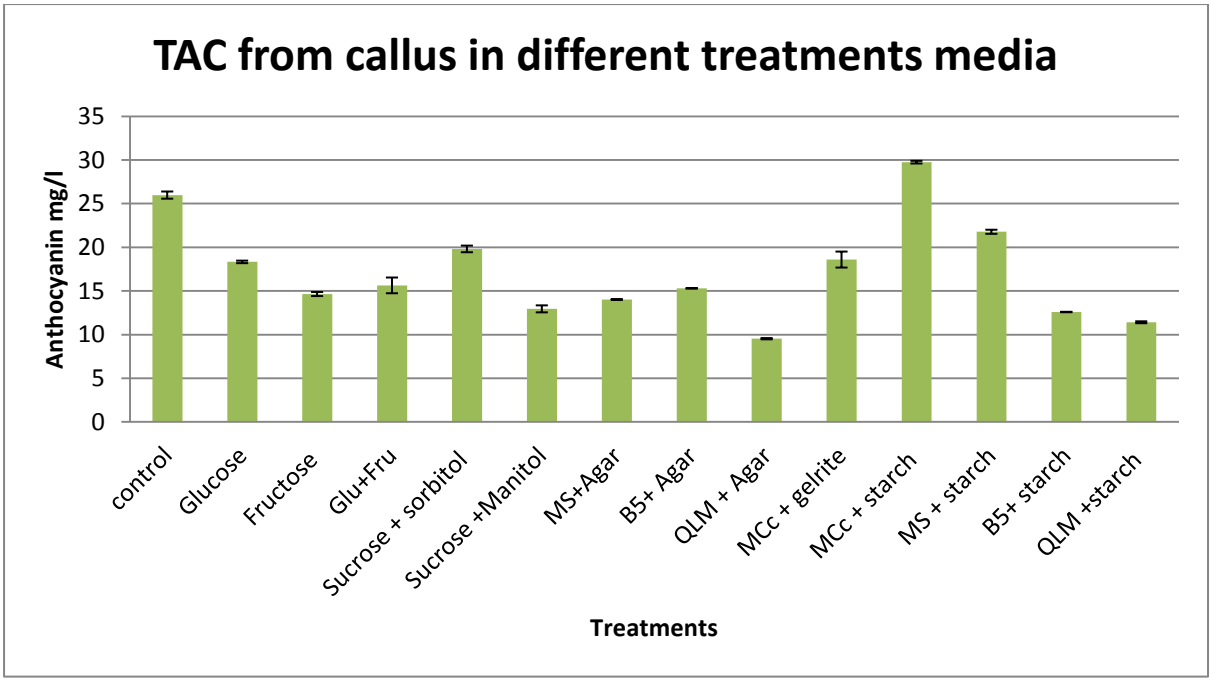


Fig 4.7: Total anthocyanin concentration (TAC) in mg/l from callus of *A. andrachne* after 25 days of culture on different media. Each value represents the mean of 3 replicates; vertical lines represent standard error of mean.

Different carbon sources and the nitrogen formula and concentration in the growth media play a major role in promoting or inhibiting the growth rate and the anthocyanins production (Deroles 2009). The TAC of *A. andrachne* from callus in the control media was 25.98 mg/l. The addition of starch (corn starch) in the presence of agar as gelling agents increased the TAC to 29.75 mg/l. Generally, starch is a polysaccharide carbohydrate that used for gelling as well as biotic elicitor on anthocyanin production pathway (Henderson and Kinnersley 1988, Vinterhalter *et al*. 2007). On contrast, the effect of using QLM media instead of MCc decreased the TAC to 9.64 mg/l. QLM contains the highest concentration amount of nitrate salts as mentioned in (Appendix 22.1) however, the nitric oxide is the end products of nitrate salts in cell cytoplasm (Lundberg and Govoni 2004) so it plays as abiotic factor which decreased the production of anthocyanin pigments (Qiao and Fan 2008).

4.3.1 The effect of carbon source (sugars) on callus growth and anthocyanin concentration

The effect of different types of sugars and the addition of an osmoticom on callus and/or anthocyanin production from cotyledonary callus of *A. andrachne* explants were investigated. The results showed that there is no correlation between the highest callus growth and the highest TAC accumulation (Fig 4.3- 4.7). Furthermore, the best callus growth was obtained

from MCc media that supplemented with sucrose and 4.0g/l sorbitol as well as media that supplemented with sucrose and 4.0g/l mannitol. This clearly shows that increasing the concentration of sugars by using alcoholic sugars increased the growth of callus. On the other hand, the TAC accumulation in callus decreased.

Results also show no significant effect on callus growth when using of sucrose alone instead of glucose + fructose. It is known that, sucrose is a disaccharide which is breakdown to give fructose and glucose. There is no significant effect between these two treatments on the growth of callus. On contrast, the highest TAC accumulation in callus from different sugar treatments came from sucrose (TAC = 26.02 mg/l). Using of glucose or fructose alone reduced the TAC to 18.33 mg/l and 14.65 mg/l respectively. Furthermore, this result agrees with other result obtained by Callebaut *et al*. (1990) in other plant, *Ajuga reptens* cell line.

4.3.2 The effect of gelling agent on callus growth and anthocyanin concentration

Three different gelling agents; agar, gelrite, and agar + starch have been used to test their effect on callus growth and TAC accumulation.

ANOVA showed high significant $p=0.000$ effect of gelling agent on callus growth within 25 days. MCc with 4.0g/l agar and 30.0g/l starch gave the highest effect, whereas gelrite gave the lowest effect on callus growth (Fig 4.3).

Starch is a polysaccharide that plays a role as a source of carbon to produce certain amount of secondary metabolites (Godoy-Hernàndez and Loola-Vargas 1997). Furthermore, the results showed that starch at 30.0g/l showed a significant effect to enhance the production of TAC in tissue (29.47 mg/l). These results are in agreement with a similar result obtained by Cai *et al*. (2012) on *Vitis vinifera* cell suspension cultures. They used different polysaccharide elicitors like chitosan, pectin and alginate and emphasize that polysaccharide significantly enhance the production of intracellular phenolic acid and anthocyanin production within cells.

4.3.3 The effect of basal media (salt) on callus growth and anthocyanin concentration

There are four different types of basal media have been used; MCc, MS, B5and QLM. They differ from each other in nitrogen formula and concentration of phosphate. Additionally, it has been approved the ratio of ammonium to nitrate in the growth media (salt) for plant tissue culture plays a marked effect on the anthocyanins production (Simoes *et al*. 2012). These different media types will affect the growth of callus fresh weight per treatment and the

accumulation of TAC within cell vacuole. The results showed that the MCc media showed the highest TAC accumulation. On the other hand, QLM media showed the highest effect on callus growth but decreased the TAC accumulation. QLM has the highest phosphate concentration 270mg/l, while MCc has an optimum level of nitrogen 786mg/l that came from two different salt ammonium nitrate and calcium nitrate (Appendix Table 22).

These results are comparable with the results achieved by Rajendran *et al*. (1992), which emphasize that the low phosphate concentration decreases callus growth and enhances anthocyanins production via nutrient starvation or osmoticom stress. Additionally, QLM contains a high level of nitrogen 2778.92mg/l which increases callus growth and suppresses the anthocyanins production. The results showed that the TAC from QLM treatment has the lowest significant value =9.64 mg/l. These results agree with the results according to Do and Cormier (1991) on *Vitis vinifera* cell suspensions culture they showed that the highest concentration of nitrogen source increases cell division and decreases anthocyanin accumulation.

4.4 The accumulation of anthocyanin in callus over time

Callus growth in (fig 4.3) showed no significant effect between sucrose and the synergistic effect of combining glucose and fructose. On the other hand, there is a significant effect on TAC accumulation between sucrose and the synergistic effect of combining glucose and fructose as illustrated on (fig 4.7). The TAC showed that sucrose on the day 25 showed the highest concentration of anthocyanin pigments. This section studied the TAC accumulation within 32 days from these different treatments (Fig 4.8). The figure shows that the synergistic effect of combining glucose and fructose gave the highest TAC on the day 16 and then it has decreased. On the other hand, the sucrose showed the highest TAC on day 25 and then it has been decreased. It was known that anthocyanin is unstable compound that produced at a specific growth phase of callus, so it reaches the peak of concentration at growth phase and then the anthocyanin has been degraded over time.

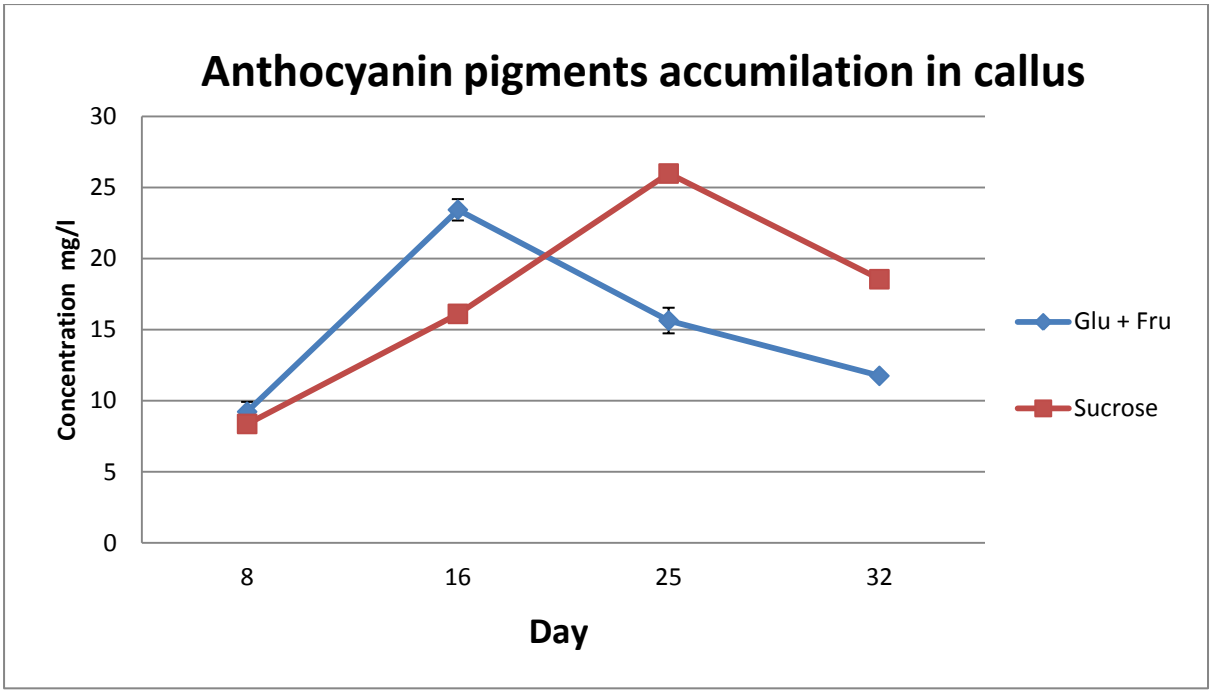


Fig 4.8 Anthocyanin accumulation in callus within 32 days on two different media

Fig 4.9 Callus growth and color from media contains Glucose + fructose after 25 days

4.5 TLC analysis of anthocyanin

The thin layer chromatography (TLC) is used to the separation and qualitative analysis of anthocyanins. The separation of total crude extract revealed on TLC plate is shown in (Fig 4.10-4.11). This section was carried out on two different systems of mobile phase:

System 1: the mobile phase composed of ethyl acetate: acetic acid: formic acid: water at 50: 5.5: 5.5: 13. As seen on (fig 4.10) plate "a" has two major bands visual one band with pale orange and the second with pale violet color. On the other hand, there is only one detected band under the UV light at 365 nm. This band refers to pale violet with R_f= 0.25-0.38. By comparing this result with the other references result for anthocyanin which illustrated in (Wagner and Bladt 1996), R_f = 0.2-0.4. From system 1 the second band is an anthocynin pigment which detected under at 365 nm UV light (Fig 4.10).

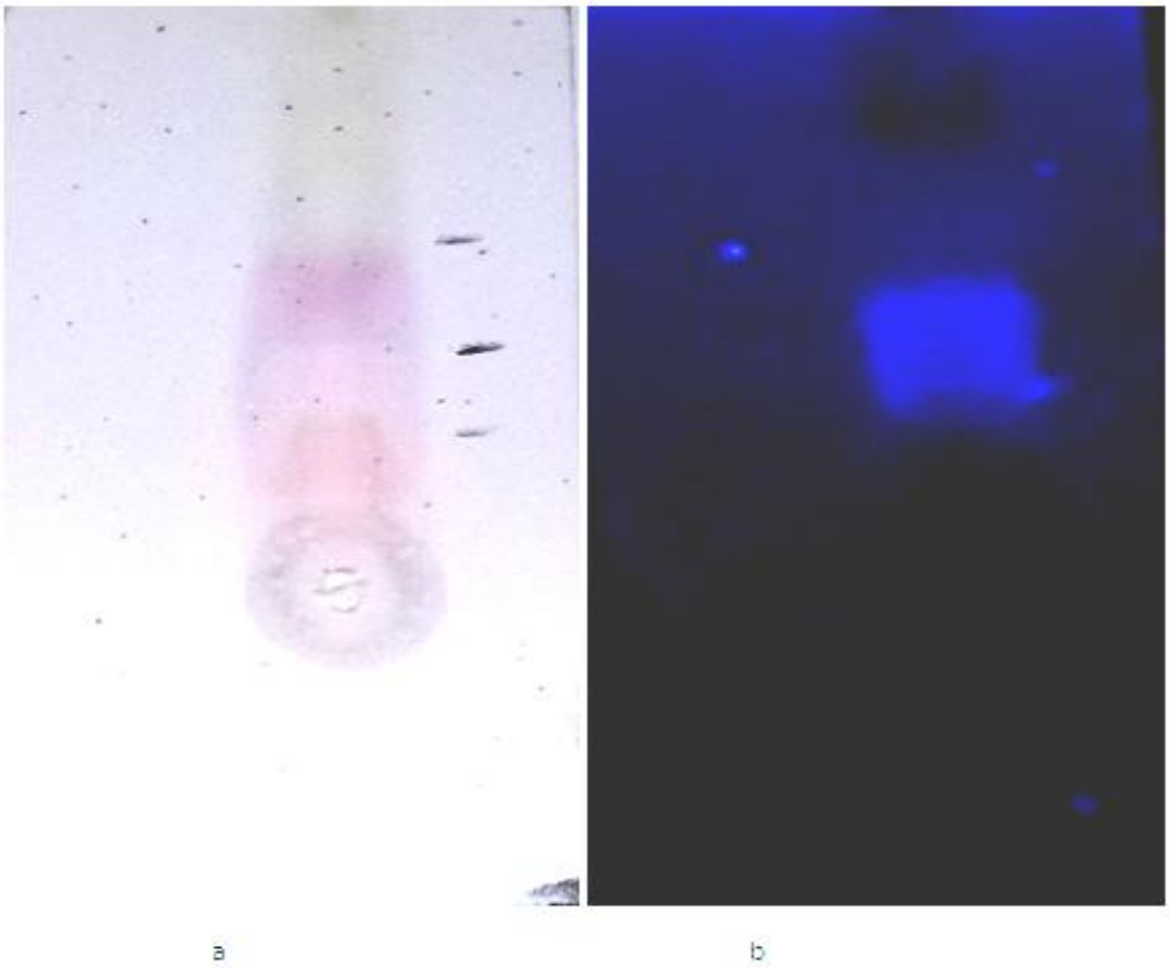


Fig 4.10: TLC plate spotted by acidified methanol extracts from *A. andrachne* callus from system 1 a) by visual evaluation there were two different pigments one was a pale orange and the second is pale violet. b) Under UV at 365 nm there is only one band can be observed.

System 2: the mobile phase composed of butanol: acetic acid: water at 25: 5: 10. As seen on (Fig 4.11) plate "a" it has two major bands visually the first one is pale orange and the second band is dark violet color. On the other hand, only one band was observed under UV light at 365 nm. This band refers to dark violet has the R_f= 0.46-0.58. By comparing this result with the other references result for anthocyanin which illustrated in (Wagner and Bladt 1996) R_f = 0.4-0.6.So, from system 2 the second band is an anthocynin pigment which detected under at 365 nm UV light (Fig 4.11).

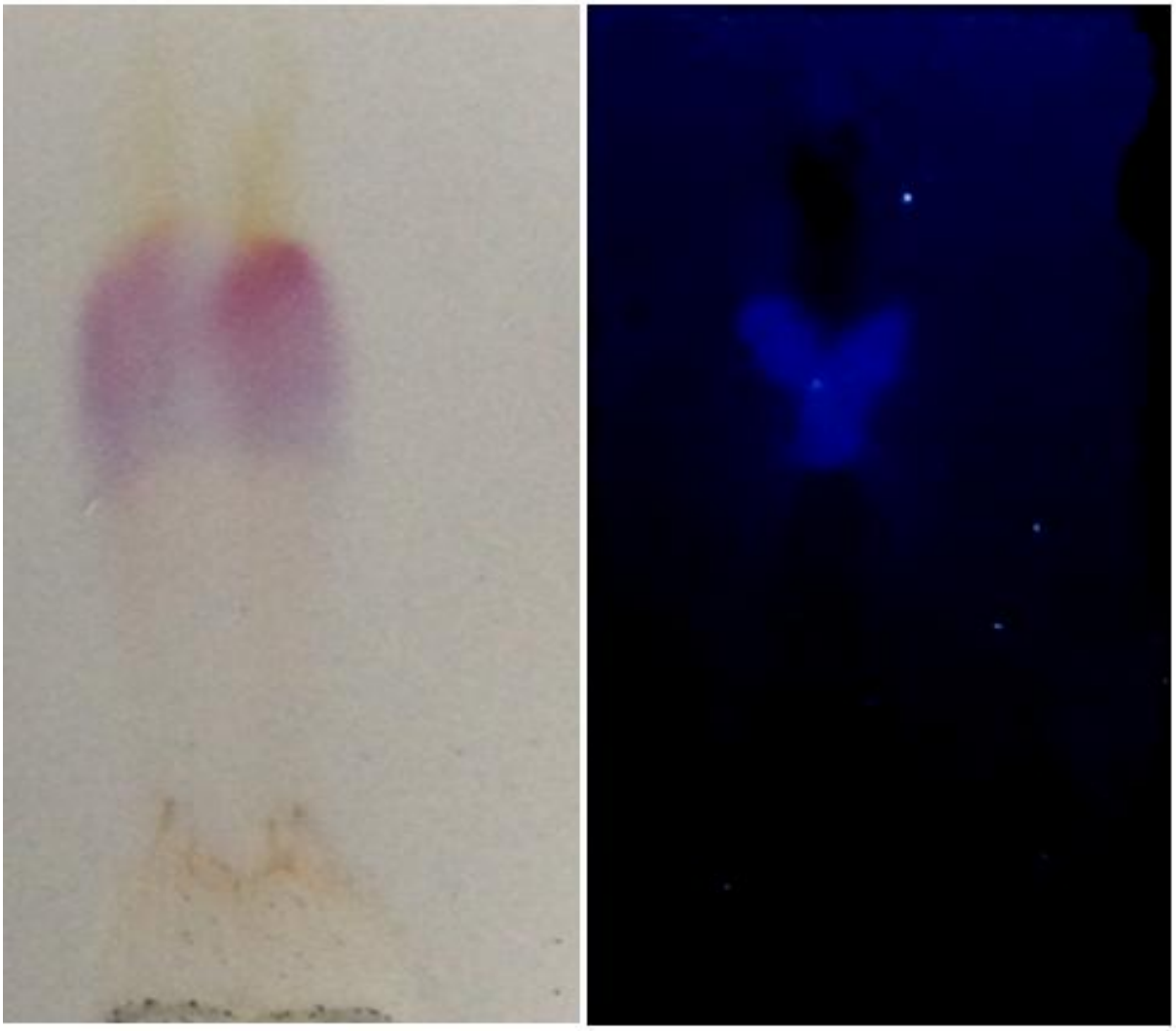


Fig 4.11: TLC plate spotted by acidified methanol extracts from *A. andrachne* callus from system 2. a) by visual evaluation there are two pigments one with pale orange and the second with dark violet. b) TLC under UV light at 365 nm where only one pigment can be observed.

These results showed that the second band from system 1 and 2 belongs to the anthocyanins compound.

4.6 Cell suspension culture

4.6.1 Initiation and determination of cell suspension culture from *Arbutus andrachne* L.

The homogenous cell suspension that produced from callus of *A. andrachne* have started to produce of anthocyanin pigments after 18 days of incubation on B5 liquid media with 0.1 mg/l NAA, 0.2 mg/l kinetin and 0.25 g/l casein hydrolysate. On the other hand, the

homogenous cell suspension that produced from callus in MCc liquid media supplemented with 2.0 mg/l TDZ and 0.5 mg/l did not produce anthocyanin pigments. It is well established in similar studies that organic, inorganic and PGRs components of the media play important role in the production and accumulation of secondary metabolites and anthocyanin pigments as well (He *et al*. 2010). The phytohormones that were used to produce anthocyanin pigments were differing between solid and liquid media (Narayan *et al*. 2005). Additionally, PGRs controlled the rate of growth and the level of differentiation of cell types. For that, the low concentration of auxin and cytokinin decreased the growth rate and enhanced the production of anthocyanin pigments at the cells level (Deroles 2009).

According to Appendix (22.4) Gamborg B5 media contained pyridoxine hydrochloride vitamin B6 which is an essential cofactor that used to synthesis different amino acid. In addition to, casein hydrolysate is protein, so it can play as a source of phenylalanine amino acid to synthesis anthocyanin pigments. This synergistic effect between media B5 and casein hydrolysate induce anthocyanin pigments from cell suspension culture of *A. andrachne*.

This experiment has studied the effect of different conditions on the growth of cell suspension culture and the production of anthocyanin pigments within the time on different osmotic stress at fixed factors like basal media, and PGR. Figure (4.12) presents the anthocyanin pigments that have been produced with different osmotic stress under different conditions. The results showed that the anthocyanin pigment was observed on sucrose with 2.0g/l sorbitol under dark condition on the day 12, while the other two media, sucrose alone, and sucrose with 2.0 g/l mannitol showed the colored pigments on the day 16 (Fig 4.12). On contrast the pigment was observed under light condition on the day 20 for all different media (fig 4.12). The highest peak of growth for induction cell suspension in all treatment was started on the day 24 (Fig 4.13). The highest TAC was observed on the day 32 under the two conditions in the control media which contained 30 g/l sucrose alone (Fig 4.14).

1) The effect of light and temperature on cell suspension growth and TAC accumulation

Temperature is one of the main factors that affecting the anthocyanin pigment accumulation in plant cell and tissue culture (Chan *et al*. 2010). Additionally, temperature showed a significant effect on the expression of anthocyanin genes (Muriithi 2009). According to Shvarts *et al*. (1997) the low temperature enhanced both the accumulation of anthocyanin pigments and chalcone synthase (CHS) gene expression in petunia plant cell culture.

It is known that light plays an important role that stimulates signal transduction and gene expression which involved in the anthocyanin biosynthesis (Mola *et al*. 1996). The results in this study came from two different conditions which combined the two factors together first is light at 24±1 °C, second is dark at 29±1 °C. Figure (4.13) showed that there is significant effect on the growth curve of cell suspension under different conditions. The best growth curve will determine under light condition with 24±1 °C. Furthermore, (Fig 4.14-15) shows the effect of these two conditions on TAC over time. The highest TAC was observed under both conditions on the day 32 with no significant effect when 30 g/l sucrose used in the media. On the other hand, the light condition at 24±1 °C showed the highest significant effect on TAC accumulation in days 20 and 24.

2) The effect of osmotic stress via alcoholic sugar on cell suspension growth and TAC accumulation

This experiment used sorbitol and mannitol respectively at 2.0 g/l concentration with 30 g/l sucrose under two different conditions. First, alcoholic sugars will increase the osmotic stress and then affect on the anthocyanin pigment biosynthesis. Figures 4.12-14-15 show that sorbitol enhanced the production of anthocyanin pigment under both conditions after two weeks of culture. Additionally, the highest peak of TAC accumulation within cell via osmotic stress appeared on the day 20 under light condition with 24±1 °C at 2.0 g/l sorbitol in addition to 30g/l sucrose. On contrast, the osmotic stress via mannitol 2.0g/l in addition to 30 g/l sucrose inhibited the production of anthocyanins. Figures 4.12-14-15 show traces of anthocyanin pigments or absence of it.

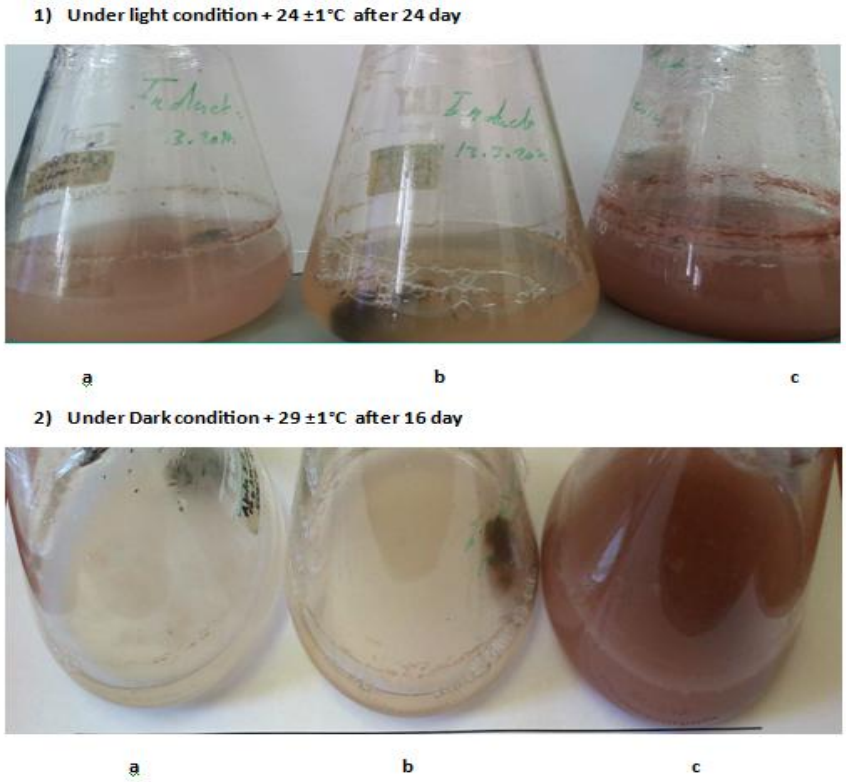


Fig 4.12: Cell suspension culture in different media under two conditions light and dark a) is control media B5+ sucrose, b) is B5 media+ Sucrose + 2.0 g/l manitol and c) is B5 media + sucrose + 2.0 g/l sorbitol.

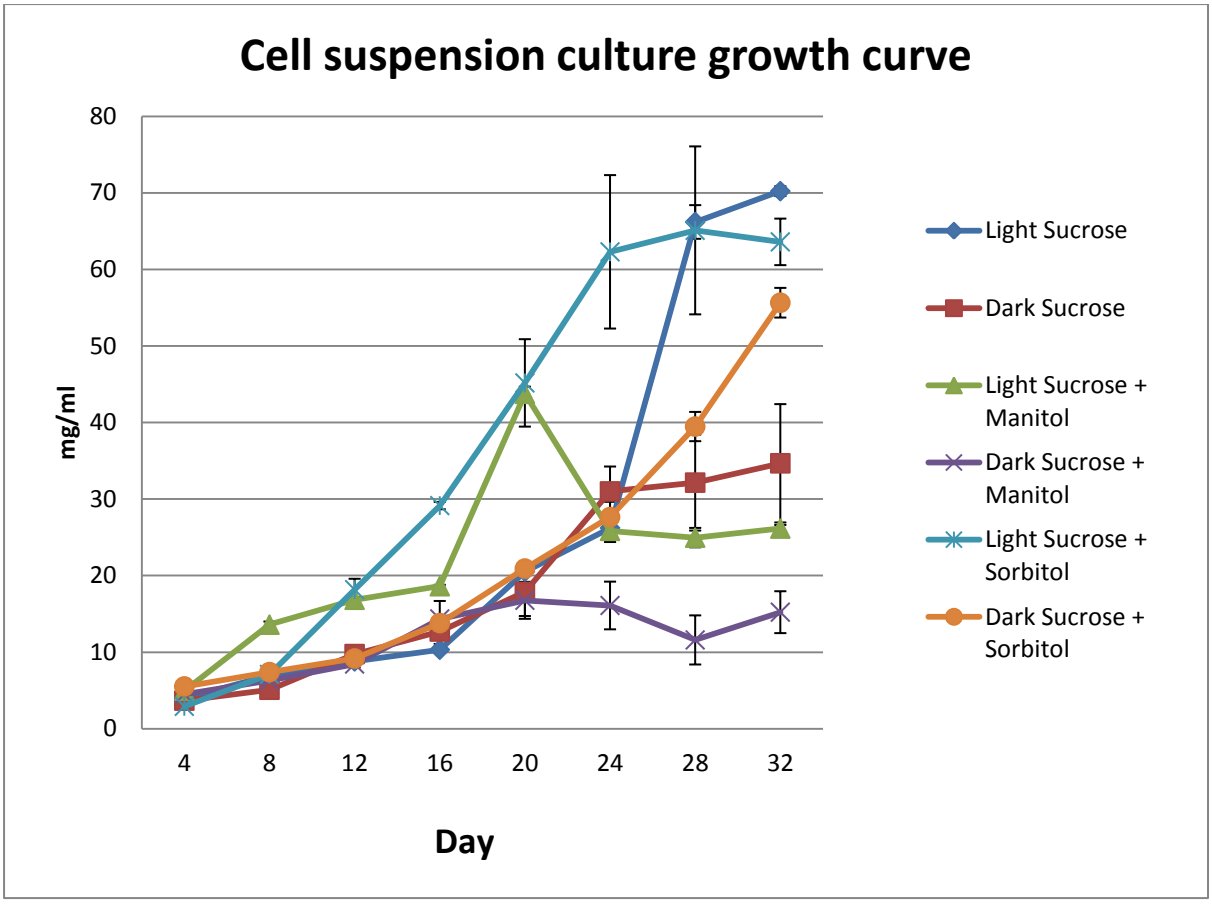


Fig 4.13: Growth curve of cell suspension culture with different osmoticoms (alcoholic sugars, sorbitol or mannitol) under two different conditions: 1) Light + 24±1 °C, 2) Dark + 29±1 °C within 32 days. Points on the curve represent values of the mean of six replicates; vertical lines represent standard error of mean.

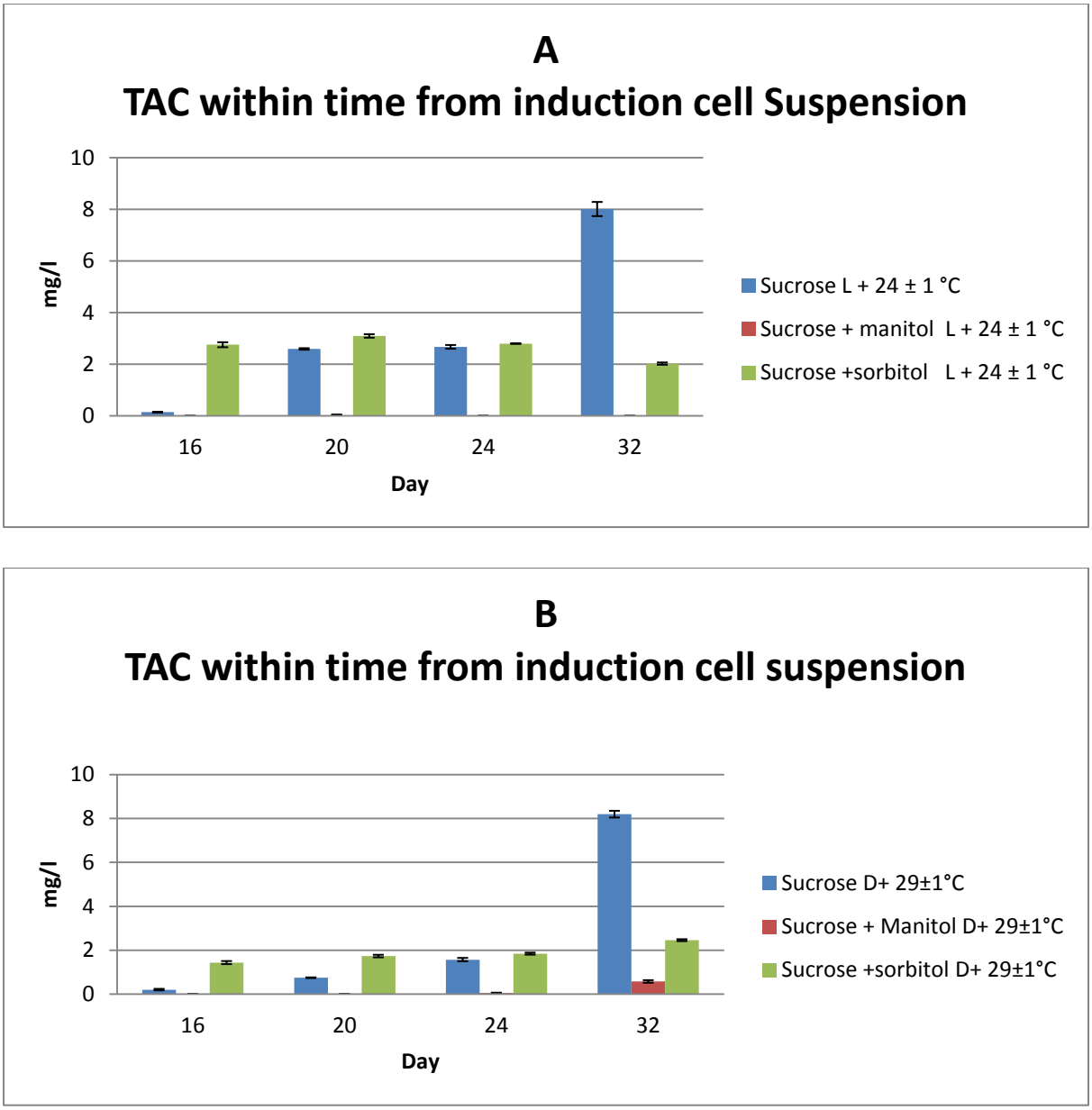
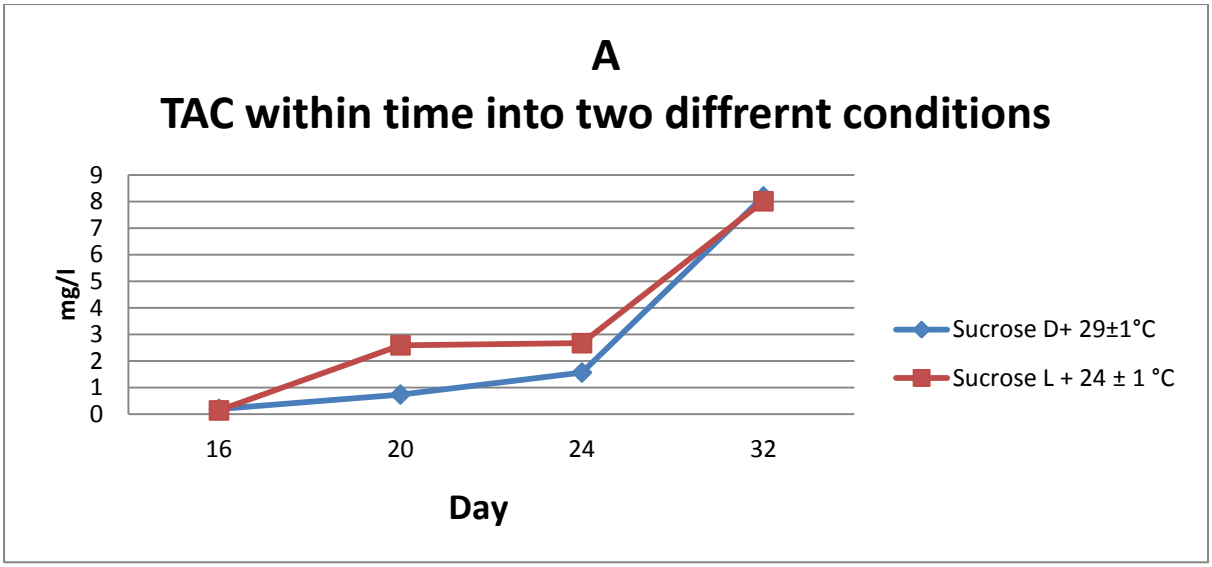
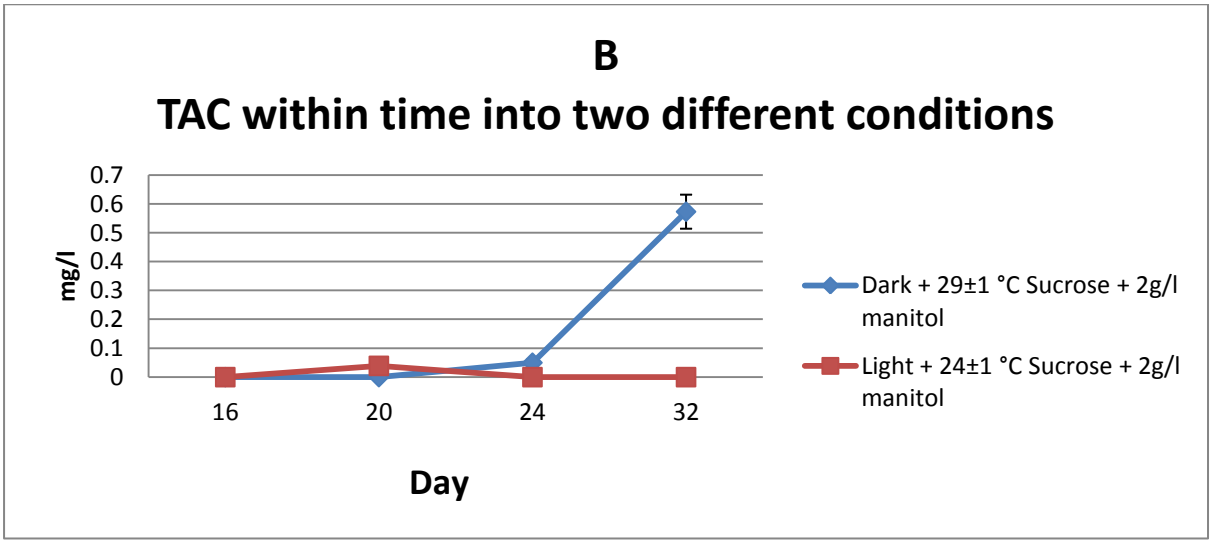


Fig 4.14: TAC in cells across time from different osmotic stress treatments by using alcoholic sugars under two conditions A) light + 24 ±1 °C temperature, B) Dark + 29±1 °C temperature. Each value represents the mean of 3 replicates; vertical lines .represent standard error in each replicates.


A
TAC within time into two diffrernt conditions
mg/l
9
8
7
6
5
4
3
2
1
0
16
20
24
32
Day
Sucrose D+ 29±1°C
Sucrose L + 24 ± 1 °C


B
TAC within time into two different conditions
mg/l
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
16
20
24
32
Day
Dark + 29±1 °C Sucrose + 2g/l manitol
Light + 24±1 °C Sucrose + 2g/l manitol

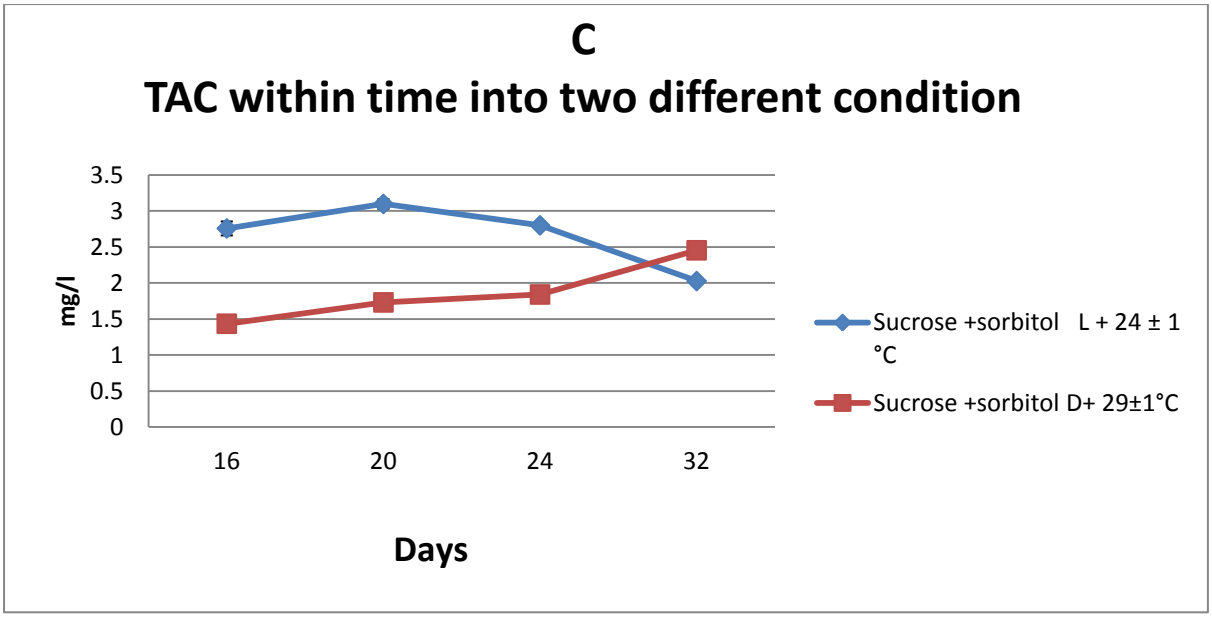


Fig 4.15: The effect of two different conditions on the TAC accumulation in cell suspension culture across time into three different treatments a, b and c.

Results showed that sorbitol enhanced the production of anthocyanin in an early stage, while mannitol inhibited the production of anthocyanin pigments. It is known that both sorbitol and mannitol are alcoholic sugars theoretically they increase the concentrations of reactive oxygen species, and decreased concentrations of nitric oxide and glutathione that may exert abiotic stress on plant *in vitro* and enhanced anthocyanin production at early stage. However, sorbitol and mannitol showed contraindication in results that mean the putative metabolic pathway of these two sugars differs from each other (Marino *et al*. 2003). Although, different enzymes are involved in putative pathway biosynthesis of sorbitol and mannitol (Marino *et al*. 2003) have stimulated different gene expression which may increase or decrease anthocyanin pigments accumulation in plant cell.

4.6.2 Anthocyanin pigment extraction from induced cell suspension culture of *A. andrachne*

The cell culture of *A. andrachne* was precipitated after centrifuge as presented in (Fig 4.16 a). The anthocyanin pigment was extracted by using 1.0% v/v acidified methanol. The clear supernatant is shown in (Fig 4.16 b). The yield extract from different induction cells under different conditions are listed on (Table 4.2). The highest extract yield was obtained from

sucrose under light condition. However, this yield did not represent the anthocyanin pigments alone and it may contain the other phenolic compounds.

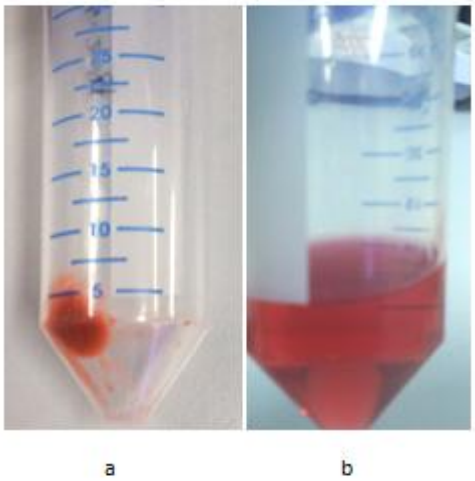


Fig 4.16: a) Cell precipitate after centrifuge and discarded supernatant media.
b) Supernatant clear extract by acidified methanol from cell on day 32.

Table 4.2: Percentage extract yield of cell suspension when 100 mg were dissolved in 3.0 ml of acidified methanol 1%HCl on day 32.

Suspension culture system	Extraction yield
Control: sucrose alone under light+ 24 ±1°C	8.83%
Induction cell suspension Sucrose alone under Dark+ 29 ±1°C	6.6%
Induction cell suspension Sucrose+ 2.0 g/l sorbitol under Dark+ 29 ±1°C	1.43%
Induction cell suspension Sucrose+ 2.0 g/l sorbitol under Light + 24 ±1°C	3.0%
Induction cell suspension Sucrose + 2.0 g/l manitol under Dark+ 29 ±1°C	2.3%
Induction cell suspension Sucrose + 2.0 g/l manitol under Light+ 24 ±1°C	5.1%

4.6.3 The growth curve of first and second subcultures cell suspension of *A. andrachne*

The curve in (Fig 4.17) shows the highest peak of cell suspension growth to find the optimum time for adding the elicitor. Figure 4.17 shows that the suitable time to add elicitors is between the days 12 to 16.

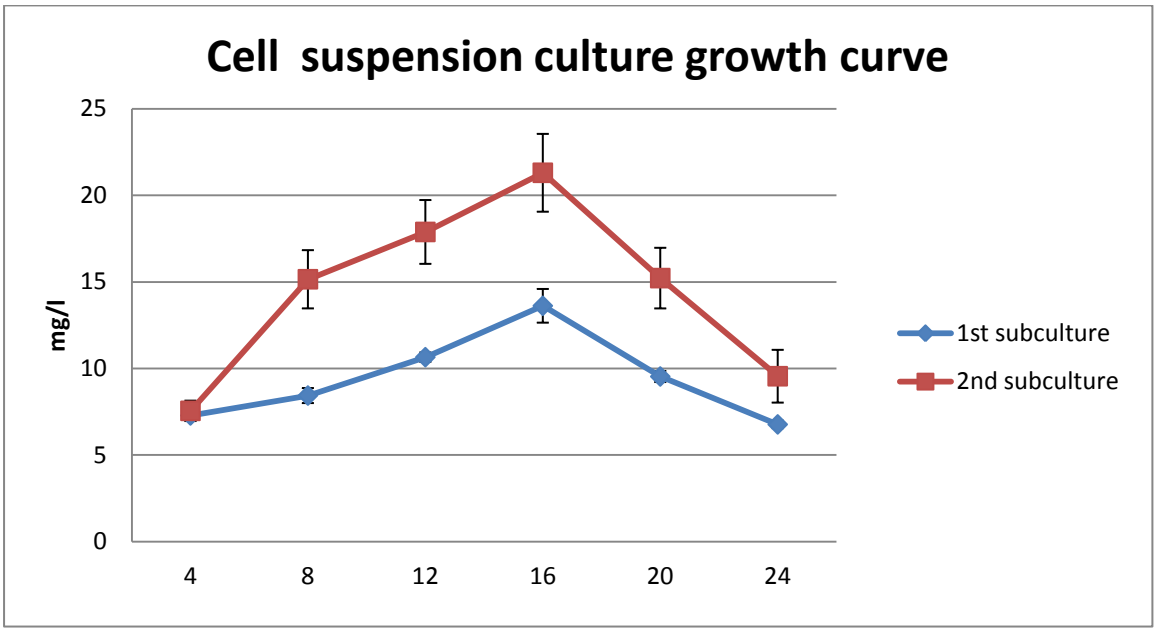


Fig 4.17: Growth curve of first and second subcultures of cell suspension from control media without alcoholic sugar within 24 day. Each value represents the mean of 6 replicates; vertical lines represent standard of mean.

4.6.4 The effect of different sorbitol concentrations on cell growth and TAC accumulation

Results from section (4.7.2) showed that sorbitol enhanced the production of anthocyanins at early stage. This section will determine the best concentration of sorbitol that enhanced the production of anthocyaninis at early stage on first subculture cell suspension of *A. andrachne*. Figure 4.18 shows that there is a difference in pigment intensity of anthocyanin from different concentrations of sorbitol. However, sorbitol is a non nutritional carbohydrate and is used to create osmotic stress and to increase anthocyanin pigments production. Figure 4.19 and 4.20 show the effect of different sorbitol concentrations on the cell suspension growth and the TAC accumulation over time. These results are in agreement with the results that adapted by Vinterhalter *et al*. (2007) that showed sorbitol (alcoholic sugar) as a significant osmotic stress that enhanced anthocyanin production in cell suspension culture. Furthermore, the highest peak of TAC accumulation was obtained on day 20 at 8.0 g/l sorbitol + 30 g/l sucrose. Moreover, there is a significant different between different concentrations of sorbitol

and the accumulation of TAC over time. The 8.0g/l sorbitol gives the highest TAC on the day 20 then; the TAC descends on the day 24.

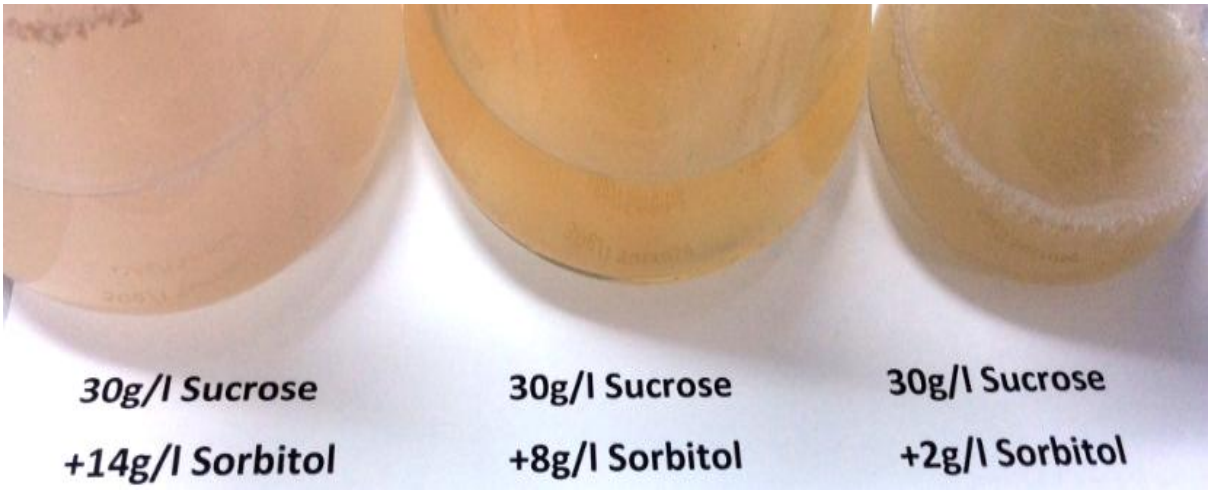


Fig 4.18: Anthocyanin pigments induction under different osmotic stress via different concentration of sorbitol in the first subculture cell suspension.

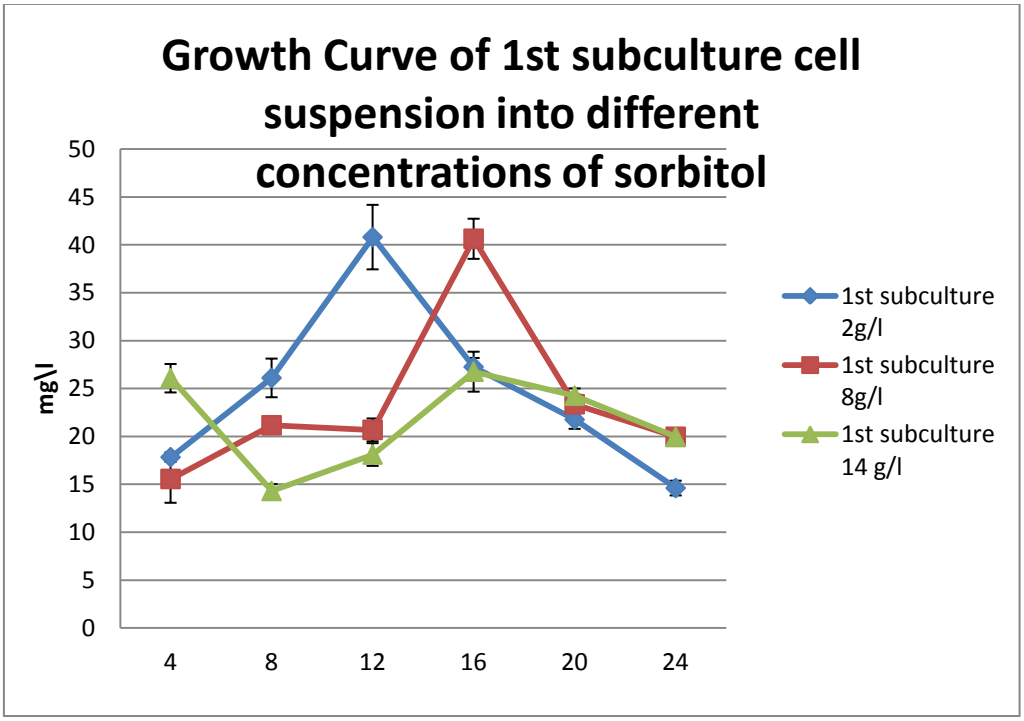


Fig 4.19: The effect of different concentrations of sorbitol (alcoholic sugar) into the first subculture cell suspension growth curve within 24 days under light condition with 24±1 °C. Each value represents the mean of 4 replicates; vertical lines represent standard error in each replicates.

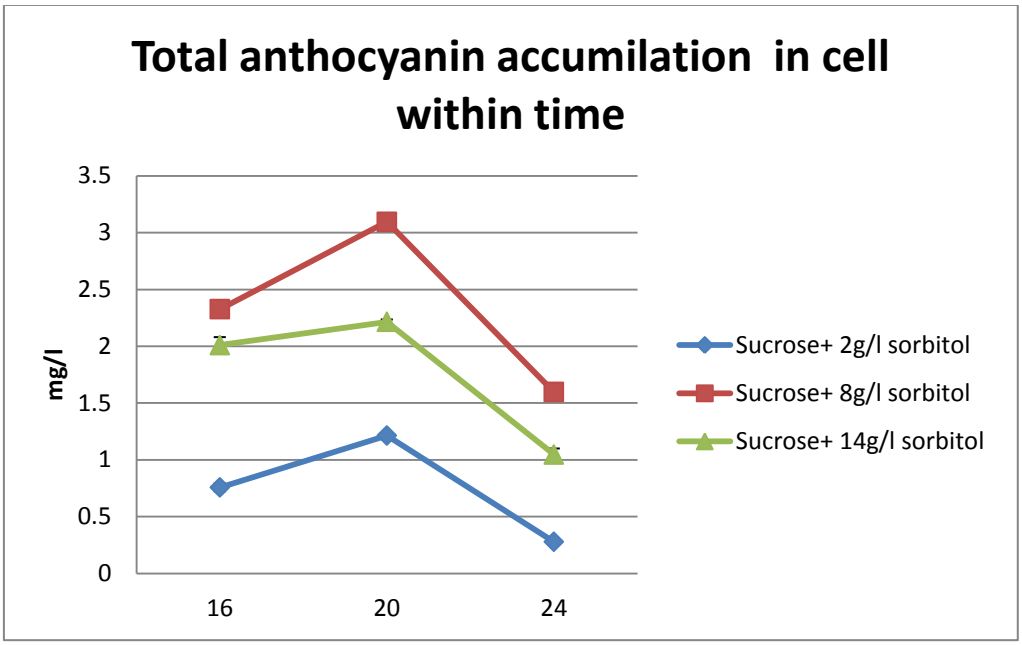


Fig 4.20: TAC accumulation in first subculture cell suspension within time into three different concentration of sorbitol (alcoholic sugar) under light condition at 24±1°C.

4.6.5 The effect of different media pH(s) on the growth of cell and TAC accumulation

The media pH is one of the physical parameters that can affect the cell growth and the production of anthocyanin pigments (Chan *et al*. 2010). Figure 4.21 showed that the alkaline pH= 8.0 changed the color of anthocyanin pigments to a pale orange color. The acidic media with pH =3.0 decreased the growth cell suspension and showed a trace amount of anthocyanin pigments on the day 20. The treatment with media pH= 5.8 showed the highest significant TAC accumulation on the day 24th, while the pH=8.0 showed the highest significant accumulation TAC on the day 20th.

Furthermore, these results showed a significant effect on cell growth at different media pH(s) and anthocyanin pigments accumulation in vacuole of epidermal cells. Although, these results did not comply with the results that adapted from Chan *et al*. (2010) on *Melastoma malabathricum* cell suspension culture which showed no significant effect after changing media pH on anthocyanins production and cell growth. Basically, the main reason that different came from is the use of variable different range of pH(s). The pH range in the

media according to Chan *et al*. (2010) was 4.25, 4.75, 5.25, 5.75, 6.25 and 6.75, while the range of pH(s) in this study was wider as illustration in section (3).

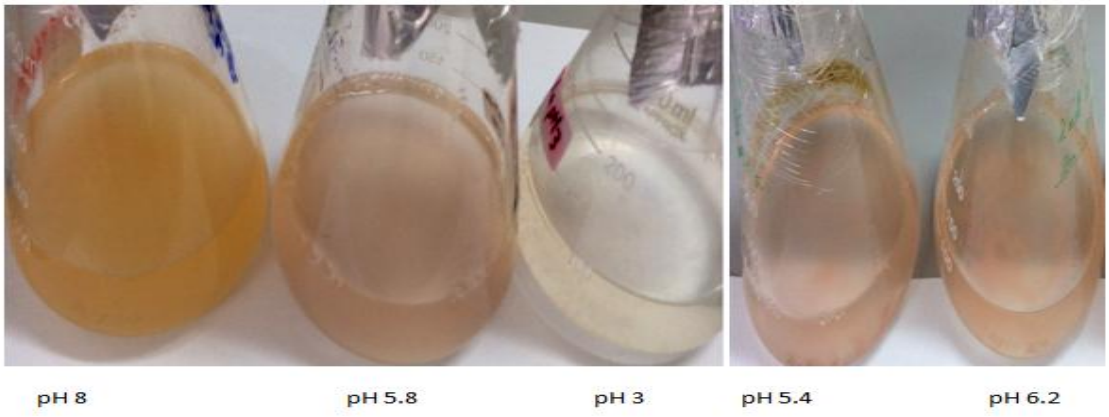


Fig 4.21: Anthocyanin pigments induction from different pH(s) into first subculture cell suspension on the day 20.

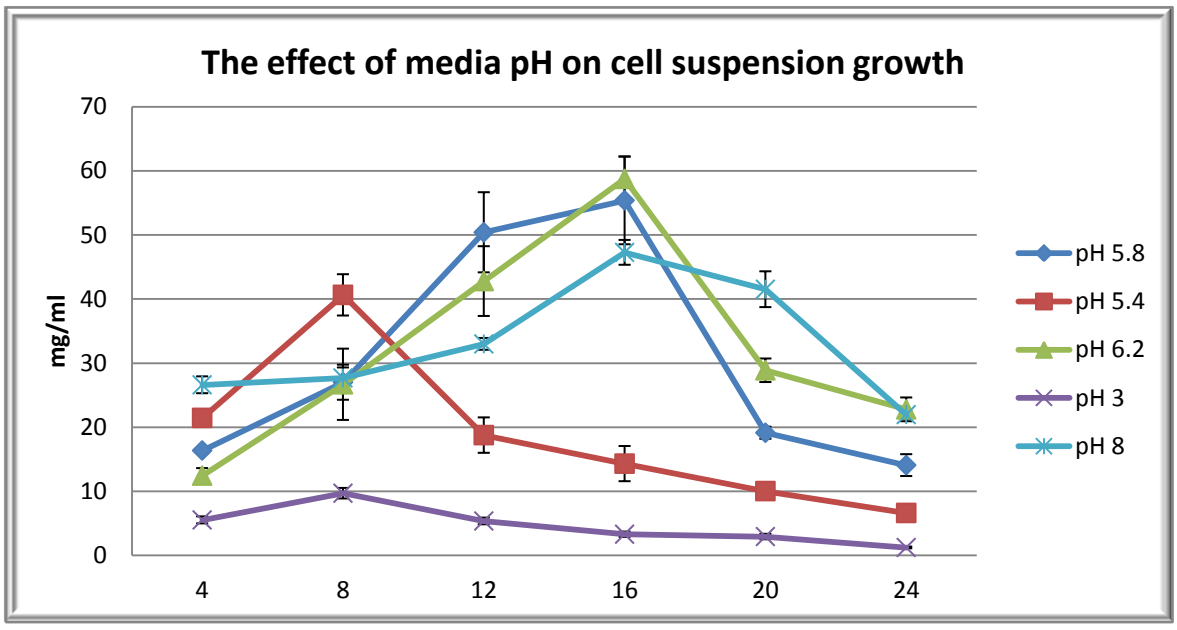


Fig 4.22: Growth of second subculture cell suspension into 5 different media pH(s). pH = 5.8 is the control one within 24 days. Each value represents the mean of 6 replicates; vertical lines represent standard error in each replicates.

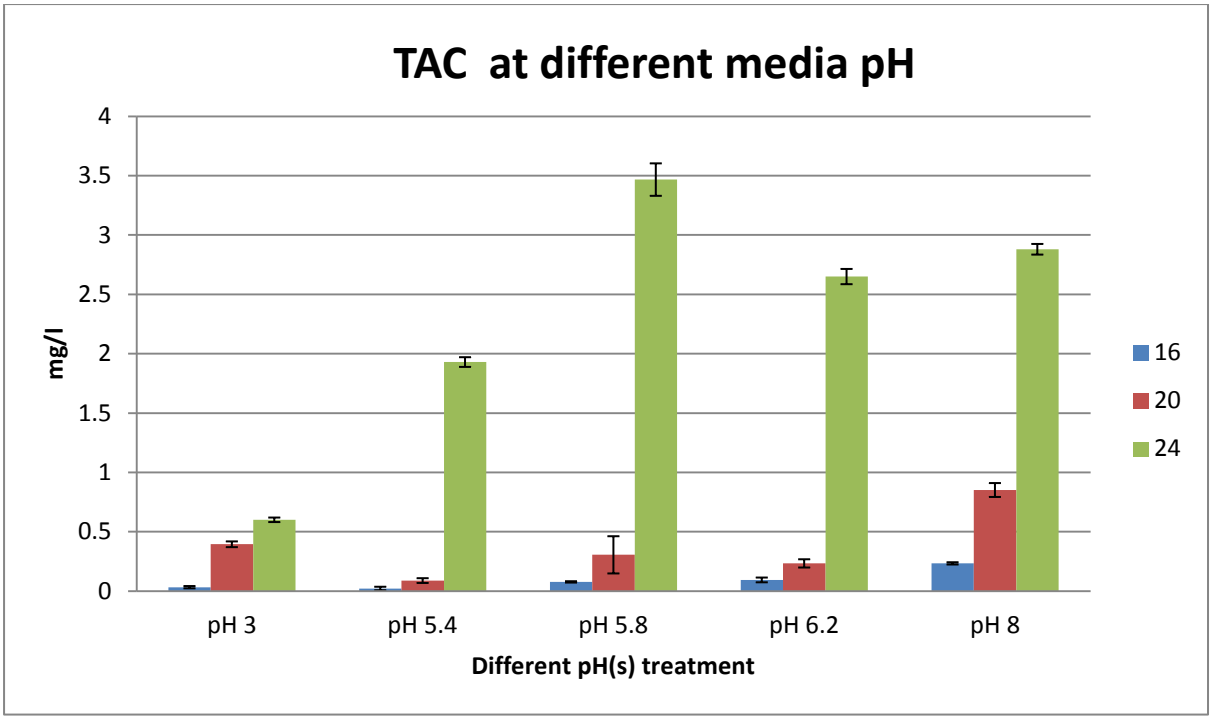


Fig 4.23: The TAC accumulation within time from 5 different pH(s) media.

4.7 Elicitation

The salicylic acid elicitor was added on the day 14th in the second subculture under light condition at two different concentrations; 1.0 and 2.0 mg/l for 96 hours (4 days). Figure 4.24-25 shows the growth curve and anthocyanin production within these four days after SA induction. The highest TAC accumulation was observed on the day 16 after 48 hours of SA induction at concentration 1.0 mg/l. SA is a simple and effective stimulant that increased the production of anthocyanin via the elevation of the enzymes (phenylalanine ammonia-lyase and chalcone flavanone isomerase), which play an important role in anthocyanin biosynthesis (Obinatai *et al*. 2003).

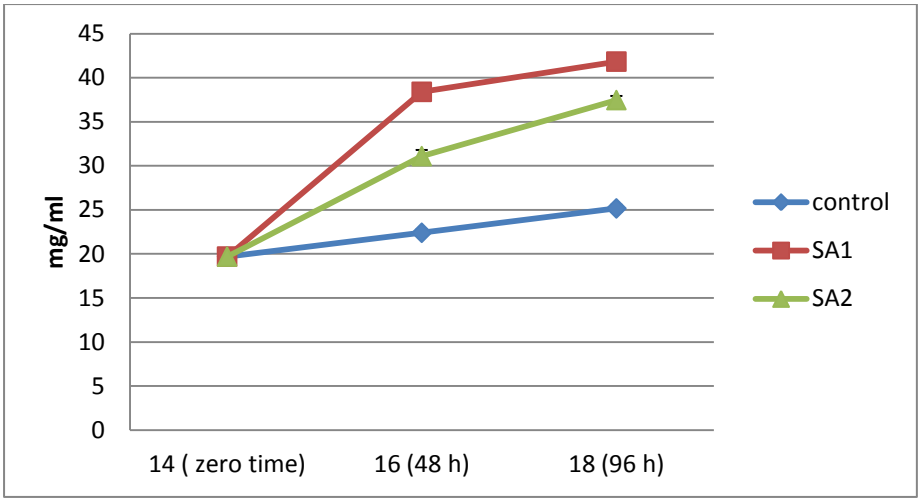


Fig 4.24: The effect of salicylic acid with two different concentrations on the growth curve of 2nd subculture suspension within 96 hour (4 days).

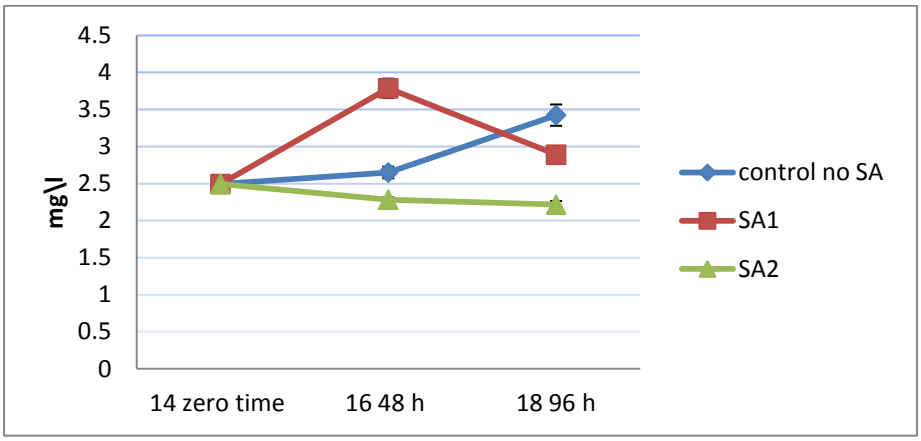


Fig 4.25: The effect of salicylic acid with two different concentrations SA1 and SA2 on the TAC accumulation in second subculture cell suspension vacuole within time.

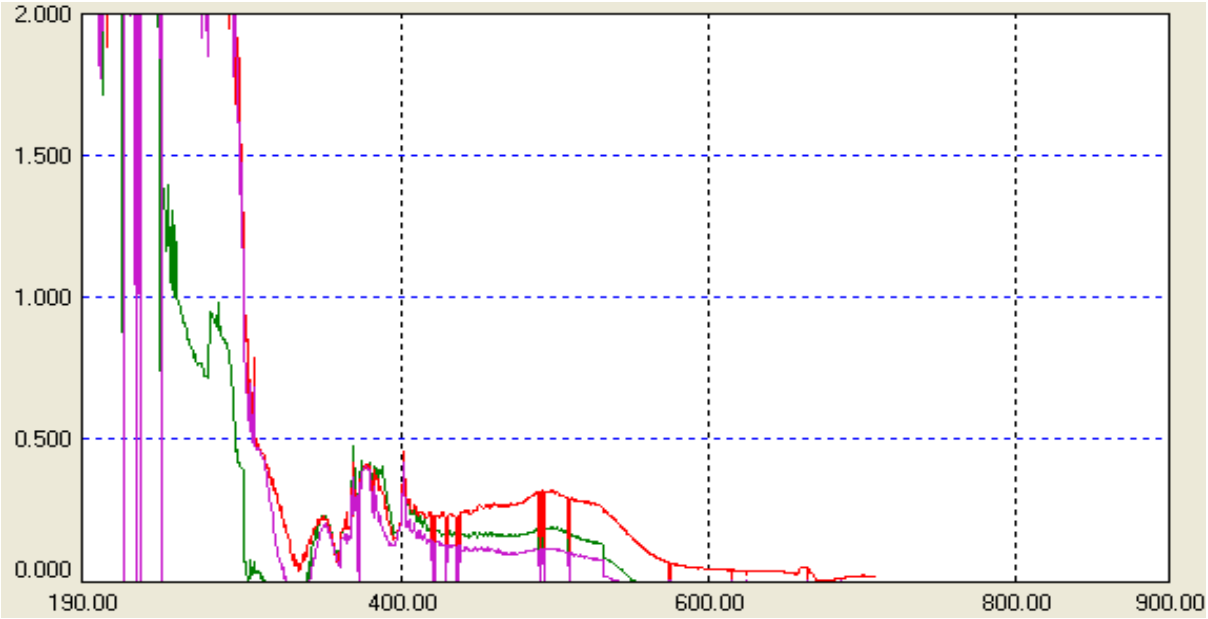


Fig 4.26: Anthocyanin UV-VIS spectroscopy spectrum from 200nm to 710nm at pH 1 for second subculture cell suspension. Green= control (no SA on day 16), Red= SA 1treatment on day 16 after 48 hours, Purple= SA2 treatment on day 16 after 48 hours.

Chapter5

CONCLUSIONS AND FUTURE WORK

5.1. Conclousions

In this study *Arbutus andrachne* callus culture technique was used as an alternative approach to produce anthocyanin pigments. The anthocyanin pigments were detected by using UV-VIS spectrometry, although the total monomric anthocyanin was determined by using pH differential methods. This study pointed out the possibility of anthocyanin pigments production after some manipulations in the growth media. The addition of starch in the presence of agar resulted in an increase of the production of anthocyanin by acting as biotic elicitor and osmotic stress. Additionally, this study has determined the best growth curve of cell suspension culture of *A. andrachne* with the highest production of anthocyanin to find the best time to insert different elicitors. Salicylic acid was used as chemical elicitor that enhanced the production of anthocyanin at 1.0mg/l concentration within 48 hours.

In the present work, the anthocyanins pigment was induced, detected and elicited in the *in vitro* growing material of *A. andrachne.*

All objectives were achieved as the following:

1. Tested the effect of different factors like carbon source, basal media and gelling agent on callus growth and total anthocyanin concentration.
2. Induced cell suspension with anthocyanins pigment from callus of *A. andrachne*.
3. Determined the best conditions to enhance the production of anthocyanins from induction cell suspension of *A. andrachne* , here in this is B5 media supplemented with 0.1 mg/l NAA, 0.2 mg/l Kin and 0.25 mg/l casein hydrolysate in addition to 30 g/l sucrose under light condition with 24 ±1°C temperature.
4. Enhanced the production of anthocyanins pigment at early stage from subculture cell suspension of *A. andrachne* by using different abiotic elicitors.
5. Detected anthocyanin pigments in callus and cell suspention by using UV-VIS and TLC.
6. Determined the total anthocyanin concentration within vacuole from different treatment by using pH differential methods

5.2 Future work

Identification and characterization of the chemical properties of the anthocyanins compound that produced by *in vitro* growing material of *A. adnrachne* using high pressure liquid chromatography (HPLC) and mass spectroscopy (MS). Additionally**,** many chemical properties of the extract can be studied like antioxidant activity, radical scavenging properties, activity of other co-occuring chemicals. Moreover, study the biological activity for the *in vitro* extract pigments are noteworthy including anticancer, antifungal or antibacterial. The production of these pigments for commercial and pharmaceutical uses with bioreactors technology can be also implemented. Finally, identifying the genetic and transcriptional profiles that drives the anthocyanins biosynthesis pathway in the *in vitro* growing material of *A. andrachne* in order to develop future transformation protocols for higher yield.

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Appendices

Appendix Table 1.

List of basal media, plant growth regulator, chemical buffer and their supplier as well as Cat number

	Supplier	Cat number
Lloyd& MCCown woody plant Basel Medium MC	Phytotechnology Laboratories	L449
Gamborg's B-5 Basel Medium	SIGMA	G893-10L
Quirin & Lepoivre Medium QLM	Duchefa Biocheme	Q0251.0050
Murashige&Skoog Medium MS	Duchefa Biocheme	M0222.0050
Sucrose crystallized	Duchefa Biocheme	S0809.1000
D-Fructose	Duchefa Biocheme	F0801.1000
D (+) –Glucose- Mohohydrate	AppliChem	A3730,0500
D-Mannitol	Duchefa Biocheme	M0803.1000
D-Sorbitol	Duchefa Biocheme	S0807.1000
Gelrite	Duchefa Biocheme	G1101.0500
European Baceriological Agar	Hy laboratories Ltd.	705437
Casein Hydrolysate	Duchefa Biocheme	C1301.0500
PVP 10 Polyvinyl Pyrrolidon 10	Duchefa Biocheme	P1368.0500
Naphtalene Acetic Acid NAA	Duchefa Biocheme	N0903.0025
Thidiazuron TDZ	Duchefa Biocheme	T0916.0250
Kinetin KIN	Duchefa Biocheme	K0905.0005
Gibberellic acid 3 GA3	Duchefa Biocheme	G0907.0005
Acetyl acetate	C5	1173
Formic acid	SIGMA ALDRICH	F0507-100ML
Acetic acid	GADOT	64197
Hydrochloric Acid 37% HCL	AR	08410501H
Butanol	C5	1120
Methanol	SIGMA ALDRICH	34860
Ethanol	SIGMA ALDRICH	34870
Silica gel 60 MN Kieselgel 60	Macherey-Nagel	815330.1
DC – Fertigfolien Alugram SIL G\UV Silica paper	Macherey-Nagel	818133
Salicylic acid	SIGMA	S7401-500G

Appendix Table 2.

Fresh weight of callus per dish from different treatment after 25 day cultivation

Media	Fresh weight sample 1	Fresh weight sample 2	Fresh weight sample 3
Control(sucrose+MCc+ Agar)	2.6	2.7	2.8
Glucose	1.3	1.8	2
Fructose	1.7	2.3	2.5
Sucrose + Sorbitol	2.9	3.5	4
Sucrose + Manitol	2.5	2.9	3
Glucose + Fructose	2.8	2.7	2.5
MS+ Agar	2.8	2.4	2.3
B5 + Agar	3.3	3.2	4.1
QLM + Agar	3.8	4.4	3.3
MCc+ Gel rite	3.4	3.7	3.5
MCc+ Agar + Starch	4.5	4.7	4.3
MS + Agar + Starch	3.6	3.8	3.9
B5 + Agar + Starch	5.1	5.6	4.6
QLM + Agar + Starch	3	1.8	2.8

Appendix Table 3.

Analysis of variance (ANOVA) for callus maintenance fresh weight (growth) per dish on control media using different source of carbon (Sugar).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	**5.424**	**5**	**1.085**	**9.344**	**.001**
Within Groups	**1.393**	**12**	**.116**		
Total	**6.818**	**17**			

Appendix Table 4.

Analysis of variance (ANOVA) for callus maintenance fresh weight (growth) per dish from control media using different gelling agent.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	**4.869**	**2**	**2.434**	**99.591**	**.000**
Within Groups	**.147**	**6**	**.024**		
Total	**5.016**	**8**			

Appendix Table 5.

Analysis of variance (ANOVA) for callus maintenance fresh weight (growth) per dish from control media using different basal media.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	**2.940**	**3**	**.980**	**6.031**	**.019**
Within Groups	**1.300**	**8**	**.163**		
Total	**4.240**	**11**			

Appendix Table 6.

Total monomeric anthocyanin concentration (TAC) from callus maintenance on day 25 after cultivation from different treatment via pH differential methods

6.1

Control (MCc+ sucrose+ agar)

PH 1 520	PH 1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
1.758	.005	.412	.256	1.597	26.67
1.723	.008	.484	.281	1.512	25.25
1.773	.026	.454	.265	1.558	26.02

6.2

Glucose

pH 1 520	pH 1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
1.439	.290	.503	.468	1.114	18.60
1.429	.303	.522	.480	1.084	18.10
1.440	.305	.544	.504	1.095	18.29

6.3

Fructose

pH1 520	pH1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
.979	-.045	.360	.2	.864	14.43
.974	-.056	.286	.161	.905	15.11
.987	-.026	.365	.215	.863	14.41

6.4

Sucrose + Sorbitol

pH 1 520	pH 1 700	pH 4.5 520	PH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
1.305	-.040	.390	.207	1.162	19.404
1.302	-.043	.362	.183	1.166	19.471
1.307	-.041	.310	.194	1.232	20.573

6.5

Sucrose + Mannitol

pH1 520	pH1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
.913	-.032	.290	.168	.823	13.743
.877	-.038	.357	.188	.746	12.457
.903	-.021	.359	.193	.758	12.658

6.6

Glucose + Fructose

pH 1 520	pH 1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
1.191	.067	.257	.025	.892	14.895
1.285	.011	.246	.016	1.044	17.434
1.188	.069	.254	.009	.874	14.595

6.7

MS+ agar

pH 1 520	pH 1 700	pH 4.5 520	pH4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
.979	.023	.468	.352	.84	14.027
1.000	.055	.376	.276	.845	14.110
.984	.045	.411	.306	.834	13.927

6.8

B5+ agar

pH 1 520	pH 1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
1.220	.292	.511	.502	.919	15.35
1.200	.270	.492	.478	.916	15.30
1.202	.265	.576	.553	.914	15.26

6.9

QLM+ agar

pH 1 520	pH 1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
.730	.062	.362	.257	.563	9.40
.718	.048	.324	.227	.573	9.57
.719	.054	.297	.209	.577	9.64

6.10

MCc+ gelrite

pH1 520	pH 1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
1.436	.121	.325	.222	1.212	20.24
1.641	.455	.250	.170	1.106	18.47
1.260	.142	.304	.209	1.023	17.08

6.11

MCc+ starch

pH 1 520	pH 1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
2.052	-.051	.440	.133	1.796	29.99
2.051	-.059	.472	.146	1.784	29.79
2.043	-.048	.471	.145	1.765	29.47

6.12

MS + starch

pH 1 520	pH 1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
1.442	-.040	.453	.248	1.277	21.32
1.443	-.055	.408	.224	1.314	21.94
1.444	-.047	.404	.235	1.322	22.08

6.13

B5 + starch

pH 1 520	pH 1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
1.049	.125	.336	.165	.735	12.57
1.045	.113	.310	.130	.752	12.56
1.044	.105	.380	.198	.757	12.64

6.14

QLM + starch

pH 1 520	pH 1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
.820	-.015	.364	.221	.692	11.56
.803	-.021	.334	.181	.671	11.20
.802	-.025	.350	.211	.688	11.49

Appendix Table 7.

Analysis of variance (ANOVA) for TAC accumulation within callus from control media by using different source carbon (sugar).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	**327.847**	**5**	**65.569**	**96.662**	**.000**
Within Groups	**8.140**	**12**	**.678**		
Total	**335.987**	**17**			

Appendix Table 8.

Analysis of variance (ANOVA) for TAC accumulation within callus from control media by using different gelling agent.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	**193.123**	**2**	**96.562**	**93.976**	**.000**
Within Groups	**6.165**	**6**	**1.028**		
Total	**199.288**	**8**			

Appendix Table 9.

Analysis of variance (ANOVA) for TAC accumulation within callus from control media by using different basal media.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	436.796	3	145.599	1096.864	.000
Within Groups	1.062	8	.133		
Total	437.858	11			

Appendix Table 10.

Callus induction cell suspension fresh growth weight from 4 replications in four days interval for thirty three two day in different osmotic stress and conditions.

10.1 light effect +24±1°C

10.1. A B5 media + 30 g/l sucrose alone

Sample	4 days	8 days	12 days	16 days	20	24 day	28 day	32 day
1	3	6	8	9.9	19.6	24	62.5	69.7
2	3.5	6.5	8.1	9.5	20	25.2	69	70.2
3	4.5	7.5	9.7	10.9	20.5	27.1	62.4	69
4	4.8	7.8	9.4	11	22	28.6	70.9	72

10.1. B B5 media + 30 g/l sucrose + 2 g/l mannitol

sample	4 days	8 days	12 days	16 days	20 days	24 day	28 day	32 day extract
1	4.8	13.9	16.9	18.2	41.6	22.2	22.9	25.8
2	3.8	12.4	15.8	18.5	42.8	24.8	24.2	27
4	5.5	14.1	17.4	18.8	45	28.4	27.8	26.8
5	5.8	14	17.3	19	45.6	28	28. 2	25

10.1. C B5 media + 30 g/l sucrose + 2 g/l sorbitol

Sample	4 day	8 day	12 day	16 day	20 day	24 day	28 day	32 day
1	2.5	5.5	15.5	28	36	43	48.8	56.9
2	2.2	5.4	16	28.9	35.6	47	43.8	60.8
3	3.2	9	20.2	29.4	50.1	79.2	80.9	70.9
4	3.8	8.5	21	30.3	59	80	86.9	65.8

10.2 Dark effect + 29±1°C

10.2. A B5 media + 30 g/l sucrose alone

Sample	4 days	8 days	12 days	16 days	20 days	24 day	28 day	32 day
1	2.9	3	8	9.8	11	23.7	19.9	20.9
2	1.9	3.9	8.9	10.5	14.3	27.6	22.8	22
3	5	7.5	10.5	13.6	21.6	34.8	43.1	50.8
4	4.9	6	11.6	16.9	24.6	37.9	42.8	45

10.2. B B5 media + 30 g/l sucrose + 2 g/l mannitol

Sample	4 days	8 days	12 days	16 days	20 days	24 day	28 day	32 day
1	3.9	5.2	7.5	9.6	12	10	5.9	10
2	4	5.8	7.9	11.1	13.4	11.7	6.2	11
3	4.8	7	9.3	18.9	22	22.9	17.5	20
4	5.3	7.2	9.1	17.8	19.7	19.8	16.8	19.9

10.2. C B5 media + 30 g/l sucrose + 2 g/l sorbitol

Sample	4 days	8 days	12 days	16 days	20 days	24 day	28 day	32 day
1	6.3	7.8	9	14.5	21.9	26.1	34.1	55.9
2	6	8	11.1	14.6	20.6	27.7	40	56.3
3	4.8	7	8.5	12.6	20.3	23.7	40.7	59.9
4	5	6.8	8.1	13.5	20.9	33.1	43.1	50.5

Appendix Table 11.

Total monomeric anthocyanin concentration (TAC) from induction cell suspension from callus within time from different osmotic stress treatment under two different conditions via pH differential methods.

11.1. Light condition + 24±1°C

11.1. A B5 media + 30 g/l sucrose

Day	pH 1 520	pH1 700	pH 4.5 520	pH4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
16	.221	.171	.155	.112	.007	.117
	.228	.177	.150	.109	.01	.167
	.227	.175	.158	.115	.009	.150
20	.361	.153	.138	.088	.158	2.64
	.362	.156	.136	.086	.156	2.60
	.360	.154	.134	.080	.152	2.54
24	.287	.156	.177	.198	.152	2.54
	.297	.155	.176	.195	.161	2.69
	.298	.153	.171	.193	.167	2.79
32	.623	-0.004	.548	.372	.451	7.53
	.624	-0.002	.492	.374	.508	8.48
	.625	-0.003	.492	.344	.48	8.02

11.1. B B5 media + 30 g/l sucrose + 2 g/l mannitol

Day	pH 1 520	pH 1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
16	.197	.166	.232	.190	-0.011	-0.1836
	.201	.170	.241	.201	-.009	-0.1503
	.194	.163	.229	.191	-.007	-0.117
20	.122	.104	.245	.231	.004	.0668
	.132	.115	.229	.214	.002	.0334
	.140	.129	.232	.222	.001	.0167
24	.225	.201	.445	.403	-0.018	-0.3005
	.235	.202	.451	.401	-0.017	-0.284
	.265	.217	.463	.404	-0.011	-0.184
32	.481	.402	.670	.466	-0.125	-2.087
	.482	.403	.671	.467	-0.125	-2.087
	.485	.407	.673	.465	-0.13	-2.171

11.1. C B5 media + 30 g/l sucrose + 2 g/l sorbitol

Day	pH 1 520	pH1 700	pH 4.5 520	pH4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
16	.598	.390	.149	.118	.177	2.95
	.580	.384	.150	.114	.16	2.67
	.579	.375	.155	.110	.159	2.65
20	.380	.133	.183	.121	.185	3.08
	.377	.130	.180	.126	.193	3.22
	.388	.149	.188	.128	.179	2.99
24	.332	.150	.210	.195	.167	2.79
	.340	.162	.209	.198	.167	2.79
	.344	.163	.212	.200	.169	2.82
32	.231	.026	.276	.197	.126	2.104
	.225	.025	.269	.190	.121	2.020
	.230	.028	.281	.196	.117	1.954

11.2 Dark condition + 29±1°C

11.2. A B5 media + 30 g/l sucrose alone

Day	pH 4.5 520	pH4.5 700	pH 4.5 520	pH4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
16	.222	.161	.231	.180	.01	.167
	.231	.163	.239	.181	.01	.167
	.226	.158	.241	.189	.016	.267
20	.280	.158	.222	.143	.043	.718
	.277	.153	.228	.148	.044	.735
	.261	.143	.221	.149	.046	.768
24	.329	.150	.215	.134	.098	1.64
	.328	.151	.216	.138	.099	1.65
	.310	.142	.212	.129	.085	1.42
32	.645	.05	.360	.238	.473	7.90
	.661	.045	.355	.234	.495	8.26
	.657	.038	.352	.237	.504	8.42

11.2. B B5 media + 30 g/l sucrose + 2 g/l mannitol

Day	pH 1 520	pH1 700	pH 4.5 520	pH4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/ l
16	.206	.145	.210	.148	-.001	-.017
	.208	.145	.212	.140	-.009	-.150
	.205	.143	.208	.145	-.001	-.017
20	.257	.161	.253	.154	-.003	-.050
	.251	.158	.257	.150	-.014	-.234
	.245	.156	.250	.157	-.004	-.067
24	.270	.191	.194	.118	.003	.050
	.278	.193	.191	.110	.004	.067
	.281	.198	.198	.117	.002	.033
32	.045	-0.068	.206	.125	.032	.534
	.049	-0.067	.150	.075	.041	.685
	.046	-0.067	.216	.133	.03	.501

11.2. B B5 media + 30 g/l sucrose + 2 g/l sorbitol

Day	pH 1 520	pH1 700	pH 4.5 520	pH4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
16	.243	.138	.130	.119	1.57	1.57
	.211	.118	.133	.120	1.34	1.34
	.212	.116	.135	.122	1.39	1.39
20	.285	.154	.156	.130	1.75	1.75
	.290	.156	.157	.132	1.82	1.82
	.241	.130	.149	.135	1.62	1.62
24	.295	.132	.189	.140	1.90	1.90
	.297	.131	.191	.137	1.87	1.87
	.296	.133	.193	.135	1.75	1.75
32	.420	.273	.443	.444	.148	2.471
	.398	.259	.434	.437	.142	2.371
	.399	.253	.438	.443	.151	2.522

Appendix Table 12.

12.1

Analysis of variance (ANOVA) for TAC accumulation in cell suspension from different osmotic stress treatment under light condition within time.

		Sum of Squares	Df	Mean Square	F	Sig.
16 day	Between Groups	14.443	2	7.221	752.755	.000
	Within Groups	.058	6	.010		
	Total	14.500	8			
20 day	Between Groups	16.128	2	8.064	1455.792	.000
	Within Groups	.033	6	.006		
	Total	16.161	8			
24 day	Between Groups	15.003	2	7.501	1394.884	.000
	Within Groups	.032	6	.005		
	Total	15.035	8			
32 day	Between Groups	104.073	2	52.037	674.771	.000
	Within Groups	.463	6	.077		
	Total	104.536	8			

12.2

Analysis of variance (ANOVA) for TAC accumulation in cell suspension from different osmotic stress treatment under dark condition within time.

		Sum of Squares	df	Mean Square	F	Sig.
16 day	Between Groups	3.615	2	1.807	301.798	.000
	Within Groups	.036	6	.006		
	Total	3.651	8			
20 day	Between Groups	4.520	2	2.260	619.445	.000
	Within Groups	.022	6	.004		
	Total	4.542	8			
24 day	Between Groups	5.587	2	2.794	356.810	.000
	Within Groups	.047	6	.008		
	Total	5.634	8			
32 day	Between Groups	94.536	2	47.268	1640.151	.000
	Within Groups	.173	6	.029		
	Total	94.709	8			

Appendix Table 13.

13.1 1st subculture cell suspension fresh weight growth from 6 replications in 4 day interval for 24 day.

Sample	4 days	8 days	12 days	16 days	20 days	24 days
1	6.3	6.8	9.5	10.9	8.4	6.8
2	7.3	8	10.5	12	9.5	6.5
3	7.6	8.5	10.6	13.6	8.8	6
4	6.6	8.3	10.8	13.5	10.1	7.5
5	7.9	9	11	13.8	10.1	7.2
6	8.0	10	11.5	17.9	10.3	6.6

13.2 2nd subculture cell suspension fresh weight growth from 6 replications in 4 day interval for 24 day.

Sample	4 days	8 days	12 days	16 days	20 days	24 days
1	5.8	10.2	11.9	13.7	9.2	5
2	5.7	10	12.5	14.8	10.8	4.5
3	8	16.2	19.6	23.8	16.8	11.9
4	8.1	16.5	20	25.2	16	11.5
5	9.	19.8	22.5	25.6	19.8	12
6	8.7	18.2	20.8	24.7	18.7	12.4

Appendix Table 14.

The effect of different concentration of sorbitol on the growth of 1st subculture cell suspension from 4 replications in 4 day intervals for 24 day.

14.1 1st subculture 30 g /l sucrose + 2g/l sorbitol

Sample	4 day	8 day	12 day	16 day	20 day	24
1	16.9	21.9	37.6	28.7	21.8	14
2	17.3	23.8	44.8	25.9	23.2	13.8
3	17.9	27.8	33	25.4	23	16.9
4	19.2	30.9	47.8	29	19	13.7

14.2 1st subculture 30 g /l sucrose + 8g/l sorbitol

Sample	4 day	8 day	12 day	16 day	20 day	24
1	11.6	19.6	18.1	35.9	25.2	20
2	10.9	21.8	20.9	40.4	24.8	18.9
3	19.7	20.7	23.8	46.1	22.5	21
4	20	22.5	19.9	40.1	20.9	19.9

14.3 1st subculture 30 g /l sucrose + 14g/l sorbitol

Sample	4 day	8 day	12 day	16 day	20 day	24
1	28.6	15.2	17.7	27.4	25	19
2	26 .4	15.8	19.8	28.9	24.9	20
3	26.8	13.3	14.9	20.7	25.1	21.9
4	22.8	12.9	20	30	22	18.9

Appendix Table 15

Total monomeric anthocyanin concentration (TAC) from 1st subculture cell suspension in different concentrations of sorbitol within time under light condition via pH differential methods.

15.1 2 g/l sorbitol

Day	pH1 520	pH1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
16	.380	.198	.450	.315	.047	.785
	.382	.199	.456	.322	.048	.802
	.378	.195	.448	.306	.041	.685
20	.249	.058	.319	.199	.071	1.186
	.256	.062	.309	.189	.074	1.236
	.261	.069	.311	.192	.073	1.22
24	.198	.159	.250	.229	.018	.3006
	.189	.144	.247	.219	.017	.284
	.193	.151	.239	.212	.015	.250

15.2 8 g/l sorbitol

Day	pH1 520	pH1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
16	.396	.198	.246	.187	.139	2.321
	.389	.189	.254	.198	.144	2.405
	.390	.195	.249	.189	.135	2.254
20	.405	.203	.301	.286	.187	3.123
	.409	.208	.299	.278	.18	3.006
	.401	.199	.303	.290	.189	3.156
24	.297	.187	.255	.235	.09	1.503
	.289	.178	.245	.238	.104	1.737
	.293	.185	.259	.244	.093	1.553

15.3 14 g/l sorbitol

Day	pH1 520	pH 1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
16	.467	.202	.258	.109	.116	1.94
	.473	.210	.255	.121	.129	2.15
	.476	.218	.253	.103	.116	1.94
20	.359	.216	.224	.216	.135	2.254
	.355	.213	.232	.221	.131	2.188
	.361	.220	.223	.214	.132	2.204
24	.312	.249	.183	.179	.059	.985
	.309	.237	.191	.188	.069	1.152
	.315	.251	.199	.195	.06	1.002

Appendix Table 16.

Analysis of variance (ANOVA) for TAC accumulation in 1st subculture cell suspension from different sorbitol concentration treatment within time.

		Sum of Squares	Df	Mean Square	F	Sig.
16 day	Between Groups	4.132	2	2.066	253.817	.000
	Within Groups	.049	6	.008		
	Total	4.181	8			
20 day	Between Groups	5.315	2	2.657	990.265	.000
	Within Groups	.016	6	.003		
	Total	5.331	8			
24 day	Between Groups	2.635	2	1.317	162.674	.000
	Within Groups	.049	6	.008		
	Total	2.684	8			

Appendix Table 17.

pH media effect on the fresh weight growth of 1st subculture cell suspension from 6 replications in 4 day interval for 24 day .

17.1 pH 5.8 regular pH

Sample	Day 4	Day 8	Day 12	Day 16	Day 20	Day 24
1	15.7	22.1	39	40	16	15.1
2	16.8	20.3	38.6	40.9	17.8	7
3	17	32	69.5	73	20. 4	19.6
4	18.5	38	70.4	79.5	22.1	12.7
5	14.8	24.5	40	48.9	20	16
6	15.3	25.1	44.9	50	19.7	14

17.2 pH 5.4

Sample	Day 4	Day 8	Day 12	Day 16	Day 20	Day 24
1	25	50.7	23.9	22.8	12.7	7.9
2	23.9	46.5	22.7	20	11.8	6.8
3	17.5	41	22.7	15.3	7.7	7.1
4	18.2	42.8	23	14.6	6	5.9
5	21.8	32	11.5	6.2	11.8	6.9
6	22.2	30.8	8.7	6.9	10.1	5

17.3 pH 6.2

Sample	Day 4	Day 8	Day 12	Day 16	Day 20	Day 24
1	11.3	38.9	60	70.5	32.5	28
2	12.6	36.8	55.9	66.8	33.8	26
3	15	35 m	40	59.1	29.6	25
4	16.5	33.9	43.9	55.8	27.9	23
5	10	11	29	49.9	21	17.8
6	9.2	12.8	27.9	50.3	28.4	16.8

17.4 pH 8

Sample	Day 4	Day 8	Day 12	Day 16	Day 20	Day 24
1	20.8	25.8	36.1	43.9	40	21.1
2	28.8	27.4	30.7	45.9	47.8	23.2
3	27.7	32	33.5	49.6	30.7	20.9
4	30	20.8	30	55.9	50	19.8
5	25.9	29.8	33.7	44.5	40.8	23.9
6	26.4	30.2	33.9	43.8	39.8	22.8

17.5 pH 3

Sample	Day 4	Day 8	Day 12	Day 16	Day 20	Day 24
1	7.6	11	6.5	3.4	1.4	1
2	5.9	9.5	7	2.5	3	1.3
3	4	5.8	4	2	2.8	1.2
4	6.3	9.8	5.8	4.9	4.7	0.8
5	4.5	10.8	4.8	3.7	2.7	1.8
6	4.8	11.2	4	3.2	2.9	1.2

Appendix Table 18.

Total monomeric anthocyanin concentration (TAC) from 1st subculture cell suspension in different pH media within time under light condition via pH differential methods.

18.1 pH 5.8

Day	pH 1 520	pH 1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
16	.050	-0.018	.230	.167	.005	.0835
	.041	-0.016	.231	.177	.003	.0501
	.051	-0.015	.228	.168	.006	.100
20	.061	-0.01	.205	.138	.004	.0668
	.062	-0.02	.206	.139	.015	.250
	.066	-0.04	.205	.135	.036	.601
24	.248	-0.053	.222	.121	.200	3.34
	.249	-0.058	.252	.169	.224	3.74
	.226	-0.056	.246	.163	.199	3.32

18.2 pH 5.4

Day	pH 1 520	pH 1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
16	.136	.061	.199	.125	.001	.0167
	.138	.062	.197	.123	.002	.0334
	.130	.o66	.190	.127	.001	.0167
20	.119	.051	.203	.141	.006	.100
	.121	.054	.200	.140	.007	.117
	.116	.048	.205	.144	.003	.050
24	.354	.085	.462	.312	.119	1.99
	.353	.086	.458	.302	.111	1.85
	.355	.084	.457	.302	.116	1.94

18.3 pH 6.2

Day	pH 1 520	pH1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
16	.054	-0.008	.215	.157	.004	.0668
	.056	-0.010	.218	.160	.008	.1336
	.052	-0.009	.211	.155	.005	.0835
20	.079	.001	.187	.120	.011	.1837
	.080	.002	.190	.125	.013	.2171
	.082	-0.001	.192	.127	.018	.3006
24	.540	.231	.345	.198	.162	2.706
	.567	.233	.359	.188	.163	2.722
	.566	.254	.360	.199	.151	2.522

18.4 pH 8

Day	pH 1 520	pH 1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
16	.081	.001	.237	.170	.013	.217
	.079	.002	.235	.173	.015	.250
	.080	.004	.233	.171	.014	.234
20	.067	-0.009	.211	.190	.055	.918
	.068	-0.008	.217	.195	.054	.902
	.069	.001	.216	.192	.044	.735
24	.648	.298	.456	.282	.176	2.94
	.655	.302	.478	.292	.167	2.79
	.645	.289	.460	.278	.174	2.90

18.5 pH 3

Day	pH1 520	pH1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
16	.066	.053	.198	.187	.002	.0334
	.060	.049	.200	.190	.001	.0167
	.068	.059	.195	.189	.003	.0501
20	.098	.043	.231	.202	.026	.434
	.089	.038	.228	.198	.021	.351
	.092	.041	.239	.212	.024	.401
24	.103	.100	.245	.278	.036	.601
	.104	.101	.246	.281	.038	.634
	.106	.103	.249	.280	.034	.568

Appendix Table 19.

Analysis of variance (ANOVA) for TAC accumulation in 1st subculture cell suspension from different pH media treatment within time.

		Sum of Squares	Df	Mean Square	F	Sig.
16 day	Between Groups	.086	4	.021	42.875	.000
	Within Groups	.005	10	.000		
	Total	.091	14			
20 day	Between Groups	1.002	4	.251	13.838	.000
	Within Groups	.181	10	.018		
	Total	1.183	14			
24 day	Between Groups	14.526	4	3.632	225.167	.000
	Within Groups	.161	10	.016		
	Total	14.687	14			

Appendix Table 20.

TAC accumulation in 2nd subculture cell suspension after induces different concentrations of salicylic acid as chemical elecitor within 96 hours.

20.1 control

Day	pH1 520	pH1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
16	.231	.012	.198	.134	.155	2.588
	.228	.013	.189	.142	.168	2.805
	.233	.015	.194	.129	.153	2.555
18	.246	-0.054	.222	.121	.199	3.323
	.246	-0.059	.252	.169	.222	3.707
	.222	-0.055	.246	.163	.194	3.24

20.2 1.0 mg/l Salicylic acid

Day	pH1 520	pH1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
16	.388	.117	.198	.138	.211	3.523
	.379	.108	.165	.129	.235	3.924
	.391	.116	.172	.131	.234	3.908
18	.361	.107	.271	.193	.176	2.94
	.359	.106	.260	.175	.168	2.805
	.360	.109	.266	.190	.175	2.922

20.3 2.0 mg/l Salicylic acid

Day	pH1 520	pH1 700	pH 4.5 520	pH 4.5 700	A = pH1($A_{520} - A_{700}$) – pH 4.5 ($A_{520} - A_{700}$)	TAC mg/l
16	.155	.004	.201	.189	.139	2.321
	.152	.001	.199	.181	.133	2.221
	.159	.010	.201	.190	.138	2.304
18	.177	-0.046	.239	.150	.134	2.238
	.173	-0.049	.235	.140	.127	2.121
	.172	-0.048	.269	.186	.137	2.288

Appendix Table 21.

Analysis of variance (ANOVA) for TAC accumulation in 2nd subculture cell suspension from different concentration of Salicylic acid treatment within time.

		Sum of Squares	df	Mean Square	F	Sig.
48 hours	Between Groups	3.684	2	1.842	75.841	.000
	Within Groups	.146	6	.024		
	Total	3.829	8			
96 hours	Between Groups	2.197	2	1.099	44.069	.000
	Within Groups	.150	6	.025		
	Total	2.347	8			

Appendix Table 22.

The contents of different basal medium QLM, MS, MCc and B5.

22.1 Quoirin & Lepoivre Medium (QLM) adopted by Plantigen Technical Data

Ingredients	milligrams/litre
Potassium nitrate	1800.00
Ammonium nitrate	400.00
Calcium nitrate	578.92
Magnesium sulphate	175.79
Potassium phosphate monobasic	270.00
Manganese sulphate.H_2O	0.76
Boric acid	6.20
Potassium iodide	0.08
Molybdic acid (sodium salt).$2H_2O$	0.25
Zinc sulphate.$7H_2O$	8.60
Copper sulphate.$5H_2O$	0.025
Cobalt chloride.$6H_2O$	0.025
Ferrous sulphate.$7H_2O$	27.80
EDTA disodium salt.$2H_2O$	37.30
myo - Inositol	100.00
Thiamine hydrochloride	0.40

22.2 Murashige and Skoog Basal Medium (MS) adopted by SIGMA-ALDRICH

Components	mg/L
Ammonium nitrate	1,650.0
Boric acid	6.20
Calcium chloride (anhydrous)	332.20
Cobalt chloride hexahydrate	0.0250
Cupric sulfate pentahydrate	0.0250
Disodium EDTA dihydrate	37.260
Ferrous sulfate heptahydrate	27.80
Glycine	2.0
Magnesium sulfate (anhydrous)	180.70
Manganese sulfate monohydrate	16.90
myo-Inositol	100.0
Nicotinic acid	0.50
Potassium iodide	0.830
Potassium nitrate	1,900.0
Potassium phosphate monobasic	170.0
Pyridoxine hydrochloride	0.50
Sodium molybdate dihydrate	0.250
Thiamine hydrochloride	0.10
Zinc sulfate heptahydrate	8.60

22.3 lloyd & MCcown woody plant medium adopted by phytotechlab

Component	mg/l
Ammonium Nitrate	400
Boric Acid	6.2
Calcium Chloride, Anhydrous	72.5
Calcium Nitrate	386
Cupric Sulfate.$5H_2O$	0.25
Na_2 EDETA.$2H_2O$	37.3
Ferrous Sulfate.$7H_2O$	27.85
Magnesium Sulfate, Anhydrous	180.7
Magnesium Sulfate.H_2O	22.3
Molybdic Acid (Sodium Salt).$2H_2O$	0.25
Potassium Phosphate, Monobasic	170
Potassium Sulfate	990
Zinc Sulfate.$7H_2O$	8.6
Glycine (Free Base)	2
Myo-Inositol	100
Nicotinic Acid (Free Acid)	0.5
Pyrodoxine.HCl	0.5
Thiamin.HCl	1.0

22.4 Gamborg B5 Medium adopted by Plantigen Technical Data

Ingredients	milligrams/litre
Potassium nitrate	2500.00
Ammonium sulphate	134.00
Calcium chloride.$2H_2O$	150.00
Magnesium sulphate	122.09
Sodium phosphate monobasic	130.42
Manganese sulphate.H_2O	10.00
Boric acid	3.00
Potassium iodide	0.75
Molybdic acid (sodium salt).$2H_2O$	0.25
Zinc sulphate.$7H_2O$	2.00
Copper sulphate.$5H_2O$	0.025
Cobalt chloride.$6H_2O$	0.025
Ferrous sulphate.$7H_2O$	27.80
EDTA disodium salt.$2H_2O$	37.30
myo - Inositol	100.00
Thiamine hydrochloride	10.00
Pyridoxine hydrochloride	1.00
Nicotinic acid (Free acid)	1.00
Sucrose	20000.00