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Ziziphus spina-christi "Christ's Thorn": *In Vitro* Callus and Cell Culture, Qualitative Analysis of Secondary Metabolites and Bioassay

By

Ali Essa Abu Allan

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:

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ABSTRACT

Ziziphus spina-christi "Christ's Thorn": *In Vitro* Callus and Cell Culture, Qualitative Analysis of Secondary Metabolites and Bioassay

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Ali Essa Abu Allan

Ziziphus spina-christi (L.) Desf. (Rhamnaceae) is a multipurpose tree distributed along the entire sahelian area from Senegal to Sudan and from middle to west Asia as well. In Palestine it occurs along the coast and in the Jordan Valley area. It has been used for different disease treatment in traditional medicine such as cholesterol reduction, treating eye inflammation and hair loss. Besides the traditional uses of this plant, recent studies reported that the extracts of every part of the plant has wide range of medical applications such as anti-diabetic, inhibition of hepatic carcinogenicity and antimicrobial activity. Moreover, important secondary metabolites were isolated from *Z. spina-christi* like Quercetin 3-O-b-D-galactoside and Kaempferol 3-O-rutinoside which are known for their antimicrobial and antifungal activity. This study was conducted to optimize a protocol for callus induction and secondary metabolites production from different tissues of *Z. spina-christi.* The highest callus induction (100%) was obtained from cotyledons and roots on MS media supplemented with 1.3 mg/l BA and 0.3 mg/l NAA with some variation in callus color and texture. Incubation in darkness had significant effects on callus induction time from cotyledons, leaves, and roots explants. Callus formation from cotyledons, leaves, and roots was observed after 8 days for all explants while in light incubation callus formed after 20, 30, 11 days respectively. Combination of 1.0mg/l BA + 2.0mg/l NAA significantly reduced the fresh weight of cotyledonary callus. It has been observed from growth curve of cotyledonary callus that callus is to be subcultured at the end of the third week of incubation for new media in both light and dark condition. Different callus lines of *Z. spina-christi* were successfully maintained for seven months on MS media supplemented with 1.3 mg/L BA and 0.3 mg /L NAA. Cell suspension was initiated on liquid MS media supplemented with 1.3 mg/l BA and 0.3 mg/l NAA, 26-day interval allowed the determination of a cell growth peak in both fresh and dry matter. TLC analysis using ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26) as solvent system detected the presence of flavoniod secondary

metabolite in different intensity from callus,*in vitro*, and *ex vitro* parts of Z. *spina-christi*. The bioassay of cotyledonary callus extract against breast cancer cell line (MCF-7) resulted at IC_{50} equals 95 µg/ml.

Keywords

Ziziphus spina-christi; *In Vitro* culture, Callus induction, Secondary metabolites, Medicinal plant

ملخص بالعربية

السدر الشوكي-كريستي"شوكة المسيح": انتاج الكالوس و الخلايا النباتية في المختبر، التحليل النوعي للمركبات الثانوية و دراسة تأثيرها البيولوجي

من قبل

علي عيسى أبو علان

السدر الشوكي-كريستي .Desf (L.) (Rhamnaceae) هي شجرة متعددة الأغراض و الاستعمالات، موزعة على طول المنطقة الساحلية بأكملها من السنغال حتى السودان وأيضا من وسط أسيا إلى غربها. في فلسطين منتشرة على طول الساحل، و في منطقة وادي الأردن. لقد تم استخدامه لعلاج أمراض مختلفة في الطب التقليدي، مثل تخفيض الكولسترول، التهاب العين، و فقدان الشعر. إلى جانب الاستخدامات التقليدية لهذا النبات، ذكرت الدراسات الحديثة إن الاستخلاصات المائية و الكحولية لديها تطبيقات طبية واسعة مثل تثبيط و مكافحة مرض السكري، علاج بعض سرطانات الكبد، و مضاد لبعض الفطريات و الميكروبات. علاوة على ذلك، تم عزل بعض المركبات الثانوية المهمة من السدر الشوكي-كريستي، مثل Quercetin 3-O-b-D-galactoside و Kaempferol 3-O-rutinoside المعروفة بنشاطها المضاد للجراثيم و الفطريات. و قد اجريت هذه الدراسة لوضع بروتوكول لتحريض إنتاج الكلوس و المركبات الثانوية من انسجة مختلفة من نبتة السدر الشوكي-الكريستي. تم الحصول على أفضل إنتاج للكالوس (١٠٠%) من النبتات و الجذور على بيئة MS بإضافة BA على مستوى ١,٣ ملغم/لتر + NAA على مستوى ٠,٣ ملغم/لتر، مع اختلاف في اللون و الملمس للكالوس. كان لمزيج BA على مستوى ١ ملغم/لتر + NAA ٢ ملغم/لتر اثر كبير على خفض وزن الكالوس للنبتات. كان للحضانة في الظلام اثر كبير على زمن التحريض للكالوس للنبتات، الاوراق، و الجذور، حيث لوحظ تشكيل الكالس بعد ٨ ايام من الحضانة لجميع الجزاء بينما في الضوء لوحظ تشكل الكالوس بعد ٣٠,٢٠,١١ يوما على التوالي. بالنسبة لمنحنى النمو لنبتات الكالوس، وجد ان نهاية الاسبوع الثالث من الحضانة هو الوقت المناسب للكالوس للنقل الى بيئة جديدة على حد سواء في حالة الضوء و الظلام. تم المحافظة على الكالوس بنجاح لمدة سبعة شهور على بيئة MS باضافة BA على مستوى ١,٣ ملغم/لتر + NAA على مستوى ٠,٣ ملغم/لتر لكل من النبتات، الاوراق، و الجذور. بدأ انشاء الخلايا للنبتات على بيئة MS باضافة BA على مستوى ١,٣ ملغم/لتر + NAA على مستوى ٠,٣ ملغم/لتر، و كان ذروة النمو للخلايا بعد ٢٦ يوما من بداية الحضانة على حد سواء بالنسبة للخلايا الطازجة و الجافة. التحليل بواسطة TLC باستخدام نظام اذابه (١٠٠,١١,١١,٢٦) ماء:حمض الفورمك:حامض الخليك الجليدي:خلات الايثيل اثبت وجود مركبات ثانوية فلوا ندية و بدرجات مختلفة في جميع انواع الكالوس و ايضا الجذور و الاوراق التي جمعت من الطبيعة و التي نمت في الانابيب. دراسة تاثير خلاصة النبتات ضد سرطان الثدي خط الخلية MCF-7 أعطى تأثير ضد الخلايا السرطانية حيث بلغت قيمة IC_{50} ٩٥ ميكروغرام/ مل.

كلمات مفتاحيه

السدر الشوكي-كريستي، الزراعة في الانابيب المخبرية، حث الكالوس، المركبات الثانوية، النباتات الطبية

DECLARATION

I declare that the Master Thesis entitled " *Ziziphus spina-christi* "Christ's Thorn": *In Vitro* Callus and Cell Culture, Qualitative Analysis of Secondary Metabolites and Bioassay " is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Ali Essa Abu Allan

Date

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DEDICATION

I dedicate this great thesis to my children and wife for their love and encouragement.

I also dedicate it for the soul of my dead father

I won't also forget my brothers, sisters and mother, whom always stood by me during thick and thin, and providing me the moral support and resources to finish my work. Their unwavering faith in me has been a source of constant inspiration for me.

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I wish to express my gratefulness to all the faculty members of the Department of Biotechnology research center at Palestine polytechnic university for their help and moral support during my project work.

ABBREVIATIONS

Abbreviation	Word or Sentence
%	percent
°C	Degree centigrade Celsius
2,4 D	2,4-dichlorophenoxyacetatic acid
ANOVA	Analysis of variance
BAP	6-Benzylaminopurine
CRD	Completely Randomized Design
DMSO	Dimethyl sulfoxide
et al.	And others
g	gram
HPLC	High Performance Liquid Chromatography
HSV-1	*Herpes simplex virus type1*
IC_{50}	Half Maximal Inhibitory Concentration
l	liter
LSD	Least Significant Difference
M	molar
m^2	Square meter
MADICO	Manasrah Development and Investment Company
MCF-7	Michigan Cancer Foundation - 7
ml	milliliter
MS	Murashige and Skooge
NAA	Naphthaleneacetic acid
PGRs	Plant Growth Regulators
pH	Potensial hydrogen
R_f	Retardation factor
rpm	Round per minute
sec.	second
TLC	Thin layer chromatography
USA	United State of America
UV	Ultraviolet
WHO	World Health Organization

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CHAPTER ONE

1. INTRODUCTION

In the long history of humankind, man used various natural products to treat different diseases. Currently, most natural products were replaced with synthetic drugs that were based on modern chemistry and biotechnology. Despite that, there are a huge growing and renewed interest in natural medicines in Western countries. Nowadays, the World Health Organization (WHO) estimates that up to 80 % of people are still relying on traditional remedies such as herbs for treating some diseases (Tripathi and Tripathi, 2003). In 2002, approximately one quarter of the best selling drugs worldwide were natural products or derived from natural products (Butler, 2004). In addition, there was a seven-fold increase in the number of people using herbal medicine between 1990 and 1997 in the United State of America (USA)(Eisenberg *et al*., 1998).

There is always an interest to find cure from nature. Natural products represent more than 50% of all therapeutic drugs used in the world today (Cragg and Newman, 2005). In the last 40 years, many active drugs derived from plants, such as Diosgenin, Reserpine, Pilocarpine, and other antihypertensive alkaloids are still used in therapy (Newman *et al*., 2000). If the scientific techniques of pharmacological evaluations are developed, a number of important drugs derived from plants will be produced, thus human health will be improved. Since cancer and microbial infections are still life threatening, an urgent need for developing a new drug will remain highly demanding. Designing a drug using classical method is becoming more challenging and less productive particularly when it comes to tackling aggressive and complex diseases like cancer. Therefore more complex molecules need to be designed which time and money is consuming. On the other hand, natural product will remain a great source for producing highly complex molecule with high therapeutic potential.

The use of plant cell and tissue culture methodology as a source of producing medicinal metabolites has a long history (Verpoorte*et al*., 2002). When plant cell and tissue culture emerged as a discipline within plant biology, researchers have encouraged utilizing plant cell capabilities for obtaining useful products (Misawa,

1994). Currently many efforts have been focused on utilizing tissue culture technique to generate active therapeutic drugs from plants with medicinal history.

1.1 Plants as a source of medication

Plants were considered as source of medication for thousands of years. There is an estimation that approximately one quarter of prescribed drugs derived from plant extracts. Many active compounds have been developed and aspirin is one of the most popular analgesic drugs that were originally derived from species of *Salix* and *Spiraea* (Katzung, 2005). Taxol, Paclitaxel and Vinblastine are derived from plant origin with high anticancer activity (Pezzuto, 1996).

Biotechnological tools are important for multiplication and genetic enhancement of the medicinal plants by adopting techniques such as *in vitro* regeneration and genetic transformations. It can also be harnessed for the production of secondary metabolites using plants cells as bioreactors (Tripathi and Tripathi, 2003). Plants which are rich in secondary metabolites are called medicinal plants and those metabolites are large and diverse array of organic compounds that appear to have no direct function in growth and development. They are biosynthetically derived from primary metabolites like amino acids, nucleotides, sugars and acyl lipids (Dubey, 2005). Secondary metabolites play an important role in providing defense against pest, pathogens and provide protection against UV radiation and stress, or could act as attractive volatile odor or pigments (Dewick, 1997; Samuelsson, 1992; Bourgaud *et al*., 2001).

1.2 Plant diversity in Palestine

In Palestine, there are many medicinal plants described for treatment of many diseases (Jaradat, 2005). Also, there are about 2600 species of plants up which more than 700 species are considered as medicinal plants (Shtayeh *et al*., 1998). Different plant species have been used in traditional medicine to treat various diseases (Palevitch and Yaniv, 1991). One of these species is the Christi's Thorn, *Ziziphus spina-christi*, which is a famous plant for its multipurpose uses in traditional medicine and also for its valuable honey.

1.3 *Ziziphus spina-christi* (L.) Desf.

1.3.1 Botanical description and geographical distribution

Ziziphus spina-christi (L.) Desf. (Rhamnaceae) is a tropical evergreen tree from Sudanese origin with edible fresh or dried fruits (Figure 1.1) (Dafni*et al*., 2005). It is known as Christ's Thorn. The Arabic name is "Sidder" and its fruit is called Nabag (Saied *et al*., 2008). This plant grows in East Africa and West Asia including Egypt, Saudi Arabia, and south Iran (Godini *et al*., 2009). It is a spiny tree that tolerates extreme heat and drought. It develops a very deep taproot system and has an amazing regenerative power (Saied *et al*., 2008). *Z. spina-christi* can be propagate by seeds, so it exhibits a broad genetic heterogeneity (Sudhersan and Hussain, 2003; Singh*et al*., 2006). The seeds are protected by hard woody coats called endocarp, which delay germination. To overcome this problem the seed coat has to be scarified before planting (Saied *et al*., 2008). Despite the traditional propagation by seeds, an alternative *in vitro* propagation for *Z. spina-christi* was reported by Sudhersan and Hussain (2003) using shoot tip and nodal segments as explants.

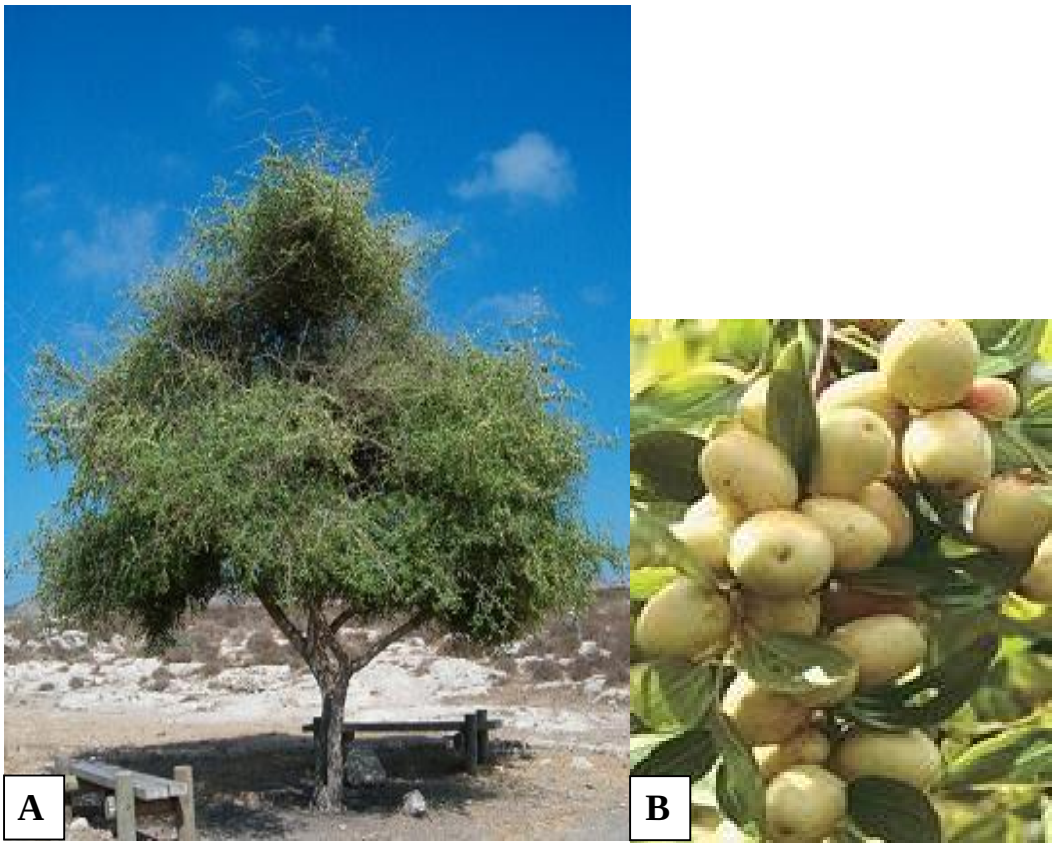

Figure 1.1 *Ziziphus spina-christi*(L.) Desf. Tree and fruit

A- Tree

B- Fruit

1.3.2 *Z. spina-christi* in Palestine

Z. spina-christi grows in Palestine in valleys and lowlands (Dafni *et al*., 2005). It has many traditional and medicinal uses around the Arabic and Islamic region. In Palestine, the leaves and young branches are used for treatment of eye inflammation; it is also used for the treatment of toothache, stomachache, and rheumatoid arthritis (Shtayeh *et al*., 1998).

1.3.3 Uses of *Z. spina-christi* in traditional medicine

In general, *Z. spina-christi* has many beneficial uses. For example the leaves are used as fodder for animals and the branches are used for fencing. The wood is used for construction and furniture. All parts of the plant (fruits, leaves, roots, bark) are used in traditional medicine. For that, it is called multiuse and multipurpose plant (Dafni *et al*., 2005; Saied *et al*., 2008; Shtayeh *et al*., 1998). There are many traditional uses for *Z. spina-christi*, the Arabs and Bedouins have used the paste of crushed roots for treatment of gum problems (Palevitch *et al*., 1985). Sinai and Negev's Bedouins have used the tea of fruits to increase milk production for nursing women and to treat liver problems (Danni *et al*., 2005). In Sudan the twigs are used externally to treat rheumatism and scorpion stings (El-Kamala and El-Halifax, 1999). Moreover, in the United Arab Emirates, the boiled leaves are used to treat hair fall (Saied *et al*., 2008).

There are many reports on medical effect of different extraction from different parts of *Z. spina-christi*. The methanolic extract of the stem bark reduces diarrhea in rats (Adzu *et al*., 2003) whereas the methanolic extract of leaves protects against hepatic carcinogenicity in rats (Abdel-Wahhab *et al*., 2007). The butanolic extract of the leaves control the glucose level in rats safely (Abdel-Zaher *et al*., 2005). The aqueous extract of the root bark has an antinociceptive activity (Adzu *et al*., 2002), and a central depressant effect (Adzu *et al*., 2002) in mice. The powder of the seeds showed high activity against *Escherichia coli* and *Bacillus subtilis* (Nazif, 2002). Furthermore, Avizeh *et al*. (2010) found that the hydro alcoholic extract of *Z. spina-christi* fruit decreases the blood glucose level in dogs. Also Waggas and Al-Hasani (2009) reported that the aqueous extract of *Z. spina-christi* fruit decreases the neurotransmitter content in the brain of male albino rats. Shahat *et al*. (2001) reported that the ethanolic extraction of fruits and the aqueous extract of the leaves showed

antiviral properties against *Herpes simplex virus type1*(*HSV-1*). Godini *et al*. (2009) reported that the hydro alcoholic extract of *Z. spina-christi* leaves induce contraction in the endothelium intact in the isolated rat aorta.

1.3.4 Chemical constituent of *Ziziphus spina-christi* and the main active ingredients

All parts of *Z. spina-christi* contain important nutrients and phytochemical compounds. The fruit is rich in carbohydrates (Saied*et al*., 2008). The seeds contain 28.5% lipid and 18.6% protein (Nazif, 2002). The leaves are rich in iron, calcium, and magnesium (Anonymous, 1992).

There are many studies that investigated the phytochemical constituents of*Z. spina-christi.* Ikram and Tomillnson (1976) reported that *Z. spina-christi* contains beotulic and ceanothic acid, Shah *et al*. (1986) also found three cyclopeptide alkaloids: Franaganine, Mauritine-C and Sativanine-A. Mahran *et al*. (1994) reported four saponin glycosides: Christinin A, B, C and D. Nawar*et al*. (1984) isolated several flavoniods from the leaves of *Z. spina-christi.* Also Weinges and Schick, (1995) separated dodecaacetylprodelphinidin B3 from the dried leaves of*Z. spina-christi.* Furthermore, Pawlowska *et al*. (2009) recognized twelve flavoniods compounds from the methanolic extract of *Z. spina-christi* fruits, Abdel-Galil and El-Jissry (1990) isolated a new peptide alkaloid spinanine-A from the stem bark of*Z. spina-christi.*

1.4 Plant tissue culture

1.4.1 Importance of tissue culture in the production secondary metabolites

Plant tissue culture is an important technique in plant biotechnology. There are many applications for plant cell culture such as micropropagation, production of secondary metabolites, and the study of plant cell genetics and pathology (Salehi*et al*., 2008). Research in the area of plant tissue culture technology has achieved the production of many pharmaceutical substances for new therapeutics (Abdin and Kamaluddin, 2006). Advances in the area of plant cell culture leads to the production of secondary metabolites, such as alkaloids, anthocyanins, saponins, flavones, and many other compounds (Ramawat, 2008). As examples Kohda *et al*. (2011) produced two lignans, Gomisin A and F in calluses of*Schisandra chinensis* Besides, Christen *et al*.

(1989) reported for the first time the production of Taxol (placlitaxel) by *Taxus* cell cultures, which is a promising anticancer agent (Jordan and Wilson, 1995).

1.4.2 Callus tissue production

Naturally, callus initiated as a response to wounding and infestations of unions (Bottino, 1981). Indeed, Callus is an unorganized tissue that can be induced synthetically on solid nutrient media. Callus can also be initiated from different explants of intact plants. The best explants can be used for callus induction are sometime young tissues of a few cell types (Yang *et al*., 2009). The growing callus can be initiated on culture media with a balance between cytokinin and auxin concentration. Callus cells increase in fresh and dry weight without differentiation, but sometime differentiation results within the tissue mass. Usually, differentiation depends on the equilibrium of plant hormones of the support medium and the physiological status of the tissue.

1.4.3 Cell suspension culture and secondary metabolites production

Suspension culture is a suspension of friable callus grown in liquid media. Suspension culture is used in a number of biotechnological applications such as inoculums for plant bioreactors, which resemble biofermentors. Valuable secondary metabolites can be extracted from bioreactors during the growth of plant cells as cell suspensions.In addition, several compounds such as Shikonin, Berberine, and Ginseng saponins have been commercially produced from *in vitro* cell cultures (Ramawat, 2008).

1.5 Qualitative and Quantitative methods for secondary metabolites investigation

The classical methods for studying secondary metabolites involve extraction, purification and characterization. Solvent is being selected for extraction based on the solubility of secondary metabolites which are mostly neither extremely polar nor non polar. Aqueous alcoholic solvents are mostly used for extraction at different temperature and time period. Using High Performance Liquid Chromatography (HPLC), column and Thin Layer Chromatography (TLC) are classically used for purification and chromatography. Chromatography is used for separating and identifying contents of a mixture in order to investigate secondary metabolite of different callus lines of *Z. spina-christi.*

1.5.1 Extraction of medicinal plants constituents

In order to investigate and study the chemical constituents of any medicinal plant, certain extraction procedure must be performed as an initial process. Two steps are usually critical for efficient extraction. First, ruptured cells should be ground or homogenized in the extraction solvent. Second, after homogenization, the extraction mixture should be allowed to stand for 24 to 48 h at a temperature that will not allow degradation of interested compounds (Cseke *et al.*, 2006). This is done simply to allow time for the solvent to penetrate all parts of the ruptured cells. There are many features affect the plant extract contents such as, the procedure used in the extraction, the constituents of the extracted plants and the nature of the solvents, organic or inorganic.

1.5.2 Thin layer chromatography (TLC)

Chromatography is one of the most useful means of separating mixtures of compounds. It is a technique to purify the components and identify them. In chromatography, the mixture is separated by differential distribution of the components between a stationary phase and a mobile phase. Thin Layer Chromatography (TLC) is one of chromatographic technique that has used in isolation and analysis of natural products. It is selected over other methods because it is the fastest, cheapest and the simplest technique used in extract analysis (Robards and Antolovich, 1997). In TLC, the adsorbent is coated on one side of a plate of glass or a strip of plastic or aluminum. Common adsorbents are silica gel and alumina (Cseke *et al*., 2006).

2. OBJECTIVES

Z. spina-christi is a highly adapted species for dry and hot climates (Saied *et al*. 2008). It grows in desert areas with annual rainfall 50-300 mm (Maydell, 1986), and found in valleys where underground water is available (Saied *et al*., 2008). In addition, *Z. spina-christi* is distributed over the whole Sahelian area from Senegal to Sudan and across many areas in Middle East, North Africa, and North West India (Arbonnier, 2004).

Plant growing in the harsh environment of desert produces various types of secondary metabolites that play a role in defense against drought, salinity and pathogens, and serve as an excellent source of bioactive metabolites such as flavonoids, alkaloids, and steroids.

The previous mentioned characteristics of *Z. spina-christi,* makes it highly variable in its phytochemical constituents and secondary metabolites production. For that, I conducted this research to investigate the following purposes:

1. Optimizing *in vitro* culture protocol for callus induction from different tissue lines of *Z. spina-christi* to be used for secondary metabolites production.
2. Compare the chemical constituents of wild type and the*in vitro* plants.
3. Bioassay of cotyledonary callus extract against breast cancer cell line MCF-7

CHAPTER TWO

MATERIALS AND METHODS

2.1 Plant material

The dried fruits of *Ziziphus spina-christi* (L.) Desf. were collected in November 2010 from MADICO (Manasrah Development and Investment Company) Date Farm near Jericho. The plant was characterized and authenticated by Dr. Rami Arafeh, Biotechnology Research Center, Palestine Polytechnic University, Hebron, Palestine. Fruit exocarp was removed manually and endocarp containing seeds were dried and stored at room temperature for initiating the *in vitro* plant culture.

2.2 Media preparation and sterilization

MS medium was used in all experiments in this study (Murashige and Skooge, 1962). As recommended by the manufacture, 4.4g/l salt was dissolved in 70% of the media final volume on a magnetic stirrer and then 30g/L sucrose (w/v) were added. The media was supplemented with the plant growth regulators (PGRs) according to each experiment's objective, and then the media was completed to the final volume with distilled water. After that the pH was adjusted to 5.8 using 1M NaOH or 1M HCl with continuous stirring. The medium was gelled by adding 0.06% (W/V) regular bacteriological agar. Finally, the medium was dispensed into 500 ml bottle (400 ml media / bottle), closed with aluminum foil and then autoclaved at 121ºC and 15 kg/cm² pressure for 20 minutes.

2.3 *In Vitro* and *ex vitro* seed germination

2.3.1 *In Vitro* seed germination

The seeds were separated from the woody endocarp, and then washed by running water for 5 minutes. After that, one drop of Tween 20 with sufficient amount of water for 60 minute with continuous shaking. The seeds were rinsed by sterile distilled water three times before disinfection by 20% v/v commercial Chlorax (5.0 % NaOCl) with continuous shaking for 15 minutes. Seeds then were washed by sterile distilled water three times under the laminar flow cabinet. Additional disinfection step was done by placing the seeds in ethanol 70% for 30 seconds. Finally, they were washed by sterile distilled water three times.

After the sterilization procedure, seeds were cultured on water gelled by agar (6.0 g/l) at pH 5.8, then was incubated in growth room at 25°C under photoperiod of 16 hours light duration with white fluorescent tubes 45 µmol / m² / sec.

2.3.2 *Ex Vitro* seeds germination

Seeds were planted on natural garden soil to obtain *ex vitro* growing plants for further experiments.

2.4 Induction and maintenance of callus tissue

2.4.1 Callus induction

The callus tissue was induced from cotyledons, leaves, and roots. The basic induction media was MS supplemented with 1.3 mg/l BA and 0.3 mg /l NAA. The inoculated explants were placed on Petri dishes 5.0 cm diameter. Cultures were incubated under photoperiod of 16 hours light duration or darkness at 25°C to test the effect of light on callus induction. Treatments were arranged in CRD with four replicates per treatment and four explants per replicate. Data for callusing percentage, fresh weight, and callus characteristics were recorded after one month of explants culture.

2.4.2 Callus subculture

Depending on the results from the callus induction experiment in the previous section, callus was subcultured on the induction media with 1.3 mg/l BA and 0.3 mg /l NAA to determine the growth curve and examine the effect of the dark and light in growth performance. Treatments were arranged in CRD with four replicates per treatment and four calli pieces per replicate each pieces weight 20.0 mg. The fresh weight was measured every week and the period of the treatment continued for five weeks.

2.4.3 Callus maintenance

After establishing an experiment for testing the best combination of BA and NAA hormones in MS medium, the callus was maintained on the same induction media and subcultured every four weeks. The callus pieces were inoculated on M1, M2, M3, M4, M5, and M6 media (Table 2.1). Treatments were arranged in CRD with five replicates per treatment and four callus pieces per replicate. Data for callus fresh weight were recorded after one month and then the data for dry weight were recorded after five days of drying the callus.

Table 2.1: Media used for callus initiation and maintenance

Media type	PGRs combination on MS media
M1	1.3mg/l BA
M2	1.3mg/l BA + 0.1mg/l NAA
M3	0.3mg/l BA + 0.3mg/l NAA
M4	1.3mg/l BA + 0.3mg/l NAA
M5	0.3mg/l BA + 0.6mg/l NAA
M6	0.6mg/l NAA

2.5 Effect of different PGRs on cotyledonary callus growth and characteristics

The aim of this experiment is to study the effect of different PGR combinations on callus growth and characteristics. Callus pieces (20.0 mg) were cultured on X1, X2, X3, and X4 media (Table 2.2). Treatments were arranged in CRD with five replicates per treatment and four callus pieces per replicate. Callus fresh weight and callus characteristics were recorded after one month

Table 2.2: List of the four media used to study cotyledonary callus growth and characteristics

Media type	PGRs combination on MS media
X1	0.5mg/l BA + 2.0mg/l 2,4 D
X2	2.0mg/l BA + 2.0mg/l 2,4 D
X3	1.0mg/l BA + 2.0mg/l NAA
X4	2.0mg/l BA + 0.5mg/l NAA

2.6 Cell suspension culture

2.6.1 Initiation and maintenance of cell suspension culture

One gram of cotyledonary callus was placed in 500 ml Erlenmeyer flasks containing 100 ml of liquid MS medium supplemented with 1.3 mg/L BA and 0.3 mg/L NAA. The flasks were maintained on orbital shaker at 120 rpm and 23± 1°C under photoperiod of 16 hours light duration to initiate the suspension cultures. The cell suspension culture was maintained by placing 5.0 ml of media containing cells into fresh liquid media. Subcultured of cells were performed after cells suspension fresh weight reached 0.55 g/5ml and this occurs after 27 days from incubation.

2.6.2 Determination of cotyledonary cell suspension growth curve

In order to quantify cell growth, one month-old suspension culture was used. From this suspension, aliquots of 3.0 ml were inoculated into 33 flasks, each contained 25 ml of MS medium supplemented with 1.3 mg/l BA and 0.3 mg/l NAA. The experiment was set in a CRD with 11 treatments (number of days in culture) and 3 replicates. Evaluations were made at 3-day intervals up to 33 days in culture. Fresh weight of cells was determined by centrifuging 5.0 ml of cell suspension at 5000 rpm for 10 minutes in a graduated centrifuge tube. The tube was gravimetrically measured after decanted the supernatant and placed in an oven at 60°C for 2 hours. In the end, the tube plus cells was weighed for dry matter determination.

2.6.3 Extraction of crude extracts from dry cotyledonary suspension cells

One gram of dried cells from cotyledons (Table 2.3) was immersed in 100 ml 90% ethanol in 250 ml Erlenmeyer flask with continuous stirring for 72 hour in the dark. After that, the extracted solution was centrifuge for 15 minute at 5800 rpm to take the supernatant. The supernatant was air dried under sterile conditions. The yield was estimated for cell suspension originated from cotyledonary callus.

2.7 Qualitative detection of biochemical's in different tissues of *Z. spina-christi* using Thin Layer Chromatography (TLC)

2.7.1 Preparation of plant material

Eight samples were used in this experiment (Table 2.3). Two samples of *Z. spina-christi* were taken from *in vitro* roots and leaves. Three samples were taken from *ex*

vitro roots, leaves, and fruits. Three samples were taken from callus of roots, leaves, and cotyledons. *In vitro* roots and leaves were collected from three months grown seedling on a half strength MS medium with 20% (w/v) sucrose. *Ex vitro* roots and leaves were collected from six months grown plants and *ex vitro* fruits were collected from MADICO farm near Jericho. The callus of roots, leaves, and cotyledons were collected from the sixth subcultured callus grown and maintained on MS medium supplemented with 1.3 mg/l BA and 0.3 mg/l NAA. Tissues were dried at room temperature for two weeks and used for further extraction.

Table 2.3 Nature and age of the plant tissues of *Z. spina-christi* used for the extraction of crude chemical.

Plant tissue	Description
In vitro roots	Three months old plant
In vitro leaves	Three months old plant
Ex vitro roots	Six months old plant
Ex vitro leaves	Six months old plant
Ex vitro fruits	Collected from MADICO farm
Roots callus	Sixth subcultured period
Leaves callus	Sixth subcultured period
Cotyledonary callus	Sixth subcultured period
Cotyledonary cell suspension	Collected 27 days after inoculation

2.7.2 Extraction procedure

From each sample, described in Table 2.3, one gram of dried powder was taken and subjected to extraction. Each sample was immersed in 100 ml of 90% ethanol in 250 ml Erlenmeyer flask with continuous stirring for 72 hours in the dark. After that, the extract was centrifuged for 15 minutes at 5800 rpm to take the supernatant. The supernatant was air dried under a chemical suction hood then the yield was calculated by measuring the weight for each plant part.

2.7.3 Extract preparation for detection of secondary metabolites

To detect secondary metabolites plant tissue samples were prepared as follows: 50.0mg of dried extracts from the eight samples and 50.0 mg of dried cotyledonary cell suspension were dissolved in 1.0 ml of 90% ethanol. Then were shaken for 24 hours. The samples were centrifuged at 5000 rpm for two minutes. Finally the supernatant from the samples were stored for further analysis.

2.7.4 Thin Layer Chromatography (TLC) analysis

The analysis of the previous samples was carried by using aluminum-backed silica-gel plates. The mobile phase which was used in the experiment is a mixture of ethyl acetate; formic acid; glacial acetic acid; water (100:11:11:26). It was examined under ultraviolet (UV) light.

2.7.5 Isolation of secondary metabolite from callus extract

The cotyledonary callus extract which weighed 62.0 mg was dissolved in 0.5 ml chloroform. The resulted solution was spotted along TLC plate. The TLC plate was placed in a chamber containing mobile phase ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26). After waiting until the solvent reached the top of the TLC plate, the plate was taken and air dried. The plate was examined under UV detector. As a result, a band appeared. This band was scratched and washed by 5.0 ml of chloroform. After that, the solution was centrifuged at 5800 rpm for two minutes in order to separate the silica and get the supernatant which contains the separated compound. The washing process was repeated three times. Finally, the supernatant was placed under a chemical hood in order to evaporate the supernatant and get the presented secondary metabolite. The yield was calculated by dividing the obtained weight by the started weight. The compound was stored for further characterization.

2.8 Cotyledonary callus extraction procedure and testing their biological activity against breast cancer cells line MCF-7

The bioassay part was carried out in University of Zurich, Institute of Organic Chemistry. Dried and powdered cotyledonary callus (4.85 g) was suspended in 150 ml of 90% ethanol and stirred at room temperature in the dark for 72 hours. The mixture was centrifuged and the supernatant was filtered through cotton. The precipitate was

further washed twice with 10 ml ethanol and solvent was evaporated till dryness. The resulted solid matter (1.2 g) was further purified by dissolving the mixture in 30 ml acetone and the insoluble solid was filtered off. Acetone was completely evaporated and the final solid matter was dried under high vacuum to obtain 350 mg as a light yellow solid. Stock solution of 50 mg/ml was prepared in DMSO and kept at -20ºC. Acetone extract was evaluated against breast cancer cell line (MCF-7) for cytotoxicity evaluation.

2.9 Measuring the cytotoxicity for breast cancer cell line MCF-7 after subjected for cotyledonary callus extract

A standard resazurin reduction assay was used to quantify metabolic activities. 5000 cells in 100µl of media were seeded in 96-well plates and grown overnight. Callus extract was serially diluted into supplemented media using a separated 96-well plate, applied to the cells and incubated for 48 hours at 37ºC with 5.0% CO_2, and 80% relative humidity. The used media and extract were then removed and 100 µl of fresh media containing 10% (V/V) of 860µM solution of resazurin was added and incubated for 2 hours. The plate was read for fluorescence in a SpectraMax M5 plate reader using excitation at 560 nm, emission at 590 nm, and a 570 nm cut-off filter.

2.10 Statistical analysis

For the tissue culture part, Complete 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 Sigma Plot version 12.0. Means were separated according to Fisher's least significant difference (LSD) test at $p \leq 0.05$ level of probability.

CHAPTER THREE

RESULTS

3.1 *In Vitro* and *ex vitro* seeds germination

The percentage of germination for *in vitro* seeds was 90% while for *ex vitro* seeds was 70% without any contamination or abnormal seedling (Table 3.1, Figure 3.1). The *in vitro* germination of seeds started after 10 days whereas that of *ex vitro* seeds started after 20 days. The difference of germination time between *in vitro* and *ex vitro* is probably due to the difference in physical culture conditions between *in vitro* and *ex vitro* treatments germination.

Table 3.1 Germination type and the percentage of germination for *Z. spina-christi*

Germination type	No. of seeds	Germination percentage	contamination	Abnormal seedling
In Vitro	50	90 %	0 %	0 %
Ex Vitro	10	70 %	0 %	0 %

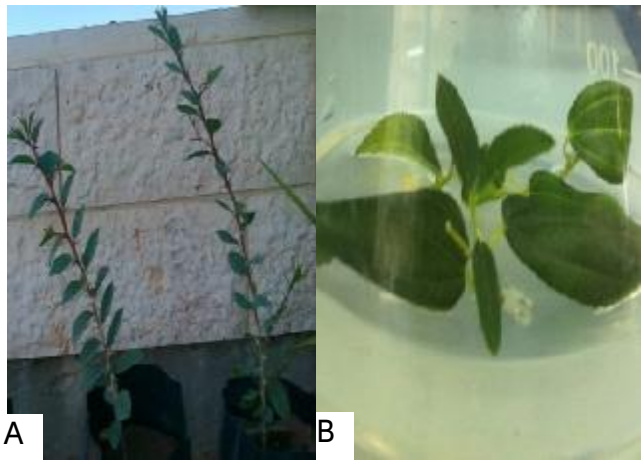

Figure 3.1 Seedlings of *Ziziphus spina-christi*

A- *Ex vitro* seedlings

B- *In vitro* seedlings

3.2 Induction and maintenance of callus tissue

3.2.1 Callus induction

The experiment was conducted to initiate and produce callus tissue under the light and dark condition on MS media supplemented with 1.3 mg/l BA and 0.3 mg /l NAA. Different tissues were used to initiate callus including cotyledons, roots, and leaves. Callus was inducted from all types of explants used in the experiment in the light and dark at different percentages. Roots and cotyledons gave 100% callus induction in the dark and light (Table 3.2). However, all of the callus tissue under dark started to grow eight days after inoculation. Under light, however, callus from roots started after eleven days of inoculation. Callus from cotyledons started after twenty days of inoculation. Moreover, callus from leaves started after thirty days from inoculation (Table 3.2). Successful fresh weight and healthy friable callus were obtained in the dark from leaves, cotyledons and roots. By contrast, healthy friable callus was only obtained from cotyledons and roots in the light (Figure 3.2, Figure 3.3). Variation in callus color and texture was observed in each treatment (Table 3.3, Figure 3.3, 3.4).

Table 3.2 Percentage of the callus inducted from different explants of Z *spina-christi* under light and dark conditions on MS media supplemented with 1.3 mg/l BA and 0.3 mg /l NAA after 4 weeks of culture.

Explants type	Induction condition	Number of explants	Number of inducted explants	Percentage of induction	Time for induction (days)	Callus characteristics
Roots	Light	16	16	100%	11	Yellowish, friable
Roots	Dark	16	16	100%	8	Brownish, friable
Leaves	Light	16	8	50%	30	Green, nodular
Leaves	Dark	16	13	81.25%	8	Very pale greenish, friable
Cotyledons	Light	16	16	100%	20	Green, friable, florescent under UV
Cotyledons	Dark	16	16	100%	8	White, friable

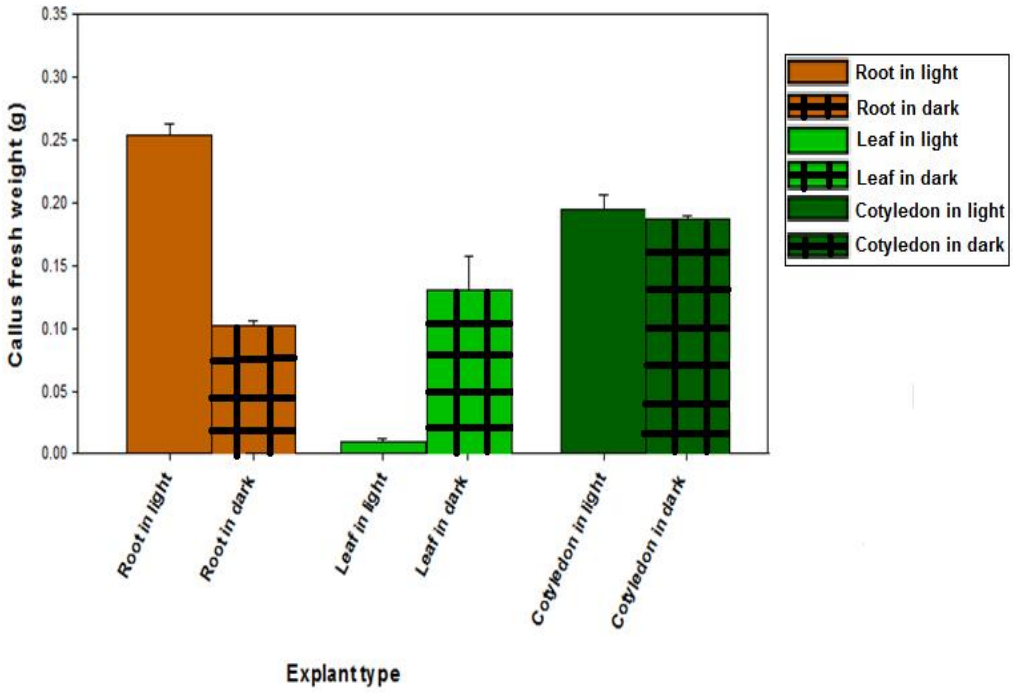

Figure 3.2 Fresh weight of callus from roots, leaves, and cotyledons of *Z. spina-christi* grown under light and dark conditions.

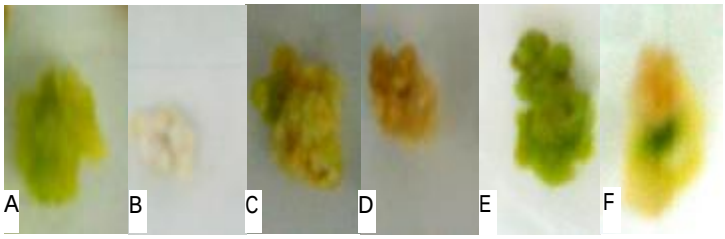

Figure 3.3 Variation in callus colors appeared in different explants of *Z. spina-christi.*

A- Cotyledonaries callus in light B- Cotyledonaries callus in dark

C- Roots callus in light D- Roots callus in dark

E- Leaves callus in light F- Leaves callus in dark

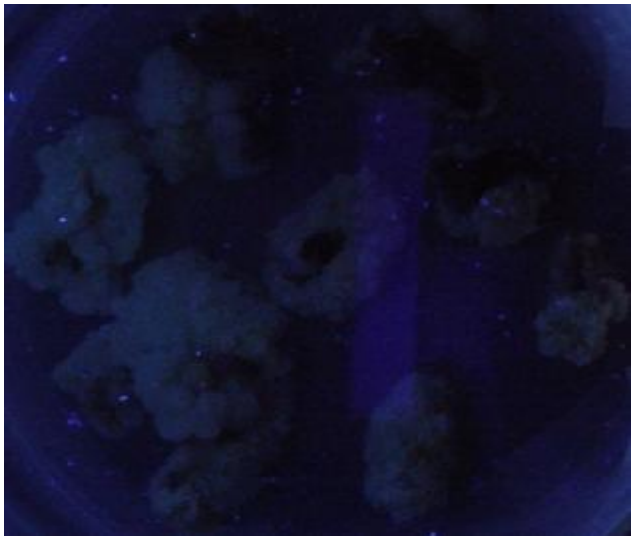

Figure 3.4 Callus of *Z. spina-christi* as appeared under UV light

3.2.2 Callus subculture

The growth curve of *Z. spina-christi* callus represented sigmoidal pattern where the three growth phases can be distinguished (lag phase, exponential phase, and stationary phase) (Figure 3.5).

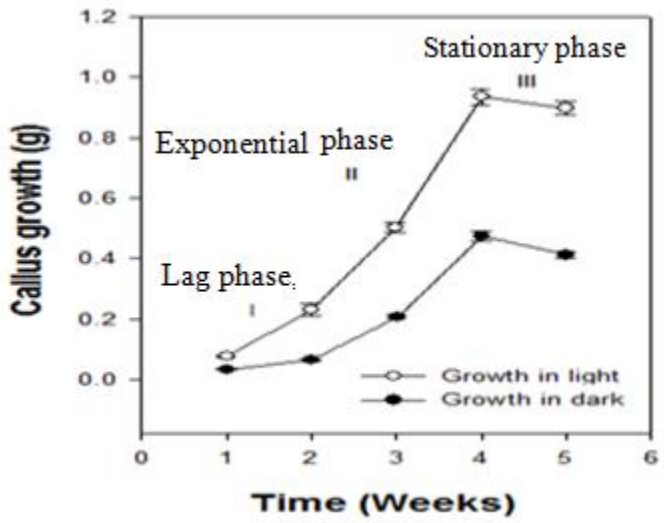

Figure 3.5 Cotyledonary callus growth curve in light and dark conditionon MS media supplemented with 1.3 mg/l BA and 0.3 mg /l NAA. I– Lag phase, II – Exponential phase, III –Stationary phase.

The results indicated that the transfer of the callus derived from different explants of *Z. spina*-christi should be carried out at the beginning of the stationary phase; (at the start of the fourth week from incubation).

3.2.3 Callus maintenance

Callus obtained from the different explants of *Z. spina-christi* was maintained for seven months on MS media supplemented with 1.3 mg/L BA and 0.3 mg /L NAA

(Figure 3.6). The highest fresh weight of cotyledonary callus was obtained on MS medium supplemented with 1.3 mg/l BA and 0.3 mg /l NAA (Figure 3.7).

Repeated subculture of callus is required for producing and maintenance it for much time for further experimental study.

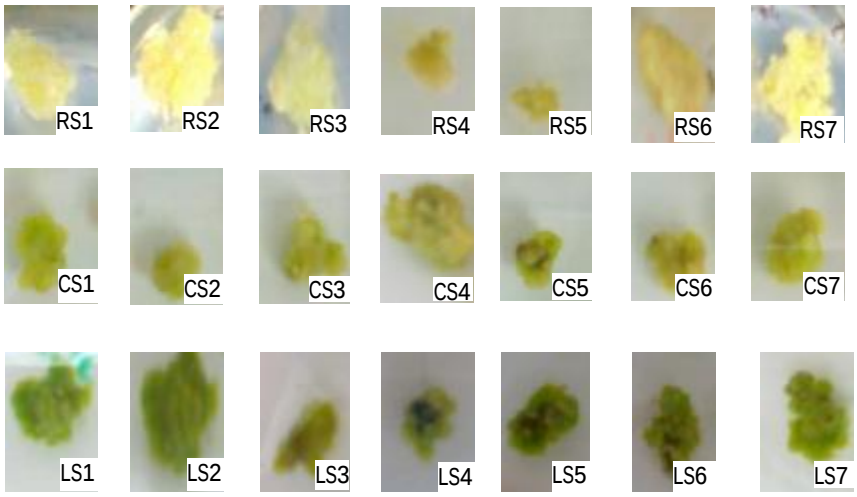

Figure 3.6 Callus maintenance of the seventh subculture of root, cotyledon and leaf explants of *Z. spina-christi*. (RS-Root subculture, CS-Cotyledon subculture, LS- Leaf subculture).

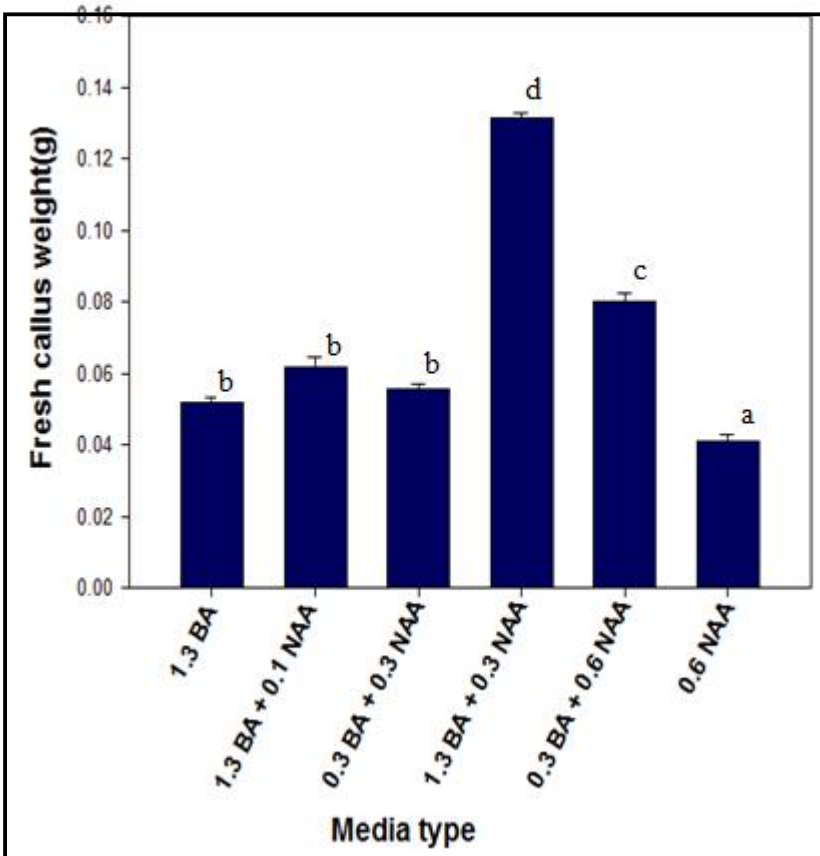

Figure 3.7 Effect of different concentration and combination of BA and NAA on cotyledonary callus growth.

3.2.4 Effect of different plant growth regulators (PGRs) on cotyledonary callus growth and characteristics

Auxins and cytokinins are the most widely used plant growth regulators in plant tissue culture and usually used together (Gang *et al*., 2003). Subsequently plant growth regulators were added into MS medium to test their effects on callus characteristics.

The results showed that the increase in the concentration of NAA from 0.5 mg/l to 2.0 mg/l reduce the cotyledonary callus fresh weight (Figure 3.8). Also it was observed that other PGRs combinations affected the cotyledonary callus color (Figure 3.9).

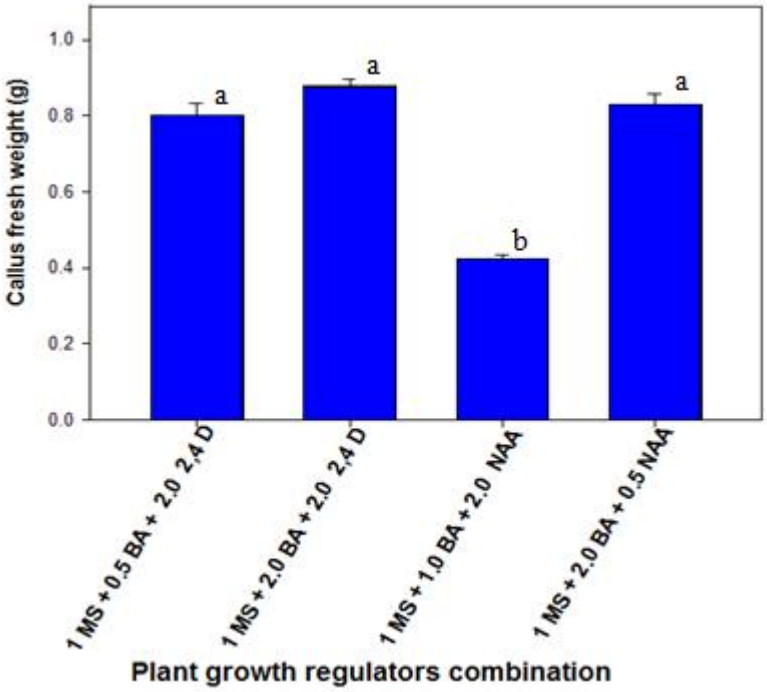

Figure 3.8 Effect of different combination of plant growth regulators on cotyledonary callus fresh weight.

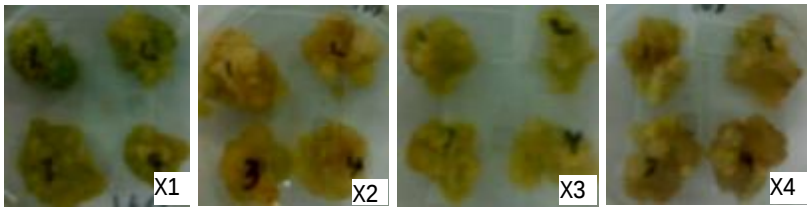

Figure 3.9 Effect of different hormonal combination on cotyledonary callus colors

X1 0.5mg/l BA + 2.0mg/l 2, 4 D **X2** 2.0mg/l BA + 2.0mg/l 2, 4 D

X3 1.0mg/l BA + 2.0mg/l NAA **X4** 2.0mg/l BA + 0.5mg/l NAA

3.3 Cell suspension culture

3.3.1 Initiation and growth determination of cell suspension culture of *Z. spina-christi*

Homogenous cell suspension culture was observed after the initiation of suspension culture from cotyledonary friable callus of*Z. spina-christi* line.

Growth determination provides important information for cell suspension maintenance and further utilization. Figure 3.10 illustrates the cell suspension growth curve. The cell growth peak observed after 26 days of inoculation in both fresh and dry matter. After that, it showed a growth decrease. These measurements were mainly used to decide the best time for medium replacement during cell suspension maintenance and also the best time for the cultivation of the secondary metabolite that produced by the cell.

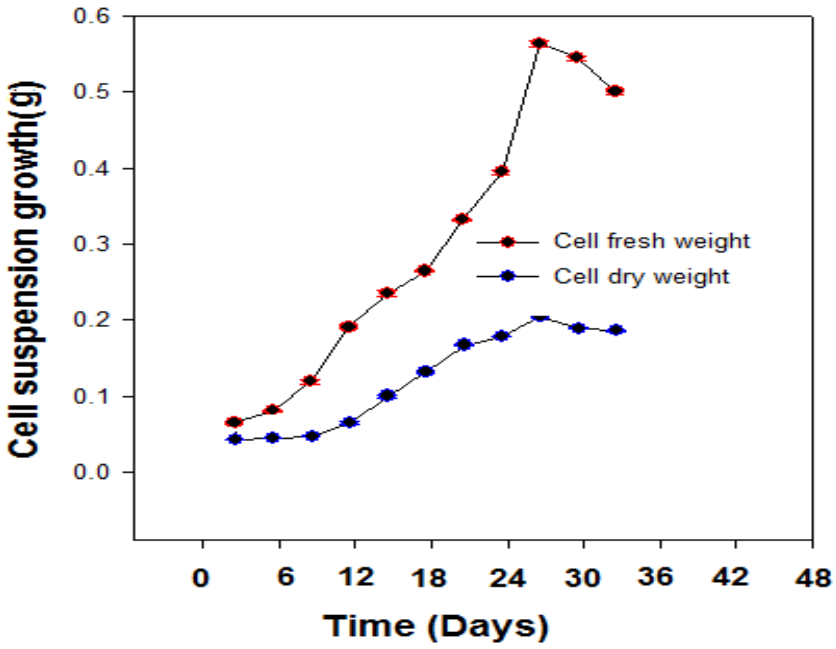

Figure 3.10 Growth curve of cell suspension culture of Z *spina-christi*, curve estimated within 36 days at fresh and dry weight basis. The lines in each spot represented the standard error.

3.4 Detection and determination of chemical constituents of *Z. spina-christi* using TLC

3.4.1 Extraction procedure

The goal of this experiment was to compare the chemical profile of different extracted tissues of *Z. spina-christi.* There is a difference in the obtained yield between plant tissues grown under *in vitro* or *ex vitro* conditions. Results showed that the cotyledonary callus has the highest percentage yield 26.1%, whereas the cotyledonary cell suspension extract has the lowest percentage yield 12.67% (Table 3.3). The percentages of extraction yield were calculated as a result of dividing the weight of the extracted powder by the weight of the extracted plant material (Table 3.3).

Table 3.3 Percentage yields of total extracts obtained from the parts used of extraction from *Z. spina-christi*. (One gram of dried powder from each plant parts was used for the extraction in 100 ml 90% ethanol)

Extracted type	Yield obtained	Percentage yield
Roots callus	0.2383 gram	23.83%
In vitro roots	0.195 gram	19.5%
Ex vitro roots	0.0924 gram	9.24%
Leaves callus	0.1315 gram	13.15%
In vitro leaves	0.244 gram	24.4%
Ex vitro leaves	0.2414 gram	24.14%
Cotyledonary callus	0.2610 gram	26.1%
Cotyledonary cell suspension	0.1267 gram	12.67%
Ex vitro fruits	0.2162 gram	21.62%

3.4.2 TLC analysis of extracted parts of *Z. spina-christi*

Extracts derived from nine tissues were investigated by TLC method. It was clear that the same band appeared in all lines in different intensities (Figure 3.9). TLC image indicated that cotyledonary and leaves callus have the most concentrated band compared to other tissue lines (Figure 3.11).

The R_f value of the produced secondary metabolite (R_f equals 0.45) was calculated by dividing the moved distance of the secondary metabolite on the moved distance of the solvent system.

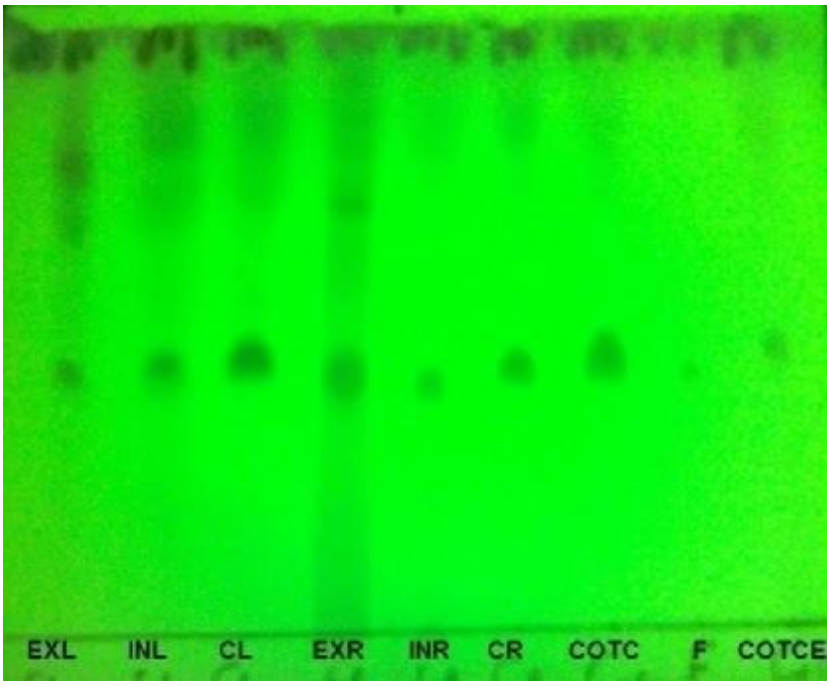

Figure 3.11 TLC plate viewed under UV detector of different ethanolic extraction lines of *Z. spina-christi*

EXL *ex vitro* leaves, INL *in vitro* leaves, CL callus leaves

EXR *ex vitro* roots, INR *in vitro* roots, CR callus roots

COTC cotyledons callus, F fruits, COTCE cotyledons cell

3.4.3 Isolation of secondary metabolite from cotyledonary callus extract

The obtained weight of the detected secondary metabolite after evaporating the solvent under chemical hood was 23.0 mg. The yield of the presented secondary metabolite was 37% resulted from dividing the obtained weight (23.0 mg) by the initial weight used (62.0 mg).

3.5 Biological activity of cotyledonary callus extract against breast cancer cells line MCF-7

The aim of this experiment was to test the cytotoxic activity of cotyledonary callus acetone extract against MCF-7 compared to blank DMSO (Figure 3.12). The tested extract presented IC_{50} value = 95 µg/ml.

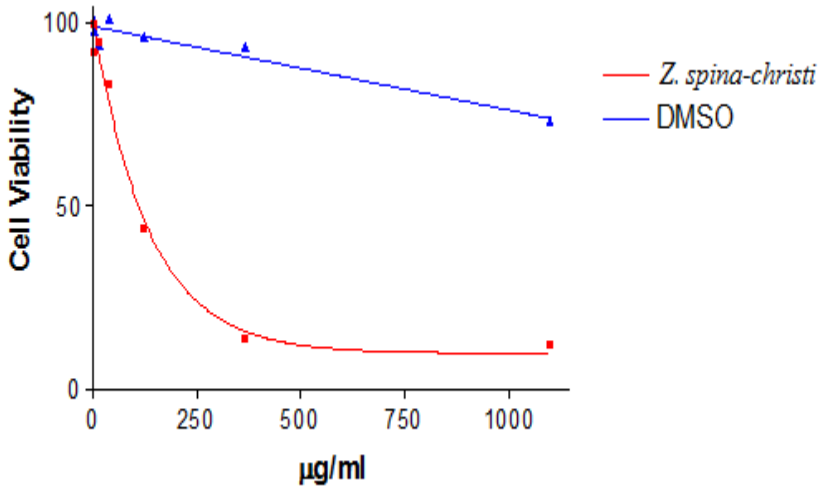

Figure 3.12 The effect of cotyledonary callus extract on the viability of the breast cancer cell line MCF-7 after 48h of co-incubation

CHAPTER FOURE

DISSCUSSION

The present investigation was undertaken on the multipurpose plant *Z. spina-christi* with more focus on callus culture characteristic and secondary metabolites production under *in vitro* conditions.

From the studies that investigated the *in vitro* propagation of Z. *spina-christi,* Sudhersan and Hussain (2002) optimized a protocol for the *in vitro* clonal micropropagation of *Z. spina-christi.* Assareh and Sardabi (2005) investigated the macropropagation and micropropagation of *Z. spina-chriti* in different PGRs combinations. Al-Sulaiman and Barakat (2010) established a protocol for multiplication of *Z. spina-christi* by shoot tip culture. However, to the best of my knowledge there are no published reports on *in vitro* factors affecting callus induction of *Z. spina-christi,* and studying the secondary metabolite production. Hence, I undertook this study to optimize a protocol for callus induction and cell suspension culture of this plant species.

Establishing *in vitro* cultures of plant using *ex vitro* plant tissues as explants could not be always accomplished. Mainly due to the high contamination rates and reduction or loss of morphogenetic ability (Tan *et al*., 2010). On the contrary, using *in vitro* plant tissue as explants for initiation of *in vitro* cultures are favored because of their high morphogenetic potential and low level of contamination (Yang *et al*., 2009). Hence, we used *in vitro* explants in the present study.

4.1 Induction and maintenance of callus tissue

It is evident from the results that different explants of *Z. spina-christi* produced callus that differs in its characteristics. However, the production of *in vitro* callus is the result of the interaction of environmental conditions and the genotype of the cultured plant cells. Morphological and phytochemical differences in callus are attributing to culture conditions, composition of the medium (Torrey, 1966), orientation of the explants (Mathysse and Torrey, 1967), and growth and morphogenesis ability of plants itself. Types and concentrations of hormones present in medium directly affect the texture, color and biochemical composition of callus (Skoog and Miller, 1957).

Chen *et al*. (2002) reported that 3/4 MS media was the best choice for callus induction of leaf explants in *Z. jujuba* 'muzao'. Sudhersan and Hussain (2003) reported that media without any growth hormones enhance shoot growth and elongation, whereas media with auxin or cytokinin induced callusing form Z. spina-christi shoot tip. The combination of BA is widely preferred with IAA or NAA for callus induction (Ripley and Preece, 1986). In the present investigation, however, six types of callus with different colors were produced from different explants in the light and dark conditions. All that was produced through using 1.3 mg/L BA and 0.3 mg /L NAA combination to initiate callus from cotyledons, leaves, and roots explants (Table 3.2, Figure 3.3, Figure 3.4). In addition to that reasonable callus fresh weights resulted from cotyledons, leaves, and roots explant (Figure 3.2). It was clear that the difference in colors, weight and textures could be attributed to difference in functional genes present in each type and as a response to chemical and physical properties of the culture conditions.

Some explants required to be cultured in the dark conditions in order to increase the callus efficiency and reduce the secretion of phenolic compounds, which generally affected the explants survival (Tan*et al*., 2010). He *et al*. (2002) found in their study of *Zizyphus jujuba* Mill. 'Huizao'that the frequency of callus induction of leaf explants in the dark treatment was 100%. They also found that 10% of the leaves during culture in the light were induced to callus with low quality and dark color. On the other hand, 78% of the leaves died without giving any callus. In my study explants were inducted in the light and dark condition. Data indicates that the dark condition was better for inducting callus tissue from leaf explants than the light (Figure, 3.2). The maintenance of callus growth was better under the light condition. Because of that the study was continued under the light condition. Additional advantage of the light conditions was the production of some secondary metabolites (Bakhshi and Arakawa, 2006; Abreu *et al*., 2005).

4.2 Effect of different plant growth regulators (PGRs) on cotyledonary callus growth and characteristics

As a result of high growth of cotyledonary callus, a trial was conducted for studying the effect of PGRs combination on callus characteristics.

Plant growth regulators influence the nature of callus. The presence of IAA or NAA in growth medium induced green colored callus from explants (Hilderbrandt*et al*., 1963). Many investigations suggested that combination of auxin and cytokinin always gave good callus proliferation and maintenance (Karami, 2008; Salmæ*t al*., 2008). Significantly in this investigation, it was found that the combination of 1.0mg/l BA + 2.0mg/l NAA in MS media reduced the proliferation of cotyledonary callus. The fresh weight of callus after four weeks was 0.42 g (Figure4.8). The combination of 0.5mg/l BA + 2.0mg/l; 2, 4 D, 2.0mg/l BA + 2.0mg/l 2, 4 D, and 1.0mg/l BA + 2.0mg/l NAA in MS media made a good proliferation of callus. The average fresh weight after four weeks was 0.8 g ± 0.05 (Figure 3.8).

Ye *et al*. (2012) in their study of the factors affecting callus induction and adventitious shoot regeneration of Zizyphus *jujube,* mentioned that greenish-yellow and compact callus was found to be the most effective for regeneration. In this study, the best healthy and friable green callus was obtained from cotyledonary explant which also was rich with UV active chemicals (Figure 3.4).

4.3 Callus subculture and cotyledonary callus growth curve for *Z. spina-christi*

There are many research reports in the literature addressed establishment of callus growth curve for different plant species. However, regarding Z. *spina-christi* it's the first time for establishment cotyledonary callus growth curve. In this study, it was established a cotyledonary callus growth curve using MS media supplemented with 1.3 mg/L BA and 0.3 mg /L NAA. The resulted curve followed a sigmoidal pattern with the lag, exponential, stationary and deceleration phases (Figure 3.5).

The lag phase -in which the cells of cotyledonary callus prepared themselves for the division- happened two weeks from inoculation of callus pieces. The exponential growth phase -period in which highest division of cells occurred- was observed between the second and third weeks after inculcation. The lag phase can be considered an energy producing phase and the exponential phase a biosynthesis phase (Shimizu *et al*., 1977).

The interval of growth deceleration was observed between the fourth and fifth weeks from inoculation. In this stage, callus should be transferred mainly because of the decreasing of the nutrients, agar dryness or accumulation of the toxic substances in

the culture medium (Abbade *et al*., 2010). In the stationary stage, the secondary metabolites also accumulate on the tissues and no cell division or weight increasing occurs (Santos *et al*., 2010).

Repeated subculturing of callus is required for producing and maintaining it for longer time. During the present study, callus growth declined on MS medium supplemented with BA or NAA alone. However BA and NAA together (the combination of 1.3 mg/L BA and 0.3 mg /L NAA) supported a healthy growth up to the seventh subculture (Figure 3.5, 3.6). This requirement is probably governed by the genotype and callus age.

4.4 Initiation and growth determination of cell suspension culture of *Z. spina-christi*

To the best of my knowledge, there is no available data or publications on the initiation and the growth of *Z. spina-christi* by cell suspension culture.

Our findings in cell suspension establishment were consistent with previous findings for callus proliferation rates using MS media supplemented with 1.3 mg/L BA and 0.3 mg /L NAA. The differences between the fresh weight and the dry weight of the suspension cells resulted from the moisture content of the tissues. The fresh weight to the dry weight ratio is a useful indicator for cell viability (Fu *et al*., 2005).

Results showed that the lag phase in all treatments lasted for 9 days before the beginning of the exponential growth phase (Figure 3.10). Furthermore, the time for subculturing the cells into new fresh liquid media was determined to be after 27 days of inoculation. This is because the early stationary phase occurs during that time. The best time to perform Subculturing was reported to be at the end of the exponential growth phase and early stationary phase of the cell growth pattern (Allan, 1991). In addition, media become exhausted due to nutrient depletion of the stationary phase. Also, cells will begin to accumulate toxic substances and growth inhibitors (Allan, 1991; Hopkins, 1995).The production of secondary metabolites in callus or suspension cultures can be obtained at the beginning of stationary phase.

4.5 TLC analysis of extracted parts of *Z. spina-christi*

The extraction of bioactive compounds from plant tissues is the first step in the utilization of phytochemicals during the preparation of pharmaceutical, cosmetic products and food ingredients. Secondary metabolites can be extracted from fresh or dried plant samples. Before extraction of plant samples, the samples usually treated by milling, grinding and homogenization which proceeded by air-drying (Dai and Mumper, 2010). Solvent extraction is the most commonly used procedures to prepare extracts from plant tissues due to their ease of use, efficiency, and wide applicability (Dai and Mumper, 2010). In this study, the nine tissues line of *Z. spina-christi* (Table 4.3) was extracted using 90.0% ethanol as a general solvent. The results showed that cotyledonary callus gave the best extraction yield. The difference in the yield was due to the type of solvents, extraction time, and sample to solvent ratio as well as the chemical and physical characteristic of the samples.

Extracts derived from the nine tissues were investigated by TLC technique. The TLC results (Figure 3.11) showed a difference in the intensity of the resulted band. The best band intensity was observed for the cotyledon line. The resulted band was isolated, for further analysis and characterization.

4.6 Biological activity of cotyledonary callus extract against breast cancer cell line MCF-7

To the best of my knowledge this is the first study using *in vitro* callus from cotyledonary *Z. spina-christi* plant for measuring the anticancer activity against breast cancer cells line MCF-7. Many reports describe that the anticancer activity of the medicinal plants is because of the presence of certain phytoconstituents, which possess strong antioxidant activities. The acetone extract of the aerial parts of *Tragia involucrate.* Linn. has cytotoxic activety aginst MCF-7 cell line with IC_{50} value equal 177 µg/ml (Joshi *et al.,* 2011). Methanol extract of *Ononis hirta* (aerial parts) and *Inula viscosa* (flowers) were active extract against MCF-7 cells with IC_{50} of 27.96 and 15.78 µg/ml respectively (Talib and Mahasneh, 2010). Cell viability was inhibited by Aesculus indica crude extract in a dose dependent manner ranging from 34.2% at 10 µg/ml to 94% at 500µg/ml (Bibi *et al.,* 2012). The results in this study revealed that

cotyledonary callus extract possessed high cytotoxic activity against breast cancer cells. The IC_{50} value was equal 95 µg/ml and this value is considered significantly cytotoxic comparing to the IC_{50} value which equal 177 µg/ml for the acetone extract of *Tragia involucrate*. So the cotyledonary callus contains secondary metabolites that have antioxidant activity, the presence of secondary metabolites confirmed through the TLC experiment (Figure 3.11).

CHAPTER FIVE

CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

From this current study, it can be concluded that:

The Christ's thorn is a multipurpose evergreen tree. All parts of *Z. spina-christi* had been used in traditional medicine. Many studies reported that *Z. spina-christi* contains different compounds that have antimicrobial, antifungal and antiviral characteristics.

Plant tissue culture can be used as a good alternative method for multiplication and callus induction of *Z. spina-christi.* The seeds are a good starting tissue for in vitro culture with a 90% percentage of germination. Sterilization and aseptic environments play critical roles in establishing and studying the factors affecting callus induction of *Z. spina-christi*.

Our results suggest that MS medium supplemented with 1.3 mg/L BA and 0.3 mg /L NAA was a good medium for callus induction from roots, leaves, and cotyledons tissues of *Z. spina-christi.* Callus obtained from roots, leaves, and cotyledons tissues of *Z. spina-christi* were maintained and subcultured for seven months on MS media supplemented with 1.3 mg/l BA and 0.3 mg /l NAA. A sigmoidal Cotyledonary callus growth curve was established on the light and dark condition. The callus must be subcultured after four weeks of inoculation.

Our results suggested that auxins and cytokinins combination play a critical role in the characteristics and the growth of callus from *Z. spina-christi.* Friable and fluorescent cotyledonary callus was observed through using MS media supplemented with 1.3 mg/l BA and 0.3 mg /l NAA. Also MS media supplemented with1mg/l BA and 2mg/l NAA reduced the cotyledonary callus fresh weight significantly.

A cell suspension culture was established for *Z. spina-christi.* The 26-day interval allowed the determination of a cell growth peak in both fresh and dry matter followed by a decrease in growth.

The cotyledonary callus extract has the highest percentage yield 26.1%, while the cotyledonary cell suspension extract has the lowest percentage yield 12.67%. TLC analysis indicated that cotyledonary and leaves callus have the most intensive band compared to other tissue lines.

The bioassay of cotyledonary callus extract against breast cancer cell line (MCF-7) showed that the cotyledonary callus extract has cytotoxic effect with IC_{50} value equal 95 µg/ml.

5.2 Future work

Further studies should be conducted to identify and characterized the obtained product from different callus line. Also more studies are required in order to investigate the factors affect the obtained product, quantitatively and qualitatively. Besides, the obtained products will be subjected for more bioassay studies regards anti-microbial, antifungal and antioxidant effects.

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APPENDICES

Table 1.

Analysis of variance (ANOVA) for callus fresh weight of *Z. spina-christi* using different PGRs after four weeks of incubation.

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	**3**	**1.846**	**0.615**	**15.720**	**<0.001**
Residual	**76**	**2.976**	**0.0392**		
Total	**79**	**4.822**			

Table 2.

Analysis of variance (ANOVA) for callus fresh weight of *Z. spina-christi* cotyledon, leaf, and root at light and dark after four weeks of incubation.

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	**5**	**0.441**	**0.0882**	**3.442**	**0.007**
Residual	**79**	**2.024**	**0.0256**		
Total	**84**	**2.465**			

Table 3.

Analysis of variance (ANOVA) for callus fresh weight of *Z. spina-christi* cotyledon, leaf, and root at light after four weeks of incubation.

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	**2**	**0.396**	**0.198**	**5.752**	**0.007**
Residual	**37**	**1.275**	**0.0345**		
Total	**39**	**1.671**			

Table 4.

Analysis of variance (ANOVA) for callus fresh weight of *Z. spina-christi* cotyledon, leaf, and root at dark after four weeks of incubation.

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	**2**	**0.0420**	**0.0210**	**1.177**	**0.318**
Residual	**42**	**0.749**	**0.0178**		
Total					

Table 5.

Analysis of variance (ANOVA) for callus fresh weight of *Z. spina-christi* cotyledon at different BA and NAA combination after four weeks of incubation.

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	**5**	**0.157**	**0.0314**	**2631.121**	**<0.001**
Residual	**114**	**0.00136**	**0.0000119**		
Total	**119**	**0.158**			

Table 6.

Analysis of variance (ANOVA) for callus dry weight of *Z. spina-christi* cotyledon at different BA and NAA combination after four weeks of incubation.

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	**5**	**2.084**	**0.417**	**4427.532**	**<0.001**
Residual	**114**	**0.0107**	**0.0000941**		
Total	**119**	**2.094**			

Table 7.

Cotyledonary callus fresh weight in light condition of twelve specimens for five weeks.

Weeks No. / Specimen No.	W1	W2	W3	W4	W5
1	0.0810	0.2720	0.4670	0.8810	0.8970
2	0.0870	0.1030	0.5400	1.0320	0.8800
3	0.0650	0.2710	0.4600	0.8710	0.8550
4	0.0880	0.2810	0.4360	1.0910	0.8960
5	0.0670	0.1890	0.5500	1.0000	0.9560
6	0.0550	0.2930	0.5200	1.0000	0.7310
7	0.0780	0.1000	0.4360	0.7860	0.9870
8	0.0920	0.2130	0.4700	0.8810	0.9210
9	0.0600	0.2810	0.6400	1.0340	0.8650
10	0.0770	0.1800	0.4670	0.8760	1.0210
11	0.0920	0.2780	0.5270	0.8900	0.8970
12	0.0620	0.2930	0.5240	0.8910	0.8670

Table 8.

Cotyledonary callus fresh weight in dark condition of twelve specimens for five weeks .

Weeks No. / Specimen No.	W1	W2	W3	W4	W5
1	0.0640	0.0880	0.2400	0.4670	0.4400
2	0.0700	0.1050	0.2200	0.5400	0.3897
3	0.0700	0.1340	0.2520	0.4600	0.4780
4	0.0560	0.0940	0.2640	0.4360	0.4040
5	0.0720	0.0790	0.2550	0.5500	0.4320
6	0.0530	0.0800	0.2240	0.5200	0.4100
7	0.0500	0.0970	0.2430	0.4360	0.4980
8	0.0570	0.0820	0.2100	0.4700	0.4780
9	0.0510	0.0850	0.2340	0.6400	0.4120
10	0.0670	0.1100	0.2590	0.4670	0.4090
11	0.0620	0.0880	0.2450	0.5270	0.4780
12	0.0680	0.0770	0.1989	0.5240	0.4540

Table 9.

Cotyledonary cell suspension fresh weight for nine specimen in three days interval for thirty three days.

Days No. / **Specimen No.**	**3 days**	**6 days**	**9 days**	**12 days**	**15 days**	**18 days**	**21 days**	**24 days**	**27 days**	**30 days**	**33 days**
1	**0.0151**	**0.0351**	**0.0765**	**0.1255**	**0.1885**	**0.2130**	**0.2789**	**0.3540**	**0.5160**	**0.5018**	**0.4732**
2	**0.0113**	**0.0267**	**0.0616**	**0.1324**	**0.1765**	**0.2200**	**0.2798**	**0.3430**	**0.5170**	**0.5054**	**0.4440**
3	**0.0123**	**0.0279**	**0.0564**	**0.1576**	**0.1987**	**0.2090**	**0.2830**	**0.3356**	**0.5090**	**0.4980**	**0.4350**
4	**0.0116**	**0.0305**	**0.0567**	**0.1363**	**0.1678**	**0.2130**	**0.2784**	**0.3543**	**0.5120**	**0.4987**	**0.4421**
5	**0.0128**	**0.0288**	**0.0678**	**0.1463**	**0.1876**	**0.2140**	**0.2754**	**0.3421**	**0.4980**	**0.4789**	**0.4650**
6	**0.0156**	**0.0276**	**0.0782**	**0.1546**	**0.1789**	**0.2121**	**0.2885**	**0.3365**	**0.5020**	**0.4876**	**0.4540**
7	**0.0118**	**0.0311**	**0.0789**	**0.1274**	**0.1987**	**0.2090**	**0.2876**	**0.3512**	**0.5320**	**0.4987**	**0.4540**
8	**0.0145**	**0.0361**	**0.0745**	**0.1444**	**0.1768**	**0.2150**	**0.2812**	**0.3420**	**0.5210**	**0.5000**	**0.4320**
9	**0.0154**	**0.0276**	**0.0654**	**0.1419**	**0.1887**	**0.2200**	**0.2802**	**0.3365**	**0.5110**	**0.4780**	**0.4560**

Table 10.

Cotyledonary cell suspension dry weight for nine specimen in three days interval for thirty three days .

Days No. / **Specimen No.**	**3 days**	**6 days**	**9 days**	**12 days**	**15 days**	**18 days**	**21 days**	**24 days**	**27 days**	**30 days**	**33 days**
1	**3.3000e-3**	**7.1000e-3**	**9.8000e-3**	**0.0182**	**0.0660**	**0.0982**	**0.1287**	**0.1412**	**0.1634**	**0.1550**	**0.1499**
2	**3.2100e-3**	**6.8500e-3**	**8.7000e-3**	**0.0211**	**0.0567**	**0.0897**	**0.1278**	**0.1401**	**0.1632**	**0.1503**	**0.1445**
3	**3.8700e-3**	**6.5400e-3**	**7.8000e-3**	**0.0318**	**0.0546**	**0.0912**	**0.1320**	**0.1398**	**0.1675**	**0.1512**	**0.1489**
4	**3.0400e-3**	**6.8700e-3**	**8.6500e-3**	**0.0200**	**0.0643**	**0.0902**	**0.1320**	**0.1389**	**0.1687**	**0.1498**	**0.1478**
5	**3.8700e-3**	**6.2300e-3**	**9.8700e-3**	**0.0325**	**0.0632**	**0.0897**	**0.1230**	**0.1401**	**0.1643**	**0.1490**	**0.1454**
6	**3.6500e-3**	**6.9800e-3**	**9.2300e-3**	**0.0215**	**0.0710**	**0.0867**	**0.1287**	**0.1400**	**0.1678**	**0.1478**	**0.1476**
7	**3.2000e-3**	**6.8340e-3**	**9.4500e-3**	**0.0287**	**0.0660**	**0.0965**	**0.1254**	**0.1396**	**0.1645**	**0.1513**	**0.1488**
8	**3.7600e-3**	**6.2340e-3**	**9.5600e-3**	**0.0300**	**0.0589**	**0.0967**	**0.1309**	**0.1378**	**0.1634**	**0.1523**	**0.1498**
9	**3.1200e-3**	**6.9870e-3**	**8.9700e-3**	**0.0298**	**0.0550**	**0.0956**	**0.1307**	**0.1404**	**0.1680**	**0.1519**	**0.1466**