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Elicitation, Analysis, and Biological Activity of Secondary Metabolites of Ziziphus spina-christi (L.) Desf. In Vitro Cultures

By

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In Partial Fulfillment of the Requirements for the Degree
Master of Science

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

Elicitation, Analysis, and Biological Activity of Secondary Metabolites of *Ziziphus spina-christi* (L.) Desf. *In Vitro* Cultures

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Elicitation, Analysis, and Biological Activity of Secondary Metabolites of *Ziziphus spina-christi* (L.) Desf. *In Vitro* Cultures

Watan Basheer Hawamda

ABSTRACT

Ziziphus spina-christi (L.) Desf. (Rhamnaceae) or “Christ's Thorn”, "Sider" in Arabic is an evergreen tree widely distributed in the Mediterranean region, North and East Africa. It has been used in traditional medicine to treat several medical conditions including dermatitis, eye inflammation, toothache, stomachache, rheumatoid arthritis and diabetes. This study investigated chemical properties and biological activity of the secondary metabolites extracted from the *in vitro*-grown cotyledonary-callus. Yield percentage of the extract was measured in callus grown on MS media in which chloroform:ethanol 3:1 gave 0.852%, 90% alcohol gave 1.648% and aqueous extract gave 0.120%. The antioxidant power of the extract was measured by ferric reducing antioxidant power (FRAP) assay after culturing the callus on three different growth media; B5, QL and MS. FRAP assay results (absorbance) were 0.189, 0.327 and 0.38 respectively indicating that growth on B5 media gave the highest antioxidant power. The effect of alcoholic extract was examined using disc diffusion assay (DDA) on four different bacterial isolates; *E. coli*, methicillin-resistant *S. aureus* (MRSA), methicillin-sensitive *S. aureus* (MSSA) and *Pseudomonas auregenosa*. Addition of 2000 µl of extract on assay discs showed inhibition zone only on MSSA. Minimum inhibitory concentration (MIC) showed positive results on *S. aureus* with MIC of 3.0mg/100µl. Other bacterial species that gave negative results on DDA were not tested by MIC. The UV active fractions was separated from the TLC plates and was applied on two cancer cell lines; A549, a human lung carcinoma, and U205 an osteosarcoma. Cell toxicity on osteosarcoma was observed at EC_{50} = 395 µg/ml. Mass spectrometry (MS) was carried out in the negative mode which gave a single peak at 485, therefore the compound is corresponded to triterpenoid acid. Salicylic acid (SA) was used as an elicitor in the cell suspension culture at three different concentrations; 25, 50 or 75 mM, HPLC analysis revealed that adding 50.0 mM SA resulted in the highest area under the curve (8575 mAU) for the total crude extract compared to control treatment (7440mAU), 25.0Mm (8143mAU) or 75mM (7483mAU).

تحفيز وتحليل ودراسة الفعالية البيولوجية للمركبات الثانوية المستخلصة من نسيج الكاللوس من نبات السدر *Ziziphus spina-christi* (L.) Desf.

ملخص

نبات السدر هو شجيرة دائمة الخضرة منتشرة على نطاق واسع في دول المتوسط وشمال وشرق أفريقيا وله استخدامات عديدة في الطب الشعبي كعلاج للكثير من الأمراض مثل التهاب الجلد والتهاب العيون والام الأسنان والمعدة والروماتزم وكذلك التهاب المفاصل كما ورد استخدامه أيضاً كعلاج للسكري. وبناءً على الأهمية الطبية لهذا النبات جرت عليها عدة أبحاث لدراسة خصائصها الكيميائية وتكثيرها على حد سواء, هذه الدراسة ركزت على المواد الثانوية المستخلصة من الكالوس المكاثر داخل الأنابيب ودراسة كميته وتحليلها ونشاطه البيولوجي. إن النسبة المؤية التي قيست من مستخلصات الكالوس النامي على بيئة (MS) بالمذيب الكلوروفورم مع الميثانول بنسبة 1/3 أعطت 0.852% ونتج عن استعمال 90% من الكحول 1.648% والمستخلص المائي 0.120, كانت قوة منع الأكسدة والتي تم قياسها عن طريق (FRAP) من الكالوس النامي على بيئة B5 (0.189) أعلى منها على بيئة QL وأعلى من بيئة MS مما يشير أن بيئة B5 أعطت أو نتج عنها أعلى كمية مانعة للتأكسد من البيئات الأخرى. كما تم فحص مقاومة المستخلص باستخدام اختبار تفشي الأقراص على أربعة سلالات من البكتيريا وهي *E.COLI, P. aeruginosa MRSS,MSSR,* وتبين أنه باستخدام 200µL من المستخلص كان هناك إبطاء للنمو أو تأخيرفي نمو البكتيريا MSSA أما بالنسبة للتركيز المثبط الأدنى MIC كان هناك نتائج ايجابية على بكتيريا MSSA بتركيز 3ملغم /100 ميكروليتر, الأنواع الأخرى والمذكورة سابقاً لم تدرس باستخدام التركيز المثبط الأدنى. ثمّ تم فصل المركب النشط تحت الضوء الفوق بنفسجي باستخدام TLC وتمت تجربته على نوعين من الخلايا السرطانية وهما سرطان الرئة والعظم وظهر تأثير طفيف على خلايا السرطانية في العظم 39µg/ml . استخدم المطياف الكتلي والذي أعطا قيمة أحادية على 485 وعلى الأغلب هذا المركب يعود إلى حامض Triterpenoid. , تم استعمال حمض الساليسيلك كمادة محفزة لإنتاج المواد الثانوية على تراكيز (0.25,0,5,0.75) ثم عن طريق ال HPLC التحقق من أن استعمال 50 مليمولر من هذا الحامض زاد من مجموع المواد الثانوية مقارنة بالشاهد.

DECLARATION

I declare that the master thesis entitled "Elicitation, Analysis, and Biological Activity of Secondary Metabolites of *Ziziphus spina-christi* (L.) Desf. *In Vitro* Cultures" is my own original work, and thereby certify that unless stated, all work contained within this theses 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.

Watan Basheer Hawamda

Date: 13th January 2015

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DEDICATION

إلى من كلّله الله بالهيبة والوقار...إلى من علمني العطاء دون انتظار ...إلى من أحمل اسمه بكل افتخار...أرجو من الله أن يمد في عمرك لترى ثماراً قد حان قطافها بعد طول انتظار وستبقى كلماتك نجوم أهتدي بها اليوم وفي الغد وإلى الأبد

والدي العزيز

إلى ملاكي في الحياة ...إلى معنى الحب والحنان والتفاني ... إلى بسمة الحياة وسر الوجود

إلى من كان دعائها سر نجاحي، وحنانها بلسم جراحي ..

أمّـي الحبيبـة

إلى من أرى التفاؤل في أعينهم، والسّعادة في ضحكاتهم

إخوتي: محمد، ومجد، وعيسى، ومحمود

إلى الأول الذي زيّن سمْعي بلقب عمّتي

بـشـيـر

إلى كل من شجّعني وآمن بي، وعلى رأسهم:

خالتي فريحة، وعمّي عاهد، ورنين

وأخيرًا

إلى كل من علمني حرفاً أصبح سنا برقه يضيء الطّريق أمامي

إليكم أهدي جهدي ونجاحي

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LIST OF ABBREVIATIONS

Word or sentence	Abbreviation
- Half Maximal Effective Concentration	MIC
- Disc Diffusion Assay	DDA
- Benzyl adenine	BA
- British pharmacopeia	BP
- Biotechnology research center	BRC
-Ferric Reducing Antioxidant Power	FRAP
- Gamborg (B5) salt mixture	B5
- High Performance Liquid Chromatography	HPLC
- Infra Red	IR
- Iron (III) Chloride	$FeCl_3$
- Naphthaleneacetic acid	NAA
- Natural products	NPs
- Mass Spectrometry	MS
- McCown woody plants media	MSS
- Muller-Hinton agar	MH
- Murashige and Skoog salt mixture	MS
- Plant Growth Regulator	PGR
- Potassium hydroxide	KOH
- Quality control	QC
- Quoirin and Lepoivre salt mixture	QL
- Rounds per minute	Rpm
- Salicylic acid	SA
- Sterile Distilled Water	SDW
- Thin Layer Chromatography	TLC
- Ultra Violet	UV
- United State Pharmacopia	USP
- Volume by volume	V/V
- World Health Organization	WHO

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Chapter 1

INTRODUCTION

For thousands of years, man used to derive his medication from natural resources, mainly those of plant or animal origins. Afterwards, the concept of traditional medicine has been recognized and became a respectable source of therapy in many civilizations and cultures. The dominant source of knowledge about traditional medicine was based on trial and error, or by chance when certain available foods showed therapeutic activities against some illnesses (Dias, 2012).

About 25% of the drugs prescribed worldwide originated from plants and 11% of the 252 essential and basic drugs defined by the World Health Organization (WHO) are exclusively of plant origin. Also significant number of synthetic drugs obtained from natural precursors (Rates, 2001). Examples of important essential drugs obtained from plants are digoxin from *Digitalis* spp., quinine and quinidine from *Cinchona* spp., vincristrine and vinblastine from *Catharanthus roseus*, atropine from *Atropa belladonna*, Taxol from *Taxus brevifolia* and morphine and codeine from *Papaver somniferum* (Rates, 2001, Gurib-Fakim, 2006).

Moreover, it is estimated that 60% of anti-tumor and anti-infectious drugs already in the market or under clinical trials are of natural origin. The vast majority of these are not yet synthesized on a commercial scale and are still obtained from wild or cultivated plants (Rates, 2001). It should be kept in mind that plant natural products can be used directly as crude extract in pharmaceutical preparations such as tinctures, fluid extracts, powder, pills and capsules, and are considered as "phyto-pharmaceuticals". Many of the crude plant extract which are used in therapeutic medications are described in the United State Pharmacopeia (USP) and British Pharmacopeia (BP); for instance *Premula* root, Ivy leaves and thyme extracts which are used in cough syrups, are described in the USP 36th edition.

Despite this, many of traditionally used bioactive natural products are still uncharacterized, which encourages researchers to invest in time and money to understand these plant secondary products and to develop methods of enhancing their yield (elicitation) and to produce large quantities for different applications.

The need for alternative methods can be explained by reviewing the story of the most important natural product-derived diterpene with anti-tumor activity found in recent years "Taxol". It can be isolated from *T. brevifolia* and *T. bacata*, however, the biggest obstacle is obtaining the material, for example in order to produce 2.5 kg of Taxol, 27 tons of *T. brevifolia* tree bark is required and 12 trees must be cut down. With to the high demand, this species of *Taxus* will soon be extinct if no alternative source of Taxol can be developed (Rates, 2001).

Biotechnological techniques and *in vitro* cell culture could solve the problem by addition of elicitors, using agitators and bioreactor at larger scale. By using these techniques, the amount of these medicinal compounds produced could be duplicated over a shorter period of time (Verpoorte, 1994, Alam, 2013). This will be of great advantage specially that many plant secondary metabolites are unique sources for pharmaceuticals, food additives, flavors and other industrial materials. However, despite the promise of these alternative drug discovery methods, there is still a shortage of lead compounds progressing into clinical trials, especially in therapeutic areas such as oncology, immunosuppressant and metabolic diseases where natural products have played a central role.

1.1 Medicinal plants in Palestine:

According to WHO estimation in 2002, 80% of the world's population in developing countries depends mainly on medicinal plants and traditional medicine practitioners to meet their primary health care needs. Although modern medicine may be available in many of these countries, the popularity of herbal medicine has persisted. This has also been the case in Palestine (Mohammed Ali-Shtayeh, 11/2011). Since Palestine is part of the Eastern Mediterranean and the Middle East, where traditional medicine, particularly herbal medicine is also widespread and appreciated in the society.

An ethnobotanical survey was carried out in the West Bank by AL-Najah National University to evaluate the relative efficacy of the plants used to treat skin diseases and prostate cancer. The following plant species were classified as popular for these uses: *Teucrium polium, Matricaria aurea, Urtica pilulifera, Paronychia argentea, Petroseli Petroselinum sativum,* and *Salvia fruticosa.* Some of the plants were claimed to be effective against cancer and prostate disorders, which include *Arum dioscorides, Urtica pilulifera, Allium sativum,* and *Allium cepa*(Raeda Tawfeq, 2008). The most commonly used plants in the north West Bank and Gaza were: *Matricaia aurea, Salvia fruticosa, Allium sativum,* and *Anisum vulgare*(Raeda Tawfeq, 2008).

Many plants including those of a medicinal value in Palestine are facing extinction threat in their natural habitats due to various human activities such as overharvesting, expansion of urbanization and agrigultural activites and the detrimental climatic and environmental changes (Raed Alkowni, 2012).

One of the known medicinal plants in Palestine is *Ziziphus spina-christi* that will be discussed in details in this study.

1.2 Ziziphus spina– christi, an overview:

The genus *Ziziphus* belongs to the family Rhamnaceae. It is a genus of about 100 species of deciduous or evergreen trees distributed in the tropical and subtropical regions of the world (Khalid, 2011a, Izuagie, 2012, Amots Dafni, 2005).

Z. spina-christi is a tree that sometimes grows to a tall tree reaching a height of 20m and a diameter of 60cm. It is reported to be native to a vast area of Africa stretching from Mauritania through the Sahara and Sahelian zones of West Africa to the Red Sea (Izuagie, 2012).

Z. spina-christi is mentioned in the three major religions including the Holy Quran. It is also known to Christians as the tree from which Jesus Crown of Thorns was made(Dafni, 2005). Its Arabic name is Sider, and in many Arab regions it is called Nubeg, Innab or Doum. In English it is called Jujube Christ thorn.

All parts of *Z. spina-christi* are used by the local Arab people to help maintain a healthy life (Korji, 2012). The ripe fruits are edible and the flowers are an important source of high value honey especially in Eritrea and Yemen and other Arabic Gulf countries.

Z. spina-christi has been used in traditional (folk) medicine for the treatment of fever, pain, dandruff, wounds and ulcers, inflammatory conditions, asthma and to cure eye diseases. It also possesses antibacterial, antifungal, antioxidant, anti-hyperglycemic, and antinociceptive activities (Basuny, 2013, Jinous Asgarpanah, 2012a, Korji, 2012, Amots Dafni, 2005). In addition to that, it has very nutritious fruits that are usually eaten fresh or dried.

In Palestine, the species is distributed along the Jordan Valley, Dead Sea shores and in the Southern Coastal Plain. It is also widely spread in Gaza Strip and other places of high temperatures. *Z. spina-christi* and other tropical trees are considered important plant communities for environmental balance in the valleys and the coast (Roubina Ghattas, 2002).

Figure1.1: *Ziziphus spina-christi* tree in Jericho/Palestine.

1.3 In Vitro culture of cotyledonary callus of Z. spina-christi.

In vitro tissue and cell culture of *Z. spina-christi* as many other medicinal plants took high attention in many studies. The main focus was to study the factors affecting it's *in vitro* culture as well as to study its secondary metabolites (Jain Monica, 2013). In addition to that, tissue culture techniques provide a continuous and reliable source of valuable plant pharmaceuticals. This method can be used for the large-scale culture of the plant tissues and cells from which the secondary metabolites can be produced and extracted(Sayeed Ahmad). On the other hand this method (*in vitro*) of obtaining secondary metabolite is favorable more than other classical methods because they are natural in origin, possible for large scale production, less toxic, safer and environmentally friendly(Alam, 2013).

Z. spina-christi can be easily propagated in vitro and can be used as a good alternative method for multiplication and callus culture of this species. The seeds are a good starting material for the *in vitro* culture with more than 90% germination(Abu Allan, 2012).

1.4 Secondary and Primary Metabolites

Metabolites are compounds synthesized by plants for both essential functions, such as growth and development (primary metabolites) those found in all plants, for example rich fuel molecules, such as sucrose and starch, structural components such as cellulose, informational molecules such as DNA and RNA, and pigments, such as chlorophyll. The other metabolites with specific functions, such as pollinator attraction or defense against

herbivory (secondary metabolites), In contrast, secondary metabolites are variously distributed in the plant kingdom, and their functions are specific to the plants in which they are found. There's no denying that many secondary metabolites found in plants has a role in defense against herbivores, pests and pathogens, many of these secondary metabolites also makes a major contribution to the specific odours, tastes and colours of plants(Bourgaud, 2001). Most of these secondary metabolites contributed in defense mechanism were derived from both shikimic acid or aromatic amino acids(Jain Monica, 2013). Secondary metabolites differs from primary metabolites in that they are not directly needed by plants as they don't perform any physiological functions. Recent advances in molecular technology and the power of large-scale genomics initiatives, are leading to a more complete understanding of the enzymatic machinery that underlies the often complex pathways of plant natural product biosynthesis. Nowadays metabolic engineering for enhancement of plant disease resistance(Bennett.R., 1994).

1.5 Elicitation and extraction of secondary metabolites:

Production of secondary metabolites through *in vitro* methods is useful for large-scale exploitation in agrochemical and pharmaceutical industries. Elicitation can be applied to increase the yields of secondary metabolites in plant cells(Ravi mahalakshmi, 2013).

In vitro growing plants or cells show physiological and morphological response to microbial, physical or chemical factors, which are known as 'elicitors'. Medicinal plant tissue culture has been introduced as a suitable method to produce valuable secondary metabolites. Also, elicitation has been proved to be an effective way to increase the secondary metabolites produced in many tissues like hairy roots, callus or cells of the in vitro grown plants (Moghadam 2013).

Accumulation of secondary metabolites often occurs in plants subjected to stresses including various elicitors or signal molecules (Heena Patel, 2013). Commonly tested chemical elicitors are salicylic acid, methyl salicylate, bezoic acid, chitosan and so forth which affect production of phenolic compounds and activation of various defense-related enzymes in plants (Heena Patel, 2013, Ben. B., 2012). A number of these elicitors and precursors such as methyl jasmonate (MJ) and salicylic acid (SA) have been used successfully for enhancing production of secondary metabolites (Moghadam 2013, Mulabagal Vanisree, 2004). SA is a well-known inducer of plant secondary metabolites

which has also been used in this study and was used in many other studies in taxol production for example (Ayatollah Rezaei, 2011).

Extraction of secondary metabolites can be defined as a method for separation of chemicals apart from plant tissues by using special solvents and techniques, the history of the extraction of natural products dates back to Mesopotamian and Egyptian times, where production of perfumes or pharmaceutically - active oils and waxes were a major business (Hans-Jörg Bart, 2011).

Many techniques are commonly used for extraction including infusion, percolation, digestion, decoction, hot continuous extraction, soxhlet counter current extraction. Solvents should be chosen carefully to be appropriate for the compound class under investigation. Common extractants are arranged from ethyl acetate to water (ethyl acetate < acetone <ethanol < methanol < acetone: water (7:3) < ethanol: water (8:2) < methanol: water (8:2) < water) in increasing order of polarity according to the Hildebrand solubility parameter. The extract can be dried using a centrifugal evaporator or a freeze-drier. A rule of thumb is that the solvent used should have a similar degree of polarity to the compounds to be extracted(Turner, 2006).

1.6 Separation technique for secondary metabolites: thin layer chromatography (TLC) and column chromatography (CC):

Thin layer chromatography (TLC) is a method for identifying substances and testing the purity of compounds. TLC is a useful technique because it is relatively quick and requires small quantities of material. Separations in TLC involve distributing a mixture of two or more substances between a stationary phase and a mobile phase. The stationary phase is a thin layer of adsorbent (usually silica gel or aluminum oxide) coated on a plate. The mobile phase is a developing liquid which travels up the stationary phase, carrying the samples with it. Components of the samples will be separated according to how strongly they adsorb on the stationary phase versus how readily they dissolve in the mobile phase. The goal of this TLC is an analytical tool is to obtain well defined, well separated spots each representing one component of the mixture.

The separated compounds can be visible by naked eye or under UV light. It is important to calculate the *Rf* factor for the separated compounds after a separation is completed; individual compounds appear as spots separated vertically. Each spot has its own *Rf* which is equal to the distance migrated over the total distance covered by the solvent.

Another separation and purification method is column chromatography (CC). This method is also used to purify individual chemical compounds from a mixture of compounds. This technique is very common and useful in organic chemistry and involves the same separation principle as TLC, but it is used to separate larger quantities. There are many fields that benefit from this technique like biology, biochemistry, microbiology and medicine, for example many antibiotics are purified using column chromatography (University of Colorado 2013).

There is an optimum flow rate for each particular separation. A faster flow rate of the eluent minimizes the time required to run a column and thereby minimizes diffusion, resulting in a better separation. In this study, a simple laboratory column runs by gravity flow was used.

1.7 Quantitative techniques: High-Pressure Liquid Chromatography (HPLC), Infra Red (IR) and Mass Spectrometery (MS):

High-pressure liquid chromatography (HPLC) is a technique used to separate the components in a mixture, to identify each component, and to quantify each component. The stationary phase is almost always solid; however, there are examples of chromatography experiments in which the stationary phase is another state. For example, in counter current chromatography (CCC), both the mobile and stationary phases are liquid.

Two phases of HPLC could be used; normal and reverse phase. A polar stationary phase and a non-polar mobile phase are used for normal phase HPLC. In reverse phase HPLC a polar mobile phase and a non-polar stationary phase is used . Reverse phase HPLC is the most common liquid chromatography method used. The R groups usually attached to the siloxane for reverse phase HPLC are: C_8, C_{18} or any hydrocarbon. Reverse phase can also use water as the mobile phase, which is advantageous because water is cheap, nontoxic, and invisible in the UV region. The most polar compounds will elute first when performing reverse phase HPLC.

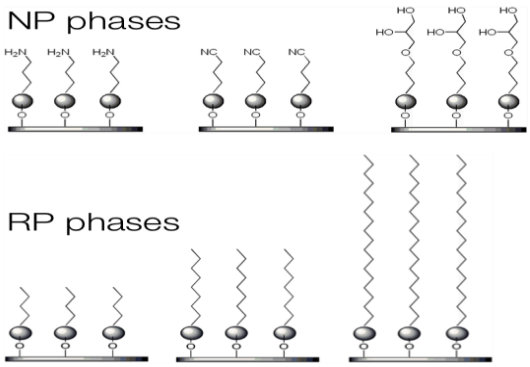


Figure 1.2: Illustration of HPLC reverse and normal phase

The use of infrared spectroscopy began in the 1950's by Wilbur kaye. It deals with the infrared region of the electromagnetic spectrum, that is light with a longer wavelength and lower frequency than visible light, it has many applications in quality control, dynamic measurement, chemical identification and others, this can be measured by three waves absorption, emission and reflection its used in both organic and inorganic chemistry in addition to that it is used by chemists to determine functional groups in molecules the principle of IR is to measure the vibrations of atoms, this lead to identify the functional groups, light atoms and strong bonds will vibrate at a high stretching frequency(White, 1989, Lau, 1999).

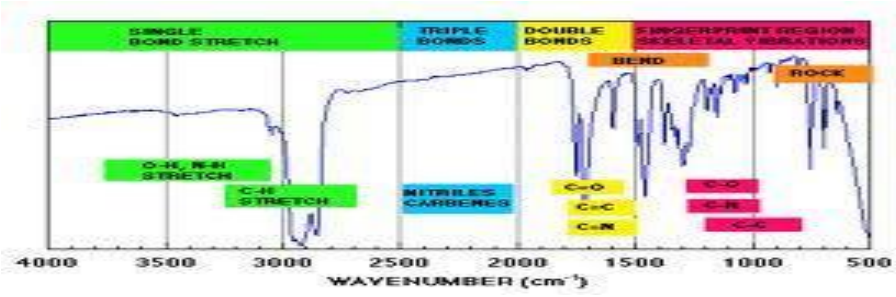


Figure 1.3:Illustration of IR spectra reads of different Carbon-Hydrogen/Carbon-Oxygen/Carbon-Nitrogen bonding

A third analytical technique that produces spectra is mass spectrometry. Atoms can be deflected by magnetic fields-provided the atom is first turned into an ion. Electrically charged particles are affected by a magnetic field although electrically neutral ones aren't. The sequence includes: Ionization, acceleration, deflection, detection. In the Ionization stage the atom is ionized by knocking one or more electrons off to give a positive ion. This works also for atoms with negative ions (chlorine, for instance) or never form ions (argon).

Mass spectrometers always work with positive ions. In the acceleration stage the ions are accelerated so that they all have the same kinetic energy. The ions are then deflected by a magnetic field according to their masses. The lighter they are the more they are deflected the amount of deflection also depends on the number of positive charges on the ion-in other words, on how many electrons were knocked off in the first stage. Also the more the ion is charged the more it gets deflected. Finally the beam of ions passing through the machine are detected electrically.

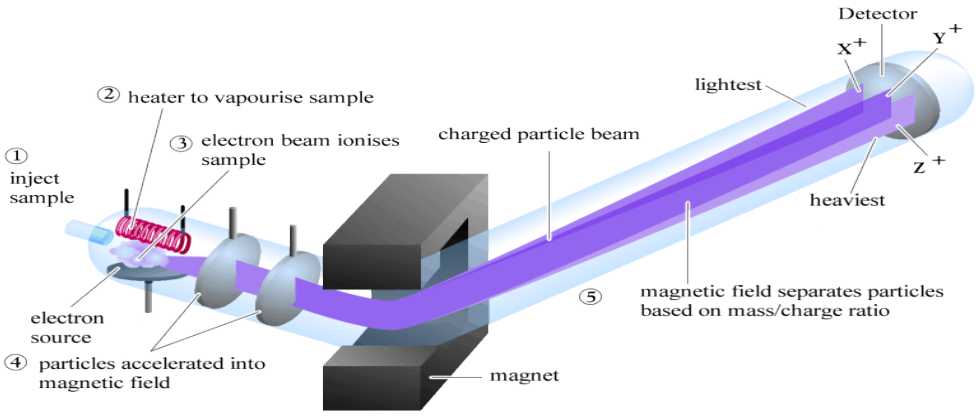


Figure (1.4) Illustration of the steps included in MS detection of unknown atoms.

1.8 Testing of antioxidant activity using Ferric Reducing Antioxidant Power (FRAP) assay.

FRAP assay is a simple automated test measuring the antioxidant capacity of foods, beverages and nutritional supplements containing polyphenols (Iris F.F. Benzie, 1996). The FRAP assay was presented as a novel method for assessing "antioxidant power". Ferric to ferrous ion reduction at low pH causes a colored ferrous-tripyridyltriazine complex to form. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions of known concentration(Iris F.F. Benzie, 1996). Absorbance changes should be linear over a wide concentration range with antioxidant mixtures.

1.9 Antibacterial activity of plant extract

Infectious diseases account for about half of the death in tropical countries. In addition, incidents of epidemic due to drug resistant microorganisms causes enormous public health concerns (Korji, 2012). This drug resistance to human pathogens is commonly reported

worldwide and its one of the biggest challenge nowadays to develop a new generation of antimicrobials (Djeussi and Simplice B Tankeo, 2013, Firdaus Jahan, 2011). In addition to that it has been known that bacteria has the genetic ability to transmit and acquire resistance to drugs. Because of this and the misuse of the available antimicrobial agents there is a big need for new active leading antimicrobial compounds and a deep understanding of the large number of organic chemicals of high structural diversity or "secondary metabolites"(Sharma, 2011). The development of resistant bacterial strains causes an increase in the number of research and papers published on antibacterial activity of plant extracts(Al-Juraifani, 2011). The demand for more drugs from plant sources is continuously increasing which necessitates screening medical plants with promising biological activity(Djeussi and Simplice B Tankeo, 2013, Dorman, 2008).

There are two common methods to test antibacterial activity of a mixture of unknown compounds; the disc diffusion method (DD), and the minimum inhibitory concentration (MIC). The aim of these two tests is to determine the lowest *ex vitro* concentration of the assayed antimicrobial agent(Ababutain, 2011).

1.10 Cytotoxicity test of plant extract

Cancer is known to be a deadly disease and difficult to cure especially at late stages. There are continuous efforts to search for efficient remedies with low side effects. However, most of anticancer drugs are known for being harmful for the patient's body and cells. Research on finding plausible anticancer compounds from plants resulted in successful achievements (Dorman, 2008). Several drugs nowadays used in chemotherapy are isolated from plant species and over 50 % of all drugs in clinical trials for anticancer are isolated from natural sources or one of their derivatives (Valko, 2006, Shah, 1989).

Many studies addressed the cytotoxicity effect of different plant extracts from different parts of *Ziziphus spina-christi* and other plants*(Abu Allan, 2012, Jafarian, 2014),* for example a for Hexane, chloroform, chloroform-methanol, butanol, methanol-water and aqueous extracts of *Z.spina-christi* a study shows that significantly and concentration-dependently reduced viability of Hela and MAD-MB-468 cancer lines. In the both cell lines, chloroform-methanol extract of *Z.spina-christi* was more potent than the other extracts(Jafarian, 2014). By reviewing the litriture no previously conducted studies targeted the anticancer activity of *invitro* propagated cotolydonary callus cell line of *Z.spina-christi* which is what this study is addressing. Other studies evaluate the anti-

cancer activity of three *Euphorbiacae* plants which was determined against seven human cancer cell lines. As a result it was shown that the ethanolic extract of *Euphorbia helioscopia* inhibited the growth of three cancer cell lines out of these seven including colon cancer (Prakash, 2013). Another study shows that after an overnight incubation with various concentrations of extracts from *A. kurdica*, produced a reduction in cell proliferation of lymphocytes cells at 24-hrs incubation(Khakdan Fatemeh, 2013).

CHAPTER 2

STUDY OBJECTIVES

General objective:

In a previous study don at PRC, Abu Allan (2012) [21] investigated some factors influencing *in vitro* culture of *Z. spina-christi*. One of his achievements was callus line derived from cotyledonary explants characterized by containing a biochemical substance that clearly reflects UV light. This research aimed at using previously developed protocol to study the secondary metabolites of *in vitro* propagated callus of *Ziziphus spina- christi* and to study the effect of chemical elicitation on total secondary metabolites beside testing the activity of theses secondary metabolites.

Specific objectives:

1- To separate secondary metabolites produced by *Ziziphus spina-christi* cotyledonary *in vitro* propagated tissue culture using different solvents.
2- To measure the antioxidant power of cotyledonary callus extract using FRAP assay.
3- To study the biological activity of ethanolic extract of these cells on different types of bacteria and to study its Cytotoxicity on two different cancer cell lines.
4- To use chemical elicitor "salicylic acid" to increase the amount of secondary metabolites produced by callus cells grown using cell culture.

Chapter 3

MATERIALS AND METHODS

The experimental work in this study was conducted in the Plant Tissue Culture Laboratory in the Biotechnology Research Center at the Palestine Polytechnic University, Hebron, Palestine. The microbiology test was done at the microbiology laboratory at Caretas Baby Hospital(Children Relief), Bethlehem, Palestine. while the analytical methods including HPLC and IR were done at Jerusalem pharmaceutical company, Al-Balou', Al-Bireh, Palestine. And finally the anticancer activity with the MS NMR was carried out in University of Zurich, Institute of Organic Chemistry by Dr Jawad Al-Zeer.

3.1 Chemicals and reagents for the *in vitro* culture:

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

3.2 Source of Plant Material

The dried fruits of *Ziziphus spina-christi* were collected in October 2012 from MADICO (Manasrah Development and Investment Company) Date Farm near Jericho. The plant was characterized 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.

3.3 Media preparation and sterilization

Four types of media were prepared according to the manufacturer's instructions; MS at 4.40gm/L, QL at 3.37gm/L, B5 at 3.16g/L and MCC at 2.3gm/L. In the preparation of each media a 3.0% sucrose (w/v) were dissolved in 70% of the media final volume then the plant growth regulators (PGRs) were added (1.3 mg/l BA and 0.3 mg/l NAA). Finally the media was brought to the final volume with distilled water and the pH was adjusted at 5.8 with 1.0M NaOH or 1.0M KCl. The medium was gelled by adding 0.8% w/v of regular bacteriological agar. Finally, the medium was dispensed into 500 ml bottles, closed loosely

and autoclaved at 121°C and 15 Psi pressure for 20 minutes. For seed germination, water gelled with 0.6% agar was prepared and was PGR free.

3.4 Seed surface sterilization and *in vitro* seed germination

In order to start the in vitro culture material, seeds were extracted from the woody endocarp manually using pliers. They were then immersed in 50 ml 20% (v/v) commercial bleach Chlorex® with 5.0 % NaOCl with continuous shaking for 15 minutes on an orbital shaker at 110 rpm. After that, seeds were washed with sterile deionized water (SDW) 3 times with shaking (2min each). Finally seeds were washed with 70% (v/v) ethanol solution for 30sec then rinsed 3 times with SDW. All previously described procedure was done under the laminar air flow cabinet. Surface sterilized seeds were cultured on plant growth regulator free gelled-water.

3.6 Growth condition:

Seeds were inoculated at the surface of gelled-water media for the purpose of germination and kept in a growth room at 24±1°C with 16:8 light:dark photoperiod under cool-white fluorescent illumination of 40-45 µmol/m²/sec of photosynthetic photon flux density (PPFD).

3.7. *In vitro* culture of callus tissue

3.7.1. Callus induction and maintenance

From the *in vitro* growing seedlings, callus was induced using only one explant source, the cotyledonary segments. Explants were cut into pieces of 1.0 cm² then pieces were inoculated on 9.0 cm Petri-dish filled with 25 ml of the media. Five explant pieces were cultured in each Petri-dish. The four types of media described above were used for the callus induction.

3.7.2 Estimation of callus growth

Callus growth was estimated according to the change in callus upper surface area across time. Surface area was measured with the image processing and Java analysis software Image-J. Measurements were taken periodically for four weeks and difference in callus total area was recorded.

3.7.3 Extraction of secondary metabolites from the cotyledonary callus

Twenty grams of fresh callus from each treatment were taken and subjected to direct extraction. Each sample was ground by a mortar and pestle then immersed in 30.0 ml of either DW or 90.0% ethanol in 50.0 ml Falcon tubes and incubated on a shaker with continuous shaking for 24 hours under dark conditions. After that, the cultures centrifuged for 15 minutes at 5000 rpm to take the supernatant. The supernatant was air dried under a chemical suction hood then the yield was calculated at percentage basis.

3.8 Cell suspension culture

3.8.1 Initiation and maintenance of cell suspension culture

One gram of friable 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 100 rpm and 23± 1°C under dark 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. Subculture was done after 30 days from incubation.

3.8.2 Extraction of secondary metabolites from cultured cells:

In order to obtain crude extracts from cell culture, 5.0gm of cells were immersed in 30 ml ethanol (95%) in 50 ml falcon tubes and incubated on orbital shaker for 24 hour under dark conditions. After that, the extracted solution was centrifuge for 10 minute at 4000 rpm to take the supernatant. The supernatant was air dried in a vacuum hood and the yield was estimated for cell suspension originated from cotyledonary callus**.**

3.9 Ferric Reducing Antioxidant Power (FRAP) assay

3.9.1 FRAP assay solution preparation

The preparation of reagents for FRAP assay followed the protocol of Iris Benzie (Iris F.F. Benzie, 1996). First acetic buffer: 3.1g sodium acetate trihydrate was prepared and dissolved in 16 ml of glacial acetic acid then the volume was completed up to 1 L of distilled water, then: TPTZ (2, 4, 6-tripyridyl-s- triazine): (M.W. 312.34), 10 mM in 40 mM HCl (M.W. 36.46) and: FeCl3. 6 H2O: (M.W. 270.30), 20 mM was prepared. Then the working FRAP solution was prepared by mixing 1, 2 and 3 in a ratio 10:1:1 respectively at the time of using.

3.9.2 Standard curve preparation (incubation time)

Five concentration of $FeSO_4$ were prepared (1.5, 1.0, 0.8, 0.4, 0.1 mM) and the absorbance at 593nm was recorded using UV spectrophotometer and treated as for sample solution .To minimize errors the same cuvett was used for all concentration and the same cell well position was used for all reads . Using Sigmaplot: a standard linear curve was plotted between the different concentration and the absorbed amount of reduced ferrous at 593nm for the five concentrations.

3.9.3 Sample preparation

FRAP solution (3.6 mL) was added to distilled water (0.4 mL) and incubated at 37˚C for 5 min. Then this solution was mixed with 80 ml of the plant water extract and incubated at 37°C for 10 min. The absorbance of the reaction mixture was measured at 593 nm(Gohari AR 2011, Iris F.F. Benzie, 1996).

3.10 Identification of secondary metabolites in callus alcoholic extract:

Alcoholic extracts were exposed to different analytical techniques as follows:

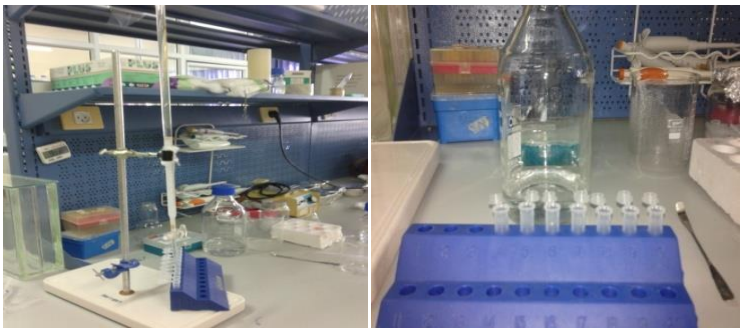
3.10.1 TLC analyses

TLC analysis was performed using precoated TLC plates (Merck, Germany Silica gel 60 F254, 0.25 mm) as stationary phase, 50 ml of DW water: Methanol: formic acid was used as a mobile phase after different experiments the ratio used for DW water: Methanol: formic acid was (57:40:3 v/v/v) respectively. Plates were run three times in the mobile phase, after each run, the plates were air-dried. And a UV active compound was observed under the UV detector. Plates were sprayed with 15 % sulfuric acid with ethanol solution then dried and exposed to temperature for five minutes on a hot plate (30°C) for visualization of other organic compounds which couldn’t be detected by the UV detector.

3.10.2 Column Chromatography (CC) analysis:

Column chromatography was established to separate the same band observed in TLC using the same mobile phase but in larger quantities for identification. Materials needed to establish the column were a piece of cotton, 1.0 gm of sterile sea sand, 5.0 gm of silica gel (stationary phase), 50.0 ml mobile phase (DW water: Methanol: formic acid (57:40:3 v/v/v) respectively), Glass burette with a stopcock, 25.0 ependruf tubes (3.0ml), Stainless steel stand (photo 1). A piece of cotton were inserted in the column for filtration followed

by a small amount of sea sand after that the silica gel (stationary phase) was poured in the column followed with a small amount of solvent(ethanol 95%). The silica gel was then backed to prevent cracking. Our sample was then poured followed by the mobile phase, and separated fractions were collected in 20 eppendorf tubes. Each fraction(2, 5 ml) was loaded at the TLC plate to look for a UV active chemical.



Figure(3.1): Left: Silica based column chromatography system. Right: fractions of the separated compound.

3.10.3 High pressure liquid chromatography (HPLC) and infra red spectra (IR).

The HPLC and IR analysis were conducted in the Quality control (QC) Lab at Jerusalem pharmaceutical company. In HPLC technique (C18, 25cm, 4.6mm i.d) column has been used, for analytical purposes two mobile phases were used with different proportions and the detection done on 3 different wavelengths; with (0.01M KH2PO4 PH6.9):CH3CN, in a ratio of (94:6), (1000:50), (1:0) and the second mobile phase was Water: Methanol: Formic acid in a ratio (50:50:0.8), for each mobile phase absorbance was detected at three wavelength 235nm, 254nm, 210nm. The parameters which give the best resolution were recorded for elicitation detection. IR spectra was recorded and compared with the library of compound exist in Jepharm QC lab.

3.10.4 Mass Spectrometric (MS) analysis:

The MS analysis was carried out at The University of Zurich, Institute of Organic Chemistry. Mass spectrometry was measured in the negative mode which gave a single peak.

3.10.5 Infra red (IR):

The IR spectra of the separated compound was compared with the library of compounds in Jepharm but no significant matching observed see appendex 4.

3.11.1Testing the biological activity of the crude extract

3.11.2 Anticancer activity

3.11.2.1 Preparation of crude extracts:

Extracts were prepared by adding the specified solvent (30 ml) to 80.0g of fresh callus material in four Falkon tubes (50 ml). The mixture was shaken for 24 hours at room temperature (23°C), centrifuged, and the supernatant was filtered through cotton using a funnel and Erlenmeyer flask. The filtrate was dried under air vacuum hood. Extraction yields was calculated by subtracting the dry weight of the natural solvent residue from the total weight of natural product extract. The extract was collected and stored at 4° to be used in the anti-cancer tests.

3.11.2.2 Cancer cell lines used in the anti-cancer test:

The extract was tested on two cancer cell lines; A549, a human lung carcinoma and U2O5, which is an osteosarcoma.

Cytotoxicity assay: As described by Al-Zeer et al. (2014), Alamar Blue- resazurin reduction assays were conducted as described (O'Brien et al., 2000). Cell suspended in 100 µl of DMEM were seeded in 96-well plates at a density of 5 × 103 cells per well and incubated for 24 hours. All extracts were serially diluted into supplemented media using a separate 96-well plate, applied to the cells, and incubated for 48 hours. Then following the incubation, 100 µl of fresh media, (containing 10% (v/v) of a 860 µM solution of resazurin in PBS) was added to the cells, and incubated for 2 to 4 h. The fluorescence intensity of the dye was then quantified by a SpectraMax M5 plate reader using excitation at 560 nm. IC50 values were calculated from the fluorescence intensity values, by using an exponential decay curve fit. DMSO was used as a negative control, whereas Nile Blue A (Lin et al., 1991) was used as a positive control(Jawad Alzeer, 2014).

3.11.2.3 Statistical analysis

IC_{50} values are defined as the concentration of the extract where there is a 50% loss of total metabolic activity as compared to untreated controls and are reported as mean ± standard deviation (SD). IC_{50} values with 95% confidence limits were calculated using GraphPad

Prism 3.3 software (GraphPad Software, Inc., San Diego, CA). *p*-Values less than 0.05 were considered to be significant(Jawad Alzeer, 2014). All experiments have been conducted in duplicate.

3.11.2. Antibacterial activity

The antibacterial activity test was done at the microbiology laboratory at Caritas Baby Hospital**.** Determination of the Relative Antibacterial Activities of *Z. Spina christi* Extracts by both Disc Diffusion Assays(DDA) and Minimum Inhibitory Concentration (MIC).

Disc diffusion Susceptibility Testing (Kirby-Bauer, Method) was performed to screen antimicrobial effects of cotyledonary callus of *Z. spina- christi* callus extracts from different medias (Ms, QL, B5, MCC). This experiment was conducted on five bacterial species; *Escherichia coli*. methicillin-resistant *Staphylococcus aureus,* (MRSA), methicillin-sensitive *S. aureus* (MSSA), and *Pseudomonas auregenosa*. The procedure used in this assay is a modification of the Kirby-Bauer tests which is commonly used as an antimicrobial susceptibility testing. The detailed procedure of the disc diffusion assay is mentioned in appendix 2**.**

The second method used is the minimum inhibitory concentration (MIC). It is conducted according to Jennifer M. Andrews protocol (M.Andrews, 2001) to calculate the minimum inhibitory effective concentration of MS extract on *Staphylococus aureus* species which has positive results using disc diffusion method described above; 2x (MH)media (OXOID) was prepared. Four rows of 10 test tubes were used; 0.5 ml of MH media were added to each tube then autoclaved (121°c/20 min/15 PSI) after cooling 0.5 ml of the extract was added to each tube then 0.5 ml of 0.5 McFarland *staphylococcus aureus.* shaked well then a serial dilution was done using 0.5ml of the mixture. Ampicillin and Kanamycin was used as a positive control while 90% ethanol as a negative control.

3.11.2.1 Microbial cultures and growth conditions

In the disc diffusion assays, the following bacterial species were chosen to test the antimicrobial activities of the extract: i) *Escherichia coli*. ii) *Pseudomonas auregenosa,* iii) *Staphylococcus aureus,* methicillin-resistant (MRSA) and *S. aureus* methicillin sensitive

strains (MSSA). The bacterial isolates were obtained from Caritas Baby Hospital. Cultures of *E. coli* bacteria were grown on MacConkey Agar media (OXOID) and *S. aureus* were grown in Sheep Blood Agar media (OXOID) for 18 hr at 37°C. The bacterial species were maintained on Mueller-Hinton Agar (OXOID) at 37°C.

In MIC Mueller-Hinton Agar (OXOID) broth medium was used and (MSSA) species were used, both Ampicillin and Kanamycin were used as a positive control and alcohol as a negative control.

3.12 Elicitation of secondary metabolites by salicylic acid:

Three different concentrations of salicylic acid were added (25.0, 50.0, 75.0) mM to the cell culture at the 16-18 days of initiation of the cell culture and the other was left as control. This was done in three replicates for each concentration and the control and in the same shaker incubator used to initiate the suspension culture and the same conditions (120 rpm and 23± 1°C under photoperiod of 16 hours light duration). After 48 hrs alcoholic extract of the four samples were prepared and column chromatography described previously was done to the three concentration samples and the control. After that fractions containing the targeted band were collected and evaporated to have a sticky gum solid extract. For elicitation detection 100.0 mg of the sample extraction was dissolved in 5.0 ml (90% v/v ethanol) and filtered using 33.0 mm filter syringe then injected in the HPLC as described previously in section 5.3.

Chapter 4

RESULTS AND DISCUSSION:

4.1 Cotyledonary callus induction and maintenance

The percentage of seed germination was very high 27/30 seeds (90%). From young seedlings, only cotyledonary explants were used to initiate *in vitro* callus tissue. Four types of media were used to support callus growth with similar PGR content (1.3 mg/l BA and 0.3 mg /l NAA). Callus growth (100%) appeared within four weeks and the subculture was carried out every three to four weeks.

Figure 4.1 Left: *In vitro* germinated seedlings. Right: Cotyledonary callus induction.

4.1.2 Estimation of callus growth

Using Imag J version 1.48 software, the change in callus top surface area was measured within three weeks, ten reads were recorded. Results show that cotyledons grown on QL media gave the highest callus growth followed by on MCC, MS and the least growth was observed on B5 media (table 4.1).

Table 4.1. Media effect on callus growth (callus surface area) after three weeks of culture. ±SE of the mean and there color/texture, 10 reads.

Media type	3rd week surface area(pixel)	SE	ST. DEV.	Callus color/texture
MS	549.6136	72.86	230.41	White / friable
B5	564.2515	58.93	186.36	Yellowish / compact
QL	**725.2876**	**53.06**	**167.78**	**Green to yellow /compact**
MCC	716.6101	22.14	70.01	White/ friable

These results show clearly the effect of salt mixture on callus initiation. The four basal salts differ from each other in salt contents and concentrations (appendix 1, table 2-4). QL media differs from the other three by containing three nitrogen salts with ammonium and nitrate formulas; ammonium nitrate, potassium nitrate and calcium nitrate. On the other hand, Gamborg's B5 media has only one source of nitrogen which is ammonium based (ammonium sulfate). It can be concluded that cotyledonary callus of *Z. spina-christi* prefers nitrogen in nitrate from rather than ammonium form. Preference of nitrate or ammonium nitrogen source in some plant species is heavily studied in plant physiology and nutrition researches(Tylová, 2010, Ricker, 1948).

4.1.3 Extraction of secondary metabolites from cotyledonary callus

The crude alcoholic and the water extracts of *Z.spina christi* was estimated at percentage basis. Fresh callus (11.0g) that grown on MS medium was soaked separately in 30.0 ml of 95%EtOAc, DW and Chlorophorm/Ethanol (3:1). Mixtures were stirred for 24 h at room temperature and the supernatant was removed by centrifugation at 5000rpm for five minutes. The solvents were air dried in the fume hood over night.

Figure (4.2): Left: callus ethanolic extraction and, right: separating the solvent from callus remains.

The percentages of extraction yield were calculated in three replicates as a result of dividing the weight of the extracted powder by the weight of the extracted plant material (Table 4.2). The highest percentage yield was for the alcoholic extract and therefore I chose it for further investigation. Furthermore, its known that most of the medical plant derived compounds are soluble in ethanol(Sultan, 2009). In my experiment it was followed by (Chlorophorm/Ethanol:3/1) and the least yield was obtained in the aqueous extract this could be an indication that the produced plants secondary metabolites are more hydrophobic and alcohol soluble than hydrophilic and water soluble components and its known that most antioxidant compound are soluble in alcohol specially ethanol more than water.

Table 4.2: The percentage yield of fresh callus for different extract grown on MS media.

Solvent	Alcohol (95%)	DW	Chlorophorm/Ethanol (3:1)
Yield (%)	**1.648%**	0.120%	0.852%

4. 2 Ferric reducing antioxidant power (FRAP) assay

4.2.1 Generating the standard curve:

Curve in (figure 9) shows the calibration and the proportional relation between five concentrations of $FeSO_4$ (1.5, 1.0, 0.8, 0.4, 0.1) mM, and the absorbance at 593nm which was recorded using UV spectrophotometer.

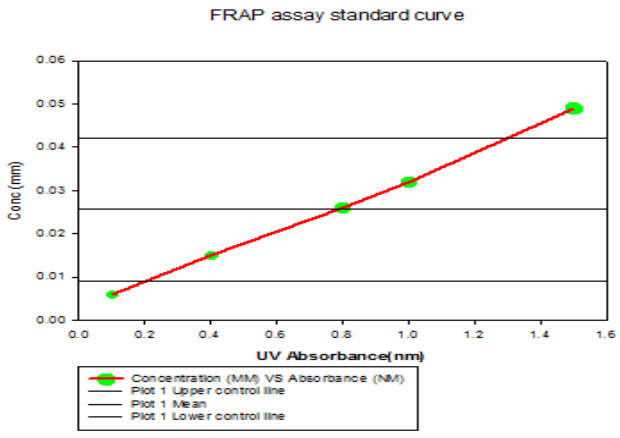


Figure4.3: linearity of FRAP (dose-response line) for standard solutions.

Using Sigmaplot, a standard linear curve was plotted across different concentration and the absorbed amount of reduced ferrous at 593nm for the five concentrations (Figure1). It is clear that there is a proportional relation between concentrations of $FeSO_4$ and the UV absorbance at 593nm.

Protocol has been adapted from Benzie and Strain(Iris F.F. Benzie, 1996). The results of FRAP assay are reported in table(3). The antioxidant activities were expressed as the concentration of antioxidant having a ferric reducing ability equivalent to that of 1 mM of $FeSO_4$. It was clear that the cotyledonary callus grown on B5 media has the highest antioxidant power (0.189) followed by those grown on QL Media and the least antioxidant capacity were for those grown on MS media, these results like other published studies indicate that the type of media and its contents affect the metabolic pathway of the plant and its ability to produce secondary metabolites(Ruiz, 2010, Frisvad, 2012)

Table 4.3: Antioxidant activity of the water extracts from cotyledonary callus grown on three different media.

Media type	**Absorbance at 593nm of extract of cotyledonary callus**
B5	0.189
QL	0.327
Ms	0.380

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity and phenolic (polyphenols) content.

4.3 Initiation of cell suspension culture and addition of the elicitor

Homogenous cell suspension culture was observed after the initiation of suspension culture from cotyledonary callus grown on MS media. Referring to Abu Allan's (2012) protocol on cell suspension growth curve, the best time to add the elicitor is between the day16 and 18 of growth since it is the time having the most viable amount of cells(Abu Allan, 2012). Tetrazolium test was used to examine the viability of cells since viable cells give pink color, this was done before starting the addition of the elicitor to ensure cells viability in the suspension.

4.4 Identification of secondary metabolites in callus alcoholic extract:

4.4.1 Thin Layer Chromatography (TLC)

A single band with R*f* = 0.73 can be observed under UV detector (figure 4.3) using a combination of the mobile phase Methanol :DW :Formic acid at 40:57:3.

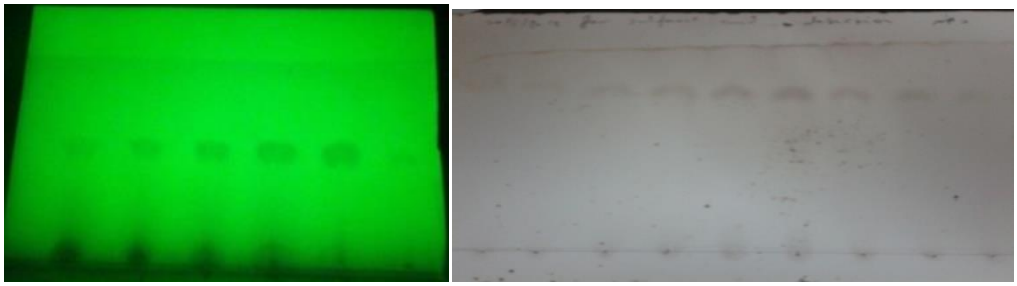


Figure 4.4: Left: the targeted compound under UV light separated with methanol: DW: formic acid **at** 40:57:3. Rf = 0.73. Right: the same plate after treating it with 15% sulfuric acid - methanol after heating.

To approve that this band is not overlapped with other non UV active bands, 15% sulfuric acid-methanol was sprayed on the silica plate and heated slowly with a hot plate for 6 min. Only one single band was observed and (figure 3.5). This test supports the presence of a single chemical compound in the extract. The full experiment is described in section 3.10.1 in chapter 2.

4.4.2 Column chromatography

The alcoholic extract was separated using column chromatography to 20 fractions (2.0ml each) and the targeted band was detected by TLC under UV light. These 20 fractions were collected and evaporated to get a total separated solid compound of 390.0 mg figure (4.4). these were used in further analyses and characterization (preserved in 4°C).

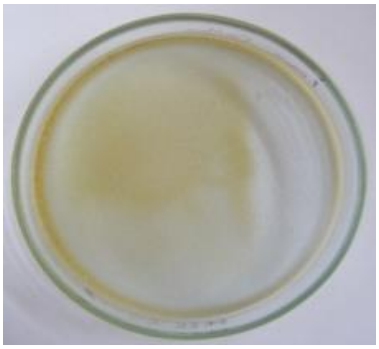


Figure (4.5): A glass Petri dish with the residues of a sticky alcoholic extract following the evaporation of the solvent.

4.4.3 Mass Spectrometry:

Mass spectrometry was done in the negative mode which gave a single peak at 485 MW. Accordingly, the most probable compound corresponds to triterpenoid acid. Since the peak is in the negative mode therefore the molecular weight is 486 and it is corresponding to either $C_{31}H_{50}O_4$ or $C_{30}H_{46}O_5$(James n. Roitman, 1978). According to TLC Many triterpenoid acids are known with similar molecular formulas e.g: methyl lycernuate A, quinovic acid, 23-deoxojessic acid, 3, 27-Dihydroxy-20(29)-lupen-28-oic acid methyl ester. Among this list Ceanothic acid has already been isolated from *Zizyphus spina-christi* (Al-Hasani, 2009, Seru Ganapaty, 2006) (Appendix 3).

4.5 Testing the biological activity:

4.5.1 Anti-cancer activity:

Cotyledonary callus extract which grown on MS media for two weeks was tested against two cancer cell lines; A549 and U205 which was carried out in University of Zurich, Institute of Organic Chemistry by Dr Jawad Al-Zeer. Only the U205 osteosarcoma was affected at EC_{50} = 395µgm/ml. Little work has been done on this cell line regarding the trial of natural anti-cancer chemicals, however, the result in this report indicate some potential therapy by *Z. spina-christi* extract. The effects of ethanolic extract on one cell line more than the other indicated sort of specificity on different cell lines to understand this more the extract should be tested on a variety of cell lines, the more the better to see if we can detect sort of selectivity toward specific cancer cells.

5.2 Antibacterial activity of the extract:

For studying the antibacterial activity of ethanolic extract of *Z. spina-christi*; disc diffusion assay (DDA) was used at first then minimum inhibitory concentration (MIC) were used to determine the exact concentration needed to kill the bacteria (Burt, 2003, Repon Kumer Saha, 2014).

In disc diffusion assay alcoholic extract of cotyledonary callus grown on four different growth media were prepared and used. Different concentrations of plant extracts (2000, 1000, 500 µl/disc) were used to test the antimicrobial activity. The results show no

significant antimicrobial effect for these concentrations on *Escherichia coli* (figure 4.6a) , *methicillin-resistant S. aureus* (MRSA) (figure 4.6b) and *Pseudomonas auregenosa* (figure 3.7c), however a small zone of inhibition was noted against *methicillin-sensitive S. aureus* (MSSA) (figure 4.6d) using the 2000µl/disc but still considered not significant because it was not a symmetric inhibition as that resulted in the positive control, 70% alcohol was used as a negative control (Rasha Saad, 2014). A study by Mohammed et al. (2013) [55], the ethanolic extract derived from *ex vitro* plant (stem bark) at 6.0mg showed 14.12±0.79 mm inhibition zone on *S. aurius* species. According to this, it is not surprising that extracts from *Z. spina-christi* obtained from other tissues have shown antibacterial properties as well. In addition to that and by reviewing the literature callus cells has the lowest amount of secondary metabolites since they are undifferentiated cells.

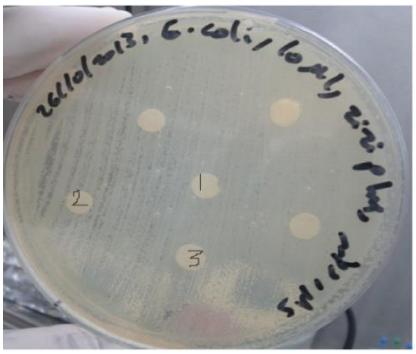


Figure 4.6a: The results of inhibition using alcoholic extract in DDA on *E.Coli* strain. Discs which are numbered (1, 2, 3) are negative control using 90% alcohol.

Figure 4.6b: The results of inhibition using alcoholic extract in DDA on MR *S. Aureus.* Discs which are numbered (1, 2, 3) are negative control using 90% alcohol.

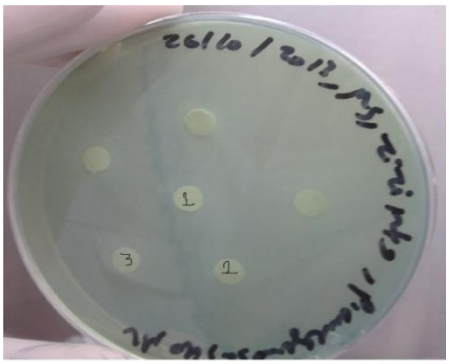


Figure 4.6c: The results of inhibition using alcoholic extract in DDA on *Pseudomonas auregenosa.* Discs which are numbered (1, 2, 3) are negative control using 90% alcohol.

Figure 4.6d: the zone of inhibition resulted using alcoholic extract in DDA on methicillin-sensitive *S. aureus.*(1, 2, 3) are negative control using 90% alcohol.

Since there are no clear zone of inhibition using disc diffusion assay, MIC test was done to determine the minimum concentration of the extract needed to inhibit the growth of *S. aureus* which gave a previous positive result. Here only MS cotyledonary extracts were used. The results showed that 3.0 mg/100 µl is needed to result in a clear solution. Which indicate that the MS crude cotyledonary extract exhibit a significant effect on *S. aureus* species (figure 4.7).

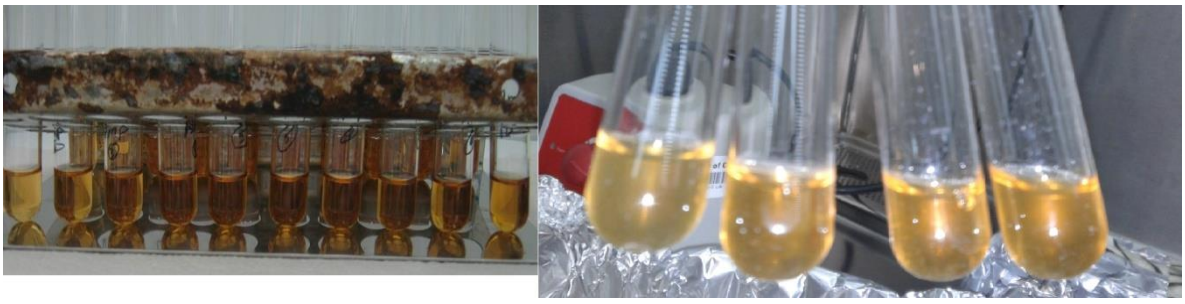


Figure 4.7: The antibacterial activity of alcoholic extract of *Z.spina.christi* cotyledonary callus using MIC method.

Compared to other studies on *Z. spina-christi*, different extracts are known to have antibacterial and antifungal activity. One study showed that aqueous extracts of leaves showed remarkable inhibition (17.67mm) at concentration of 200mg/ml on *S. aureus* (Khalid, 2011b). In other study, it is shown that the aqueous extract of *Z. spina-christi* from stem bark possesses significant antibacterial activity against *S. aureus, Bacillus subtilis, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella para typhi B and Klebsiella pneumonia*(Jinous Asgarpanah, 2012b). A third recent study shows that the antibacterial activity of the ethanol extract of the leaves of *Zizphus spina-christi* shows zone of inhibition of 12.0mm, 14.0mm on *Salmonella typhimurium* at 90.0mg/ml, 120.0mg/ml and 10.0mm, 11.0mm, 15.0mm on *S. aureus* at 60.0mg/ml, 90.0mg/ml and120.0mg/ml respectively and 11.0mm, 12.0mm, 13.0mm, 17.0mm on *Shigella spp* at 30-120.0mg/ml but no activity was observed for *P. aeruginosa* (S.M. Dangoggo, 2012).

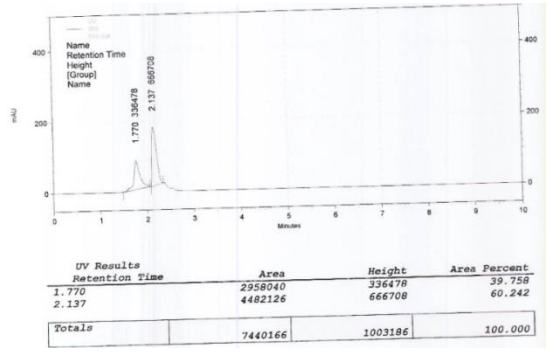
According to a recent literature review, this is the first study to address the properties and bioactivities of a specific callus line derived from cotyledonary tissues of this medicinal tree.

4.6. Elicitation using salicylic acid and HPLC analysis of cells' extract:

The same UV active organic compound separated by column chromatography was tested with HPLC in this section, the HPLC was done on the same mobile phase for TLC (water: methanol: formic acid) but the separation was not that sufficient by using this mobile phase (figure 3.9). The constituents and ratio of the mobile phase was changed until a sufficient reasonable separation was achieved by the following mobile phase:monopotassium phosphate: acetonitrile.

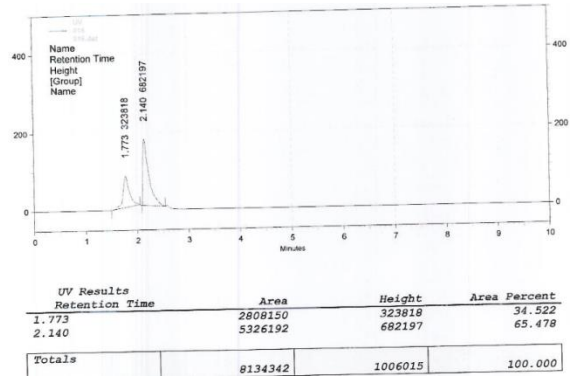
Salicylic acid was used as a secondary-metabolites elicitor between the 16^{th} and 18^{th} day of the culture. It was added to the cultures at different concentrations (0.0, 25, 50 or 75) mM. Results show a change in the total concentration (area under the curve) of the targeted compound with different salicylic acid treatments. The HPLC chromatograms figure (4.7) depicted a total area for the control treatment = 7440.166mAU, with 25.0mM SA the total area increased to 8134.342, with SA at 50.0mM the area =8574.684, and the highest SA treatment with 75.0mM resulted in decrease of total area to = 7483.754. Accordingly, treatment of cell suspension with 50.0mM SA showed the maximum elicitation of

secondary metabolites and the next concentration (0.75mM) has inhibitory effect. This is a typical result that can be obtained by the treatment with abiotic source of stress.



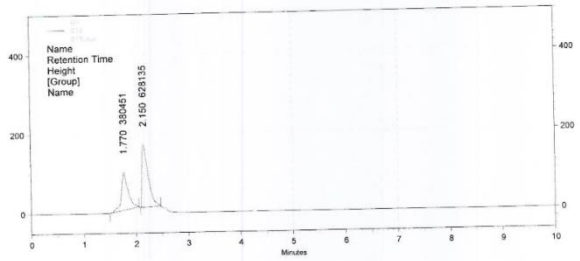
UV Results Retention Time	Area	Height	Area Percent
1.770	2958040	336478	39.758
2.137	4482126	666708	60.242
Totals	7440166	1003186	100.000

A



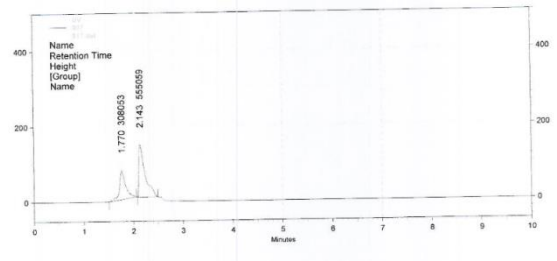
UV Results Retention Time	Area	Height	Area Percent
1.773	2808150	323818	34.522
2.140	5326192	682197	65.478
Totals	8134342	1006015	100.000

B



UV Results Retention Time	Area	Height	Area Percent
1.770	3382247	380451	39.445
2.150	5192437	628135	60.555
Totals	8574684	1008586	100.000

C



UV Results Retention Time	Area	Height	Area Percent
1.770	2636835	308053	35.234
2.143	4846919	555059	64.766
Totals	7483754	863112	100.000

D

Figure 4.8: HPLC chromatograms of the extracted band. Absorbance was set at 254 nm using (water: methanol: formic acid) as a mobile phase. A: area for the control treatment 0.0 MmSA, B: area for treatment with 0.25mM SA, C: area for treatment with 0.50mM SA, D: area for treatment with 0.75mM SA.

Chapter 5

Conclusion remarks and future work

In secondary metabolites production, in vitro studies have always the superiority over traditional (ex vitro) studies in plant research since the plant gives continuous and reproducible amounts independently of the season or external environment . One important aspect in *in vitro* studies is the possibility of culturing tissues that are difficult to obtain or grown in natural or external conditions such as root culture and cotyledonary tissue culture. This is the first study that addressed the *in vitro* callus and cells derived from cotyledonary explants of the medicinal tree *Z. spina-christi* since this callus line differs from others in containing a UV active organic compound(s) that can be clearly seen under UV light. The results in this study showed the possibility of increasing its concentration by elicitation. Also this unique secondary metabolite possesses antioxidant activity depicted by FRAP assay. In the literature *Z.spina christi* is known to have many antioxidant compounds including Alkaloids, Flavonoids and polyphenols (Basuny, 2013) but in cotolydonary callus further studies are recommended to analyze and understand the source of this antioxidant power and compare it with other *exvitro* studies.

The alcoholic extract showed significant anti bacterial effect on *Staphylococcus aureus,* a gram positive bacteria, it is an approach nowadays to use biotechnological techniques including bioreactors and different types of elicitors to increase the amount produced by plant cells of these active antibacterial secondary metabolites. In addition to that in this study the alcoholic extract showed a kind of specificity effect against the osteosarcoma cell line U205 at high concentration in the Cytotoxicity assay compared to human lung carcinoma (A549).

The sequence of analytical and separation techniques which was conducted from TLC to Column chromatography, HPLC and MS indicated the presence of a triterpenoid acid in the alcoholic extract, probably one of the following: methyl lycernuate, quinovic acid, 23-deoxojessic acid or 3,27-Dihydroxy-20(29)-lupen-28-oic acid methyl ester, for future analysis a comparison of a standard compound of each of them is needed to determine the exact compound separated in our extract.

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Appendices

Appendix 1

Callus surface area from ImageJ results within five weeks.

Table 1: Row data for growth changes of surface aria of cotolydonary callus grown on MS media

Petri dishes	MS W5	MS W4	MS W3	MS W2	MS W1
1	1131.627	908.175	461.354	375.35	398.708
2	865.026	429.349	312.922	251.741	307.145
3	1182.91	942.175	686.693	342.518	260.18
4	693.397	572.393	686.693	382.384	451.317
5	1490.737	1252.596	817.632	579.462	477.172
6	1403.793	1041.663	761.444	484.878	396.492
7	1428.059	1152.503	822.242	614.496	461.373
8	903.532	323.256	225.36	311.072	304.052
9	577.664	384.052	293.787	311.633	257.938
10	661.048	677.139	428.009	439.611	406.74
Mean	1033.7793	768.3301	549.6136	409.3145	372.1117

Table 2: Row data for growth changes of surface aria of cotolydonary callus grown on B5 media

Petri dishes	B5 W5	B5 W 4	B5 W3	B5 W 2	B5 W1
1	400.58	368.487	258.199	259.968	247.972
2	976.109	955.202	680.554	536.459	471.142
3	445.871	436.711	386.554	402.103	409.547
4	509.178	484.485	398.43	372.625	388.237
5	864.941	765.183	612.747	508.191	250.144
6	828.301	768.898	514.511	377.367	442.434
7	989.637	996.716	874.372	572.819	416.644
8	847.565	786.856	686.908	489.987	310.102
9	731.621	623.252	727.144	398.93	431.053
10	743.757	873.892	503.096	484.168	355.135
Mean	733.756	705.9682	564.2515	440.2617	372.241

Table 3 : Row data for growth changes of surface aria of cotolydonary callus grown on QL media

QL W5	QL W4	QL W3	QL W2	QL W1
not available	1199.111	868.444	737.339	564.495
not available	1412.27	876.328	651.23	440.616
not available	1245.274	789.178	687.171	483.413
not available	1046.783	872.178	510.945	448.743
not available	1234.785	911.66	751.498	572.706
not available	682.33	428.76	373.858	270.548
not available	692.143	577.664	356.726	294.762
not available	994.152	723.114	559.444	414.721
not available	965.369	673.11	447.393	451.659
not available	1063.091	532.44	499.542	439.379
not available	1053.5308	725.2876	557.5146	438.1042

Table 4: Row data for growth changes of surface aria of cotolydonary callus grown on MCC media

MCC W5	MCC W4	MCC W3	MCC W2	MCC W1
not available	not available	743.334	686.228	559.773
not available	not available	740.132	617.071	518.376
not available	not available	795.277	640.132	437.011
not available	not available	754.75	667.211	551.774
not available	not available	757.039	639.758	575.832
not available	not available	699.751	654.731	495.026
not available	not available	553.016	495.018	382.477
not available	not available	645.712	597.478	486.473
not available	not available	722.21	657.529	530.04
not available	not available	754.88	610.439	520.916
not available	not available	716.6101	626.5595	505.7698

Table5. Components of MS and B5 media salt mixture in mg/L.

Component (mg/L)	M&S mixture	Gamborg B5 medium
Ammonium nitrate	1650.0	
Ammonium sulphate		134.0
Boric acid	6.2	3.0
Calcium chloride anhydrous	332.2	113.24
Cobalt chloride • $6H_2O$	0.025	0.025
Cupric sulfate • $5H_2O$	0.025	0.025
Na_2-EDTA	37.26	37.3
Ferrous sulfate • $7H_2O$	27.8	27.85
Magnesium sulfate	180.7	17.099
Manganese sulfate • H_2O	16.9	10.0
Molybdic acid (sodium salt) • $2H_2O$	0.25	0.25
Potassium iodide	0.83	0.75
Potassium nitrate	1900.0	2500.00
Potassium phosphate monobasic	170.0	
Sodium phosphate monobasic		130.5
Zinc sulfate • $7H_2O$	8.6	2.0
myo-Inositol	100.0	100.0
Nictotinic acid (free acid)	0.5	1.0
Pyridoxine • HCl	0.5	1.0
Thiamine • HCl	0.5	10.0

Table 6. Components of McCown Woody Plants salts and vitamins in mg/L

Components (mg/L)	WPM salts
Ammonium nitrate	400
Boric acid	6.2
Calcium chloride, anhydrous	72.5
Calcium nitrate	386
Cupric sulfate·$5H_2O$	0.25
Na_2 EDTA	37.3
Ferrous sulfate·$7H_2O$	27.85
Magnesium sulfate	180.7
Manganese sulfate·H_2O	22.3
Molybdic acid (Na salt)·$2H_2O$	0.25
Potassium phosphate, monobasic	170
Potassium sulfate	990
Zinc sulfate·$7H_2O$	8.6

	mg/l	µM
Glycine	2.00	26.64
Myo-Inositol	100.00	554.94
Nicotinic acid	0.50	4.06
Pyridoxine HCl	0.50	2.43
Thiamine HCl	1.00	2.96

Table 7. Quoirin & Lepoivre Basal Salt Mixture in mg/L.

Ammonium Nitrate	400
Boric Acid	6.2
Calcium Nitrate	833.77
Cobalt Chloride•$6H_2O$	0.025
Cupric Sulfate•$5H_2O$	0.025
Na2 EDTA•$2H_2O$	37.3
Ferrous Sulfate•$7H_2O$	27.8
Magnesium Sulfate, Anhydrous	175.79
Manganese Sulfate•H_2O	0.76
Molybdic Acid (Sodium Salt) •$2H_2O$	0.25
Potassium Iodide	0.08
Potassium Nitrate	1800
Potassium Phosphate, Monobasic	270
Zinc Sulfate•$7H_2O$	8.6

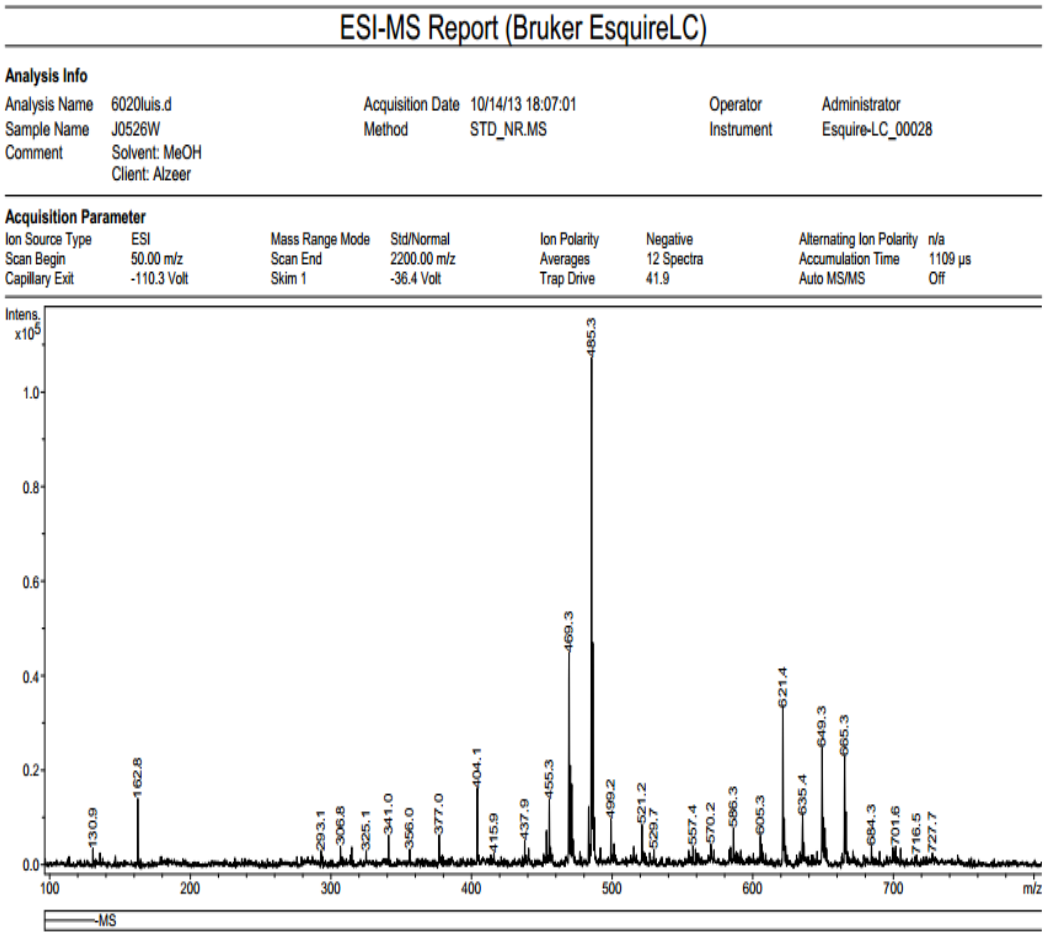
Appendix 2

Procedure of Standard Disc Diffusion Method.

1- Cultures were prepared in Caretas microbiology lab by transferring one loop of stock bacteria which are kept in – 80° and streaked onto culture media (MacConkey Agar media for e-coli and Sheep Blood Agar media for other microorganisms) to obtain isolated colonies.
2- These cultures incubated in 37° overnight and subcultures were obtained by transferring one colony from this 18 hr incubated cultures to fresh culture media.
3- After incubation at 37°C overnight, 1 or 2 well-isolated colonies were selected with an inoculating loop, and transferred to a tube of sterile saline (≈ 2 ml) and vortex thoroughly. The bacterial suspension should then be compared to the 0.5 McFarland standard.
4- If the bacterial suspension does not appear to be the same density as the McFarland 0.5, turbidity can be reduced by adding sterile saline or increased by adding more bacterial.
5- A sterile cotton swab was dipped into the suspension. Then pressed firmly against the inside wall of the tube just above the fluid level, the swab was rotated to remove excess liquid.
6- Inoculated culture was dispersed by streaking the sterile swab over the entire sterile agar surface (Muller Hinton Agar) by rotating the plate 60° each time to ensure the inoculum uniformly spread. Eventusally , swab all around the edge of the agar surface.
7- The inoculated plates were allowed to sit for 5-10 minutes to let the broth absorb into agar.
8- Standard size blank Whatman filter paper discs (6.00 mm. in diameter) sterilized by autoclaving and dried at 60°C for 1 hour, were saturated with the tested extracts.
9- Three concentrations of each extract types were used in this test, 2000 µl,1000µl, 500µl.
10- For control discs, 2000 µl of 90% alcohol were added to sterilized filter discs separately.
11- The discs were air dried at room temperature and under laminar flow to remove any residual solvent which might interfere with the determination.
12- Standard antibiotic discs of Ampicillin, Gentamicine, and Sulphamethoxazole/ trimethoprim were used for positive control.
13- The discs were placed on each plate (4 to 5 discs per 25 ml plate) and then gently pressed to ensure contact with gar surface. Plates were incubated for 24hr at 37°C.
14- At the end of the incubation period the antibacterial activity was evaluated by measuring the inhibition zone by using a ruler. Results should be expressed as mm. The zone of inhibition was considered as an indicator for the antimicrobial activity.
15- Eventually plates were sterilized and discarded properly as biohazard material in a special container.

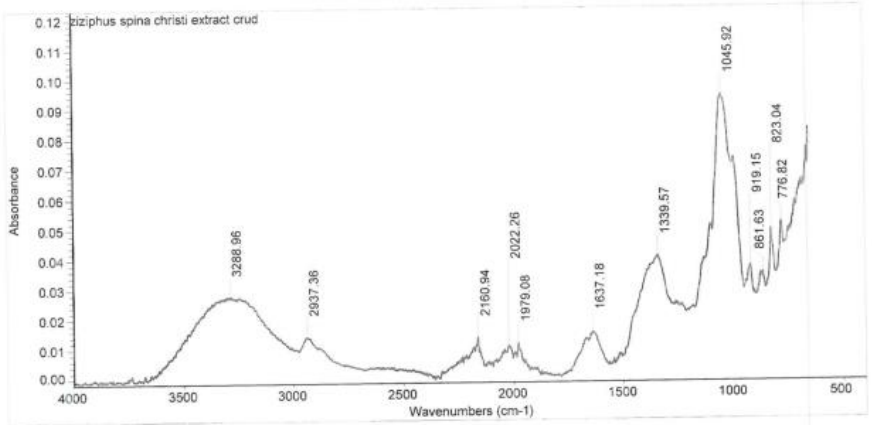
Appendix 3:

MS read which was measured in the negative mode which gave a single peak at 485.



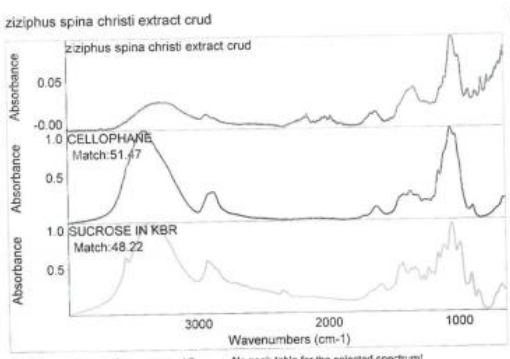
Appendix 4:

IR results of the crud cotyledonary callus extract of *Ziziphus spina-christi* and the comparison of the library compounds in Jerusalem Pharmaceuticals:



Mon Jan 13 22:44:23 2014 (GMT+02:00)
FIND PEAKS:
Spectrum: ziziphus spina christi extract crud
Region: 4000.00 400.00
Absolute threshold: 0.008
Sensitivity: 50
Peak list:

Position:	659.27	Intensity:	0.0760
Position:	776.82	Intensity:	0.0511
Position:	823.04	Intensity:	0.0491
Position:	861.63	Intensity:	0.0344
Position:	919.15	Intensity:	0.0367
Position:	1045.92	Intensity:	0.0935
Position:	1339.57	Intensity:	0.0401
Position:	1637.18	Intensity:	0.0149

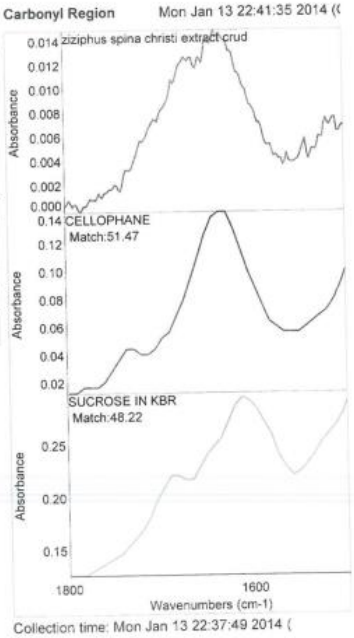


Number of sample scans: 16
Number of background scans: 16
Resolution: 4.000
Sample gain: 1.0
Optical velocity: 0.4747
Aperture: 80.00

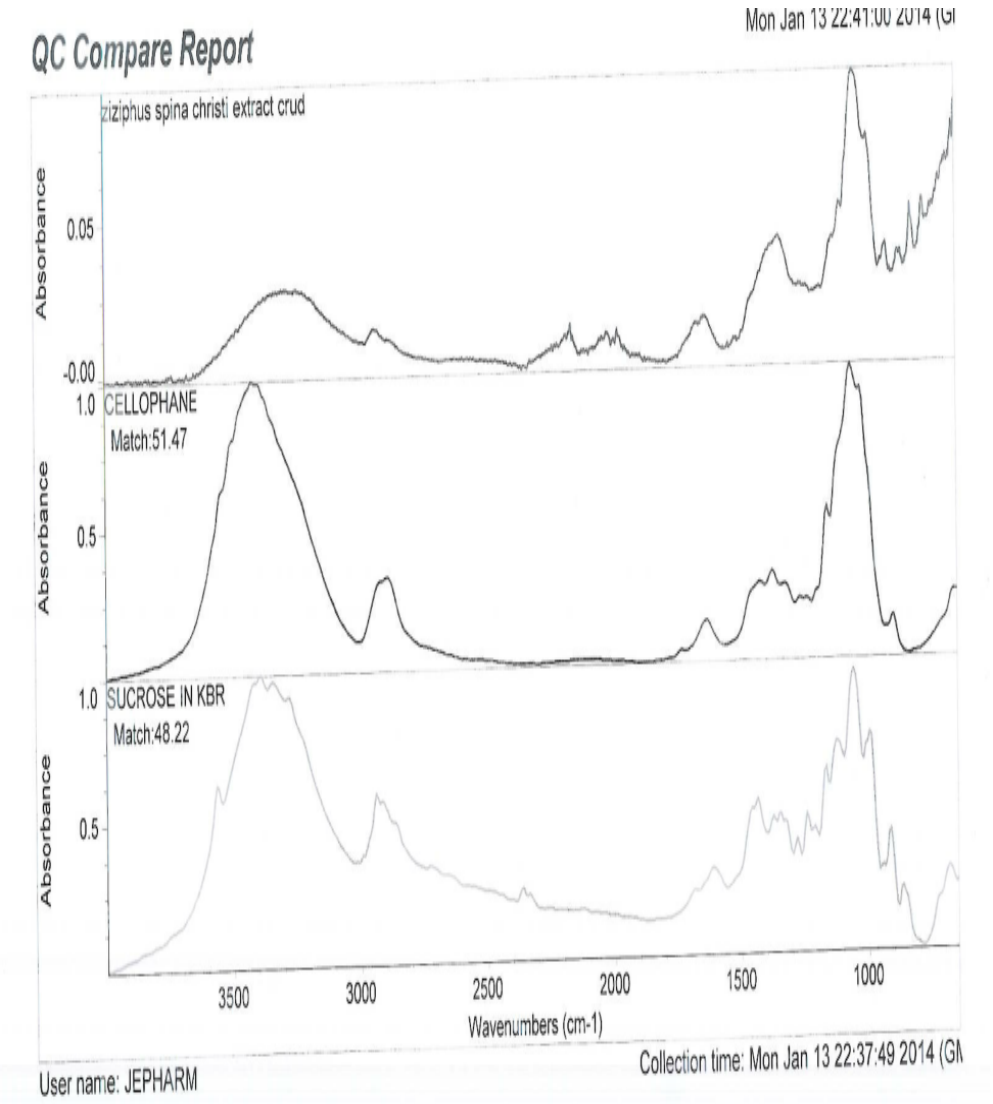
No peak table for the selected spectrum!

User name: JEPHARM

Comments:



Collection time: Mon Jan 13 22:37:49 2014



All figures and TLC results will be given upon request