Bioassay of Anti-tumor Activity of Black Cumin Oil (Nigella sativa) and Bee Honey, Using the Indigenous Strain of Agrobacterium tumefaciens “SDB0012”

Hagir Abdalrhman Mohammed Gsmalseed
B.Sc (Honours) in Food Engineering Technology
Faculty of Engineering and Technology
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<td>Dr. Mutaman Ali bd Algadir Kehail</td>
<td>Co-Supervisor</td>
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Date: November 2014
Bench Top Bioassay of Anti-tumor Activity of Black Cumin (*Nigella sativa*) Oil and Bee Honey, Using the Indigenous Strain of *Agrobacterium tumefaciens* “SDB0012”

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<td>Dr.</td>
<td>External Examiner</td>
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<tr>
<td>Dr. Yasir Mohamed Abd Elrahim</td>
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Date: 13 November 2014
DEDICATION

To My parents, brothers and sisters
ACKNOWLEDGMENTS

Firstly, praise and thanks to Allah or giving power, health ability to achieve this humble and with his will this achievement was patience, and properly till completing this study.

Sincere appreciation and thanks are extended to my major supervisor Dr. Mohammed Taha Yousif. Deep thanks and appreciation are due to my co-supervisor Dr. Mutaman Abd ElGadir who helped me a lot to conduct the research

I would like also to acknowledge Salah Ahmed Mustafa and Ustaz Hassan Anssary for their technical assistance. Thanks are extended to Algum Company in Khartoum for providing me some chemicals to conduct my research.
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ABSTRACT

Sudan is endowed with natural products having anti-bacterial, anti-fungal and anti-cancer activities. Among these, bee honey and black cumin oil are recommended by some local botanist to be used as antibacterial and anti-cancer production; in addition to other health uses. In this study both bee honey, crude oil of black cumin and a mixture of them at a rate of 1:1 were studied for antibacterial and anti-tumor activities using the potato disc bioassay at the Microbiology Laboratory, Faculty of Engineering and Technology, University of Gezira. The indigenous strain SDB0012 used in this study was kindly provided by the National Institute for Promotion of Horticultural Exports-University of Gezira. Whereas, bee honey and black cumin oil were collected in Wad Medani market. Moreover, the crude oil of the black cumin was subjected to fractionation using thin layer chromatography and potato disc bioassay to identify the active anti-tumor components. In these trials, the Complete Randomized Design was used with three replicates. Results indicated that both bee honey and the crude extract of the black cumin had no anti-bacterial activities against the indigenous strain of *Agrobacterium tumefaciens* “SDB0012”, which characterized by having resistance against penicillin (30 mg), chloramphenicol (30 mg) and ciprofloxacin (30 mg). Both bee honey and black cumin had anti-tumor activities with inhibition of 40% and 55%, respectively. The mixture of bee honey and cumin oil extract (1:1) gave complete inhibition of tumor on the potato discs. Only one out of the eight components separated from the crude extract of black cumin in thin layer chromatography showed complete inhibition of tumors and was considered as the active anti-tumor component of the black cumin. Results recommended further fractionation of the fraction 2 (Rf 0.147) of black cumin oil should be run to reach a stable homogenous component after successive fractions followed by bioassay. Moreover, animal experimentations (in mice having induced cancer) should be conducted to study the effect of the end product in-vivo.
التقييم المعمى للنشاط المثبط للأورام لزيت الحبة السوداء وعسل النحل باستخدام السلالة المحلية من بكتريا Agrobacterium tumefaciens "SDB0012".

هاجر عبد الرحمن محمد قسم السيد

خلاصة البحث

وهب الله السودان منتجات طبيعية لها أنشطة مضادة للبكتيريا ومضادة للفطرية ومضادة للسرطان. وبين هذه المنتجات يوصي العشاقين بعسل النحل وزيت الكمون لاستخدامها كمنتجات مضادة للجراثيم والسرطان بالإضافة إلى استخدامات صحية أخرى. هدفت هذه الدراسة إلى تقييم كل من عسل النحل، وزيت الكمون الأسود وخليطه منهما بمعدل 1:1 كنشاط مضادة للبكتيريا والمضادة للأورام باستخدام تقنية التقييم الاحيائي بأقران البطاطس في مختبر الأحياء الدقيقة في كلية الهندسة والتكنولوجيا، جامعة الجزيرة. البكتيريا الزراعية المستخدمة في هذه الدراسة هي السلالة المحلية "SDB0012" التي تم عزلها من جزيرة توتى بولاية الخرطوم وتحديدها في جامعة الجزيرة (Yousf et al., 2011). تم تجميع عسل النحل وزيت الكمون الأسود من عطاريه بمدينة حمداني. كما تم فصل المكونات الفعالة لزيت الكمون باستخدام كروماتوغرافيا الطبقة الرقيقة و اختبارها على أقران البطاطس بحثًا عن المكونات المضادة للأورام. في هذه التجارب، تم استخدام التصميم العشوائي الكامل استخدم ثلاثة تكرارات. وأشارت النتائج أن كلا من عسل النحل وزيت الكمون الأسود ليس لديها نشاط مضاد للتكرير البكتيريا ضد السلالة المحلية المستخدمة في هذه الدراسة والتي تميز بمقاومة ضد البنستي (30 مل), الكلورامفينيكول (30 ملغ) و سيبروفلوكاسين (30 ملغ). بلغت نسبة تثبيط انتشار الأورام 40، 55 لكل من عسل النحل وزيت الحبة السوداء، على التوالي. كما أعطي خليط من عسل النحل وزيت الكمون (1:1) تثبيط كاملا للأورام على أقران البطاطس. أظهر مكون واحد من نواتج الفصل الكروماتوغرافية الثمانية من زيت الكمون المكون رقم 2 من بداية الفصل (Rf 0.147)، تثبيط كامل للأورام إذا اعتبر المكون النشط المثبط للأورام في زيت الحبة السوداء. بناء على النتائج المتحدث عنها، أوصت الدراسة بدراسة وزنها لاستخدام حيوانات التجارب لدراسة اثره على تثبيط الأورام فيها.
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CHAPTER ONE

INTRODUCTION

The search for anticancer agents from plant sources started in earnest in the 1950s with the discovery and development of vinca alkaloids, vinblastine and vincristine and isolation of cytotoxic podophyllotoxins. The development of new screening technologies led to the revival of collections of plants and other microorganisms in 1986 with a focus on the tropical and subtropical regions, the derived clinical anti-cancer agents have, as yet, reached the stage of general use, but a number of agents are in preclinical development. Several plant derived compounds are successfully used in cancer treatment such etoposide derived from *Podophyllum peltatum* and *Podophyllum emodi*. Etoposide produces high cure rates in testicular cancer when used in combination with bleomycin (also derived from a natural product) and cisplatin (Igram, 1997).

Sudan is rich in medicinal plants with a wide biodiversity among these plant species. The crown gall tumor assay (CGTA) is one of several bench top bioassays recommended for the rapid screening of plants against anti-cancer activity. The inhibition of crown gall tumors on discs of potato (*Solanum tuberosum* L.) tubers shows an apparent correlation with anti-tumor activity. The rationale for the use of this type of bioassay is that the tumourogenic mechanism initiated in plant tissue by *A. tumefaciens* is in many ways similar to that of animals. Several plant species with anticancer activity have already been discovered using this bioassay. In this regard, the indigenous strain of *A. tumefaciens* SDB0012 has the ability to induce tumors growth on roots of some plant species such as pigeon pea, melon, sorghum, tomatoes and on potato discs (Yousif *et al.*, 2011 a). The surface of the tumors was smooth with no shoot growth which recognizes the octapine-type tumors. Addition of the bacterial suspension to small discs of potato (1.5 cm in diameter) resulted in growth of a number of large 21 days after inoculation. Therefore, this bacterial strain is rapidly used at the Microbiology laboratory of the Faculty of Engineering and Technology for detection of natural products having anti-tumor activity. Physical analytical methods such as chromatography are useful for determining sensitivity to the chemical complexities found in crude botanical extract. Use of more precise techniques such as HPLC, gas chromatography and gel electrophoresis were suggested to identify the active ingredients possessed in the promising extracts and to study polymorphism among constituents.
of these extracts. As some products have more than one active compound and their combined effects may be responsible for inhibition of growth of the tumors Elsedig (2007).

**The main objectives**

The main objectives of this study were to examine anticancer activity of bee honey and cumin and the combination of them at a rate of 1:1 using potato disc bioassay; In addition to determination of the active anticancer component(s) of the cumin crude extract using potato disc bioassay and thin layer chromatography technique.
CHAPTER TWO

Literature Review

2.1. *Agrobacterium tumefaciens*

*Agrobacterium tumefaciens* is a member of the family *Rhizobiaceae*. These bacteria are Gram-negative and grow aerobically, without forming endospores. The cells are rod-shaped and motile, having one to six peritrichous flagella. Cells are 0.6-1.0 µm by 1.5- 3.0 µm and may exist singly or in pairs. In culture on carbohydrate-containing media, cells produce large amounts of extracellular polysaccharides, giving colonies a voluminous slimy appearance. Recent classification of the species *Agrobacterium* has been undertaken by use of ribosomal RNA sequencing as a taxonomic tool. The resulting nomenclature placed the former species, *A. tumefaciens* biovar 1, *A. radiobacter* biovar 1, and *A. rhizogenes* biovar 1, within the new taxon *Agrobacterium tumefaciens* (Schaad et al., 2001). It is the cause of the crown gall disease. Yet, the mechanism that bacterium uses to parasitize plant tissue involves the integration of some of its own DNA into the host genome resulting in unsightly tumors and changes in plant metabolism, it is now used as a tool for engineering desired genes into plants (Horst, 1983).

*Agrobacterium tumefaciens* is cosmopolitan in distribution, affecting dicotyledonous plants in more than 60 different plant families. Crown gall can be found most often on stone fruit and pome trees as well as brambles and several species of ornamental plants (Agrios, 1988). It affects hundreds of species, particularly fruits, nuts and ornamental plants such as roses. The disease recently caused severe damage and economic losses among walnut tree growers in California. Once *A. tumefaciens* infects a plant, the bacterium travels throughout the root system and can wipe out an entire crop. The disease is difficult to control and the only option for farmers is to destroy the plants (Schaad et al., 2001).

2.1.1. Genetic transformation using *Agrobacterium tumefaciens*

*Agrobacterium tumefaciens* has the ability to transfer a particular DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease (Binns and Thomashow, 1988). The T-DNA contains two types of genes: the onco-genes, encoding for enzymes involved in the synthesis of auxins and cytokinins and responsible for tumor formation; and the genes encoding for
the synthesis of opines. The latter compounds are produced by condensation between amino acids and sugars, they are synthesized and excreted by the crown gall cells and consumed by A. tumefaciens as carbon and nitrogen sources. Outside the T-DNA, are located the genes for the opine catabolism, the genes involved in the process of T-DNA transfer from the bacterium to the plant cell and the genes involved in bacterium-bacterium plasmid conjugative transfer (Zupan and Zambrysky, 1995).

Virulent strains of A. tumefaciens and A. rhizogenes, when interacting with susceptible dicotyledonous plant cells, induce diseases known as crow gall and hairy roots, respectively. These strains contain a large megaplasmid (more than 200 kb), which play a key role in tumor induction and for this reason it was named Ti plasmid, or Ri in the case of A. rhizogenes. Ti plasmids are classified according to type of opines produced and excreted by the tumors they induce (Hooykaas and Schilperoort, 1992). During infection the T-DNA, a mobile segment of Ti or Ri plasmid, is transferred to the plant cell nucleus and integrated into the plant chromosome. The T-DNA fragment is flanked by 25-bp direct repeats, which act as a cis element signal for the transfer apparatus. The process of T-DNA transfer is mediated by the cooperative action of proteins encoded by genes determined in the Ti plasmid virulence region (vir genes) and in the bacterial chromosome (Zupan and Zambryski, 1995). The Ti plasmid also contains the genes for opine catabolism produced by the crown gall cells, and regions for conjugative transfer and for its own integrity and stability. The 30 kb virulence (vir) region is a regulon organized in six operons that are essential for the T-DNA transfer (virA, virB, virD, and virG) or for the increasing of transfer efficiency (virC and virE) (Jeon et al., 1998). Different chromosomal-determined genetic elements have shown their functional role in the attachment of A. tumefaciens to the plant cell and bacterial colonization: the loci chvA and chvB, involved in the synthesis and excretion of the b-1,2 glucan, the chvE required for the sugar enhancement of vir genes induction and bacterial chemotaxis (Cangelosi et al., 1990 and 1991); the cel locus, responsible for the synthesis of cellulose fibrils, the pscA (exoC) locus, playing its role in the synthesis of both cyclic glucan and acid succinoglycan and the att locus, which is involved in the cell surface proteins (Matthysse, 1987).

The initial results of the studies on T-DNA transfer process to plant cells demonstrate three important facts for the practical use of this process in plants transformation. Firstly, the tumor formation is a transformation process of plant cells resulted from transfer and integration of T-DNA and the subsequent expression of T-DNA genes. Secondly, the T-DNA genes are transcribed only in plant cells and do not play any role during the transfer process (Hooykaas and Schilperoort, 1992). Thirdly, any foreign DNA placed between the T-DNA borders can be transferred to plant cells, no
matter where it comes from. These well-established facts, allowed the construction of the first vector and bacterial strain systems for plant transformation (Torisky et al., 1997).

2.1.2. The indigenous strain of Agrobacterium tumefaciens “SDB0012”

An Agrobacterium isolate was extracted from the root of a mulaita plant (Sonchus cronutus) grown in Tutti Island in Khartoum State-Sudan following Schaad et al. (2001) and purified in five successive rounds of selection and identified at the National Institute for Promotion of Horticultural Export-University of Gezira, since 2005 (Elsedig, 2007). It was subjected to different biological and molecular tests. These included expression of enzymes, production of acids from carbohydrates, resistance to different herbicides, sensitivity to antibiotics, type of tumors and plasmid(s) characteristics. Qualitative tests showed positive results for oxidase, urease, and catalase enzymes. This strain successfully utilized fructose, lactose, raffinose, sucrose and manitol as sources of carbon. Whereas, other carbohydrates such as starch, glucose, galactose and maltose were not utilized (Yousif et al., 2011a). It showed resistance against glyphosate (the active ingredient of roundup) and 2,4-D herbicides and sensitivity to pendimethalin, the active ingredient of stomp (Yousif et al., 2011b). The tumor caused by this strain showed shoots like growth on the surface of potato discs, which indicates the presence of nopaline-type plasmid(s). This strain showed resistance against penicillin (30 mg), chloramphenicol (30 mg) and ciprofloxacin (30 mg). Therefore, it was described as a multi-resistant or a superbug bacterium. Results indicated the presence of two distinguished plasmids of close molecular weights ranging between 2.0 and 2.4 kb, while molecular weight of the indigenous isolate of Escherichia coli was estimated to be 2.0 kb. The presence of the two plasmids in the bacterial cell of SDB0012 might explain the presence of more than one.

2.1.3. Crown gall tumors growth on potato discs induced by A. tumefaciens

Crown gall is a neoplastic disease of plants induced by specific strains of the gram negative bacterium, the A. tumefaciens. The bacteria contain a large Ti (tumor – inducing) plasmid which carries genetic information (T-DNA) that transforms plant cells into tumorous cells. The inhibition of crown gall tumors on disc of potato (Solanum tuberosum L.) tubers showed an apparent correlation with compounds and plant extracts known to be active (in vivo, murine leukemia) anti tumor assay. In 1980 potato disc method was modified and tested the effectiveness of the developed method adopting an appropriate statistical evaluation of the modified assay as a prescreening activity in crude
plant extracts. The modified assay was initially performed using a series of natural antitumor compounds, aqueous plant extracts and on ethanol extracts of seeds of 41 *Euphuriaceae* ssp (Ferrigin *et al.*, 1980).

According to Elsedig (2007), the indigenous strain of *A. tumefaciens* “SDB0012” has the ability to induce tumors growth on roots of some plant species such as pigeon pea, melon, sorghum, tomatoes and on potato discs. Addition of the bacterial suspension to small discs of potato (1.5 cm in diameter) used as control resulted in a high tumors growth 21 days after inoculation. The overall mean of tumors was found to be four large tumors /disc of 1.5 cm in diameter. The surface of the tumors was smooth with no shoot growth which recognizes the octapine-type tumors. This bacterial strain was further used to be applied on potato discs treated with plant extracts and natural products to assess their anti-tumor activity (Yousif *et al.*, 2012).

### 2.1.4. Use of the biological assays to evaluate botanicals

Bioassays offer a special advantage in the standardization and quality control of heterogeneous botanical products. Such products can be “heterogeneous” due to presence of mixtures of bioactive components either from the same or from purposefully mixed botanical sources. Physical analytical methods, such as chromatography, are useless for this purpose as they are usually insensitive to the chemical complexities found in crude botanical extract. Most often a desired biological response in due to only one compound but a mixture of bioactive plant components and the relative proportion of a single bioactive compound can vary from batch to batch while the bioactivity still remains with tolerable limits. Thus, physical or chemical analysis of a single component in such mixtures is not completely satisfactory. The potato disc bioassay technique was developed as to aid drug discovery work with botanicals. This method was used over the past 15 years, and was apparently adaptable to the purpose of standardization or quality control for bioactive components in such heterogonous botanicals (Jerry *et al.*, 1998). The indigenous strain of *A. tumefaciens* SDB0012 was effectively used to be applied on potato discs treated with plant extracts and natural products to assess their anti-tumor activity (Yousif *et al.*, 2012).
2.2. Anti tumor response of some natural product

Natural product being synonymous with secondary metabolite, and these organic substances are of relatively small molecular weight (<3,000) and of considerable structural diversity. Such compounds tend to be in the correct chiral form to exhibit biological activity, and it has been postulated that, this facilitates species survival by repelling or attracting other organisms (Williams et al., 1989). For over 40 years, natural products have played a very important role as established cancer chemotherapeutic agents, either in their crude or synthetically modified forms. For example, antitumor antibiotics from microbes include the anthracyclines (such as doxorubicin), bleomycin, dactinomycin (actinomycin), and mitomycin C. In turn, members of four classes of plant-derived compounds are used widely as antitumor agents, namely, the bisindole (vinca) alkaloids, the camptothecins, the epipodophyllotoxins, and the taxanes (Kinghorn, 2008). In addition, there are several examples of promising natural product-derived anti-neoplastic agents currently in advanced clinical development or recently approved, not only from microbes and plants, but also of marine origin (Cragg et al., 2005). Of a total of 155 anti-cancer agents approved for use in Western medicine and Japan since the 1940s, 47% were classified as either natural products (14%), semi-synthetic derivatives of natural products (28%), or otherwise derived from natural products (5%) (Newman and Cragg, 2007). The clinically used anticancer agents of natural origin, as well as those compounds of this type in advanced clinical trial, are known to exhibit considerable structural diversity (Kinghorn, 2008).

Among the largest groups of taxonomically identified classes of organisms that may be studied as sources of new anticancer drugs are arthropods, higher plants, and marine invertebrates. In addition, natural product researchers have examined other taxonomic classes of organisms found all over the world, including algae, bacteria, fungi, and even terrestrial vertebrates. However, it must be pointed out that natural product drug discovery for anticancer agents requires special procedures involved with sample collection, inclusive of the development of “benefit-sharing” agreements with source countries, whether the samples are of marine or terrestrial origin (Tan et al., 2006). There is a tendency for natural product chemists to specialize on the types of organisms they work, such higher plants or marine fauna, due to the different methods of organism collection and work-up in the laboratory. However, there is increasing evidence that the same secondary metabolite of significance as a potential anticancer agent may be produced by more than one type of organism (Eyberger et al., 2006).
Recent developments in anti-cancer technology allow isolation chemists working in a natural product laboratory to isolate only a few milligrams of a promising lead compound of novel structure. This would then be tested against a number of human cancer cells, such as the U.S. National Cancer Institute (NCI) 60-cell line panel (Shoemaker, 2006), the application of which may also yield valuable information on mechanism of action (Zhou et al., 2000). Compounds may also be shown to be active in a mechanistic *in-vitro* assay germane to cancer chemotherapy. Ideally, the *in-vitro* activity of the new compound should be evaluated in an *in-vivo* model such as a xenograft of a human tumor in an immune-deficient mouse, but this may require over 100 mg of pure compound. Lead compound scale-up usually may be performed by re-isolation using a further collection of a plant or a marine specimen, or by additional fermentation if the compound is of terrestrial microbial origin. Since these procedures may be quite time-consuming, steps can be taken to make a more rapid decision as to whether a given lead compound natural product has the potential for further development as an anticancer agent. For example, the hollow fiber assay was developed at the NCI (Hall *et al*., 2000) as a relatively rapid *in-vivo* test for use prior to traditional xenograft assays (Kingham *et al*. 2003).

In recent years, a number of natural products isolated from Chinese herbs have been found to inhibit proliferation, induce apoptosis, suppress angiogenesis, retard metastasis and enhance chemotherapy, exhibiting anti-cancer potential both in vitro and in vivo. These natural products are also reviewed for their therapeutic potentials, including flavonoids (gambogenic acid, curcumin, wogonin and silibinin), alkaloids (berberine), terpenes (artemisinin, β-elemene, oridonin, triptolide, and ursolic acid), quinones (shikonin and emodin) and saponins (ginsenoside Rg3), which are isolated from Chinese medicinal herbs. In particular, the discovery of the new use of artemisinin derivatives as excellent anti-cancer drugs iCurr Opin Drug Discov Devel (Pazdur, 2009).

### 2.3. Cancer

A tumor (or tumour) is commonly used as a synonym for a neoplasm a solid or fluid-filled (cystic) lesion that may or may not be abnormal growth neoplastic cells that appears enlarged in size. Tumor is not synonymous with cancer. While cancer is by definition malignant, a tumor can benign, pre-malignant, or malignant, or can represent a lesion without any cancerous potential. The term tumor/tumour is derived from the Latin word for “swelling”. A neoplasm can be caused by an abnormal proliferation of tissues, which can be caused by genetic mutations. Not all types of
neoplasms cause a timorous overgrowth of tissue (Volokh, 2006). Cancer can be detected in a number of ways, including the presence of certain signs and symptoms, screening tests, or medical imaging. Cancer is usually treated with chemotherapy, radiation therapy and surgery (Pazdur et al., 2009). Cancer is primarily an environmental disease with 90-95% of cases attributed to environmental factors and 5-10% due to genetics (Kleinsmith, 2006). Many mutagens are also carcinogens, but some carcinogens are not mutagenic (Kinzler and Vogelstein, 2002). Physical inactivity is believed to contribute to cancer risk not only through its effect on body weight but also through negative effects on immune system and endocrine system. Worldwide approximately 18% of cancer deaths are related to infectious diseases. Radiation is a more potent source of cancer when it is combined with other cancer-causing agents. Global cancer rates have been increasing primarily due to an aging population and lifestyle changes in the developing world (Pazdur et al., 2009).

2.3.1. Cancer development

Cancer develops when cells in a part of the body begin to grow out of control, usually due to a mutation in the DNA. Although there are many kinds of cancer, they all appear to start because of out-of-control growth of abnormal cells. Normal body cells grow, divide, and die in an orderly fashion. Because cancer cells continue to grow and divide, they are different from normal cells. Cancer cells are very similar to cells of the organism from which they originated and have similar (but not identical) DNA and RNA. This is the reason why they are not very often detected by the immune system, in particular if it is weakened. Cancer cells usually have an increased ability to divide rapidly and their number of divisions is not limited by telomeres on DNA (a counter system to limit number of divisions to 40-60). This can lead to the formation of large masses of tissue and in turn may lead to disruption of bodily functions due to destruction of organs or vital structures. Cancer cells are formed from normal cells due to a modification/mutation of DNA and/or RNA. These modifications/mutations can occur spontaneously or they may be induced by other factors such as: nuclear radiation, electromagnetic radiation (microwaves, X-rays, Gamma-rays, Ultraviolet-rays… etc.), viruses, bacteria and fungi, parasites (due to tissue inflammation/irritation), heat, chemicals in the air, water and food, mechanical cell-level injury, free radicals, evolution and ageing of DNA and RNA… etc. (Marek, 2006). All these can produce mutations that may start cancer. Cancer can be called therefore "Entropic Disease" since it is associated with the increase of entropy of the organism to the point where the organism cannot correct this itself. Cancer cells are formed continuously in the
organism (it is estimated that there are about 10,000 cancer cells at any given time in a healthy person). The question is why some of these results in macroscopic-level cancers and some don't. First, not all damaged cells can multiply and many of them die quickly. Those which have the potential to divide and form cancer are effectively destroyed by the various mechanisms available to the immune system. This process takes place continuously. Therefore cancer develops if the immune system is not working properly and/or the amount of cells produced is too great for the immune system to eliminate. The rate of DNA and RNA mutations can be too high under some conditions such as: unhealthy environment (due to radiation, chemicals..etc.), poor diet (unhealthy cell environment), people with genetic predispositions to mutations and people of advanced age (above 80). Instead of dying, they outlive normal cells and continue to form new abnormal cells. Cancer cells can sometimes travel to other parts of the body where they begin to grow and replace normal tissue. This process, called metastasis, occurs as the cancer cells get into the blood stream or lymph vessels of our body. When cells from a cancer, like colorectal cancer, spread to another organ like the liver, the cancer is still called colorectal cancer, not liver cancer. Cancer cells develop because of damage to DNA. DNA is in every cell and directs all activities. Most of the time when DNA becomes damaged, the body is able to repair it. In cancer cells, the damaged DNA is not repaired. People can inherit damaged DNA, which accounts for inherited cancers. More often though, a person’s DNA becomes damaged by exposure to hazardous chemicals, like cigarette smoke. Cancer usually forms as a tumor. Some cancers, like leukemia, do not form tumors. Instead, these cancer cells involve the blood and blood-forming organs and circulate through other tissues where they grow (Marek, 2006).

2.3.2. Differences between cancer and tumor growth

Plants can get tumors. However, in general it is not as harmful to plants as to animals. This is because plant cells have a cell wall and this cell wall inhibits the cells from moving. So while a plant can get uncontrolled division of cells and make a tumor, without cell movement, the cells can’t metastasize. The tumors tend to remain localized. By and large, plants just live with their mutations and with tumors. Additionally in animal cells, the mobility of cells requires mechanisms that prevent in appropriate cell movement and the disruption of patterning. This is usually accomplished by killing the cell using a mechanism called apoptosis, or programmed cell death. Animal cells require survival factors from neighboring cells to stay alive. With no such signal, the cells undergo apoptosis. While plants have capacity for apoptosis (for instance apoptosis is used during the formation of leaf lobes and
the abscission of leaves in the fall), they also have the ability to police cell fate by de-differentiating and reassigning the cell’s fate. In culture, individual cells can not only survive but are toti-potent, able to regenerate the entire plant. Instead of killing a cell off if in the wrong place, the plant will try to “reform” it to the appropriate fate.

One bacterial disease of plants, called crown gall, is caused by tumorigenic strains of the Gram-negative bacterium *Agrobacterium tumefaciens*. The bacterium overwinters in infested soils. When host plants are growing in infested soils, the bacterium enters the roots or stems near the ground through wounds caused by factors such as freeze damage, grafting, or mechanical injury. The bacterium finds plants by detecting phenolic substances produced by wounded plant cells. Once inside the plant tissue, the bacterium moves from cell to cell, stimulating surrounding host cells to divide at a rapid rate. The bacterium does this by transferring a piece of its own DNA into the plant cell. This piece of genetic information does not come from the chromosome of the bacterium but from a separate piece of DNA called a plasmid. The *A. tumefaciens* plasmid is called the tumor-inducing or Ti-plasmid, and the piece of DNA that is transferred to the plant is called the T-DNA. Following transfer to the plant, the T-DNA becomes integrated into the chromosomes of the plant cell. Genes on the T-DNA cause the plant cell to divide repeatedly, forming the gall or mass of undifferentiated tissue, and to produce chemicals called opines, which are used by the bacterium as food. The bacterium itself lives and multiplies in the intercellular spaces of the gall. These galls are one instance of tumors in plants (Huntington.org, 2012)

2.4. Examples of potential natural products with anti-cancer activities

2.4.1. Nigella sativa L.

The seeds of *Nigella sativa* L. (family: Ranunculaceae), commonly known as (black seeds, black cumin, nigella, fennel flower, nutmeg flower, Roman coriander or “Habbatul Barakah”) have long been used in folk medicine in the Arabian Gulf region, Far East Asia and Europe. The Prophet Mohammad has described the healing powers of the Black Seeds against a variety of diseases. According to common Islamic and Arabic belief “Habbatul Barakah” is a remedy for all ailments (universal healer). Black Seeds were also mentioned as the curative “black cumin” in the Holy Bible and described as Melanthion by Hippocrates and Dioscorides and as Gith by Pliny (Worthen *et al.*, 1998). In the traditional system of medicine practiced in the Arabian Gulf region, Black Seeds is recommended for a wide range of ailments, including fever, cough, bronchitis, asthma, chronic
headache, migraine, dizziness, chest congestion, dysmenorrhea, obesity, diabetes, paralysis, hemiplegic, back pain, infection, inflammation, rheumatism, hypertension, and gastrointestinal problems such as dyspepsia, flatulence, dysentery, and diarrhea. It has been used as a stimulant, diuretic, emmenagogue, lactagogue, anthelmintic, and carminative (Nadkarni, 1976). Black Seeds have also been used externally where they are applied directly to abscesses, nasal ulcers, orchitis, eczema, and swollen joints. Many of the folk medicinal claims of Black Seeds use have been scientifically tested. Over 150 studies have been conducted over the last five decades to investigate chemical and pharmacological properties of Black seeds. Phytochemical studies of Black Seeds showed the presence of >100 constituents many of which have not been chemically identified nor have they been pharmacologically tested. A combination of fatty acids, volatile oils, and trace elements are believed to contribute to the pharmacological activity of Black Seeds. The results of extensive pharmacological studies justify the broad, traditional therapeutic value of Black Seeds. These studies demonstrated that Black Seed have an analgesic activity (Abdel-Fattah et al., 2000), antilipemic (Hassanin and Hassan, 1996), postcoital contraceptive, diuretic and antihypertensive (Zaouï et al., 2000), bronchodilatory and calcium antagonist (Gilani et al., 2001), histamine release inhibitor (Chakravarty, 1993), hepatoprotective (Daba and Abdel Rahman, 1998), anthelmintic (Akhtar and Riffat, 1991), antifungal (Khan et al., 2003) and antimicrobial against a wide range of organisms (Sokmen et al., 1999) anticancer (Worthen et al., 1998) and anti-inflammatory activities (Tarig et al., 2007).

Some researchers found that cumin oil, even in very low concentrations (less than one tenth of one percent) stopped the growth of invasive forms of toxic yeasts. In addition, cumin oil completely stopped the production of harmful fungal toxins such as aflatoxin, one of the deadlies toxins known. It was also found that, cumin oil blocked the growth of fungus in very low concentrations. Cumin oil is also an aggressive anti-fungal agent which can completely stop fungal growth in modest concentrations. Numerous researchers identified the ability of cumin oil to increase the activity of glutathione-S-transferase, a well-studied protective liver enzyme (Igram, 1997). This enzyme helps the liver detoxify toxic chemicals. Therefore, taking cumin oil internally may help the liver remove cancer-causing chemicals and other toxins. On the other hand, cumin oil significantly inhibited DNA cell damage caused by aflatoxin. This demonstrates how cumin oil can protect cells and organs from damage by toxic chemicals its powerful detoxification properties.

As a potent antioxidant, Indian researchers found that cumin oil is a potent antioxidant, even more powerful than the highly regarded turmeric root. They found that cumin oil had a strong ability
to prevent oxidative damage. Cumin was superior in blocking oxidation and toxic cellular damage, more powerful than other famous spices such as garlic and onion. In animal studies, Indian researchers found that cumin had valuable anti-cancer actions (Igram, 1997)

2.4.2. Honey

Honey is a supersaturated solution of sugars, to which the main contributors are fructose (38%) and glucose (31%). It also has a wide range of minor constituents, many of which are known to have antioxidant properties (Antony et al., 2000). These include flavonoids and phenolic acids, certain enzymes (glucose oxidase, catalase) and ascorbic acid, Maillard reaction products, carotenoid-like substances, organic acids (Cherchi et al., 1994) and amino acids and proteins (White, 1978). The natural antioxidants, especially flavonoids, exhibit a wide range of biological effects, including antibacterial, anti-inflammatory, anti-allergic, antithrombotic and vasodilatory actions (Cook and Sammon, 1996).

Honey has been used as a food since the earliest times. Only in recent years, however, has evidence emerged of its antioxidant capacity. It is also used as a food preservative (Meda et al., 2005), preventing deteriorative oxidation reactions in foods, such as lipid oxidation in meat and the enzymatic browning of fruits and vegetables (Chen et al., 2000). Antioxidants specifically retard deterioration, rancidity or discoloration due to oxidation caused by light, heat and some metals. Nevertheless, the antioxidant activity of honey varies greatly depending on the floral source (Gheldof and Engeseth, 2002) and external factors such as the season and environment, and finally its processing.

Honey is reported to contain at least 181 substances (White, 1975) and is considered as part of traditional medicine. Apitherapy has recently become the focus of attention as a form of folk and preventive medicine for treating certain conditions and diseases as well as promoting overall health and well being. It has been reported to be effective in gastrointestinal disorders, in the healing of wounds and burns (Subrahmanyam, 1991), as an antimicrobial agent and to provide gastric protection against acute and chronic gastric lesions (Ali, 1995).

The water soluble derivative (WSDP) and related polyphenolic compounds of honey showed significant anti-metastatic effect (P<0.01 and P<0.001) given either before or after tumor-cell inoculation. Oral or systemic application of WSDP or caffeic acid significantly reduced subcutaneous tumor growth and metastatic effect (P<0.05) when applied before tumor-cell inoculation (oral dose: 2 g/kg for mice or 1 g/kg for rats) once a day for 10 consecutive days. Royal
jelly did not affect metastasis formation when given intraperitoneally or subcutaneously however, intravenous administration of royal jelly before tumor-cell inoculation significantly (P<0.05) inhibited metastasis formation. When mice were given 105 tumor cells intravenously immediately after bee venom injection the number of tumor nodules in the lung was significantly lower (P<0.001) than in untreated mice or mice treated with bee venom subcutaneously where local presence of bee venom in the tissue caused significant delay in subcutaneous tumor formation. These findings clearly demonstrate that anti-tumor and anti-metastatic effects of bee venom are highly dependent on the route of injection and on the close contact between components of the bee venom and tumor cells. These data show that honey bee products given orally or systemically may have an important role in the control of tumor growth and tumor metastasizing ability (Orsoli et al., 2004).

2.5. Thin layer chromatography analysis

Thin-layer chromatography (TLC) is a chromatography technique used to separate non-volatile mixtures. It can be used to monitor the progress of a reaction, identify compounds present in a given mixture, and determine the purity of a substance. Specific examples of these applications include: analyzing ceramides and fatty acids, detection of pesticides or insecticides in food and water, analyzing the dye composition of fibers in forensics, assaying the radiochemical purity of radiopharmaceuticals, or identification of medicinal plants and their constituents (Reich and Schibli, 2007). A number of enhancements can be made to the original method to automate the different steps, to increase the resolution achieved with TLC and to allow more accurate quantitative analysis. This method is referred to as HPTLC, or "high-performance TLC"). TLC technique is used to isolate and purity compounds from Impatiens balsimina with anti-tumor activity. Also Kasetsart (2010) used TLC technique to investigated the anti-oxidant activity of Acanthopanax trifoliatus and Toddalia asiatica.
CHAPTER THREE
Materials And Methods

This study was conducted at the Microbiology Laboratory of the Faculty of Engineering and Technology in 2013-014. It aimed at screening bee honey and cumin seeds (*Nigella sativa*). The potato disc bioassay was used to estimate anticancer activity of these using the indigenous strain of *Agrobacterium tumefaciens* “SDB0012”. Latter, the crude extract of cumin was subjected to fractionation using thin layer chromatography (TLC) techniques to identify the anti-tumor active components. Five sub-cultures (Sc-1 to Sc-5) of this bacterium were made into NASA medium to prepare the original supplied slant of *Agrobacterium tumefaciens* strain SDB0012. The bacterium culture was maintained throughout the study by routine sub-culturing under aseptic microbiological methods.

3.1. *In-vivo confirmatory test of the bacterial strain SDB0012*

The preserved bacterium inocula at the Microbiology Laboratory of the Faculty of Engineering was added to the selective *A. tumefaciens* differential growth yeast manitol broth media “YMP” for two days in a conical flask 500 ml. The medium was then subjected to centrifugation at 5000 rpm for 10 minutes. The resulted pellet was dissolved in 10 ml distilled water. *Cajanus cajan* seedlings grown in small pots contained sterilized soil (1:1; clay: sand) were injected with the prepared inoculums, at 1 ml of the inoculum per plant at stem base and allowed to continue growth for 21 days after injection plants were observed for the growth of tumors. The plants were observed for the presence and absence of tumors. Inoculum samples were extracted from the tumors grown on the root surface of the injected plants and added to petri dishes contained NASA medium. As follows: A bacterial suspension of *A. tumefaciens* was prepared from two of the selected subcultures of the original *A. tumefaciens* supplied slant of indigenous isolate. The growth medium Yeast Manitol Broth (YMP) was prepared in flasks, the flasks were plugged with cotton, covered with aluminum foil and sterilized in an autoclave for 15 minutes. The medium was allowed to cool and a loop of *A. tumefaciens* from storage cultures on agar slant was added to the (YMP) media using aseptic techniques. The flasks were then placed on an orbital shaker at speed of 5000 rpm for 48 hours at 30 C for the growth the bacterium (Jerry *et al.*, 1988).
3.2. Preparation of NASA Media

It is the selective media for growth of Agrobacterium. It contains nutrient agar and sucrose. Nutrient agar ingredients included petit digest of animal tissue 5.0 – beef extract 1.0 – yeast extract 1.0 – sodium chloride 5.0 – agar 15 – (g/L). The medium was prepared by adding 20 g sucrose and 28 g nutrient agar to 1000 ml of distill water in a conical flask. The mixture was boiled in a water bath until the agar melted. Plug the flask with cotton, cover with aluminum foil, and sterilize in an autoclave for 15 minutes at 121°C and pressure 15 psi. 15 ml sterilized media solution which contain an antibiotic (chloroamphenicol at concentration of 100 μl/L) was poured per Petri dish and was left to cool and solidify.

3.3. Preparation of Yeast Manitol Broth (YMP) media

A. tumefaciens was maintained in the selective differential growth media “YMP” for A. tumefaciens. It is composed of 10 g manitol, 0.5 g Di-potassium hydrogen orthophosphate, 0.5 g yeast extract, chloramphenicol at the concentration of 0.1 mg/1, 0.2 g Magnesium sulphate hepta-hydrate and 0.1 g sodium chloride. This ingredients were being stirred between additions. The medium was then sterilized by autoclaving for 15 minutes, at 121 °C and 15 psi.

3.4. Samples selection

Computer surveys were conducted for gathering information on promising natural compounds act as anti-cancer agents. Honey and Black Cumin oil were selected for use in this study on the basis of information gathered from traditional folk medicine practioners’ questionnaire in Gezira State in a previous study conducted by Dawei (2007).

3.5. The potato disc bioassay

Experiments were conducted to estimate anti-tumor activity of honey, cumin and honey-cumin mixture (1:1), in a complete randomized design (CRD) with three replicates. Each treatment consisted of two Petri-dishes, each containing four potato discs. One drop (0.03 ml) of the prepared inoculums of the A. tumefaciens was added onto each potato, then one drop of honey and/or black cumin extract (0.03 ml) was added to the top of the potato disc. The potato discs which were used as negative control were treated with sterilized water. The potato discs used in bioassay were prepared
two days before performing each assay. The prepared potato discs were inoculated for 21 days with the 24 hours old *A. tumefaciens* “SDB0012” inocula in NASA medium.

**3.5.1. Preparation of potato discs.**

Red skinned potatoes tubers (*Solanum tuberosum*) were used for preparation of potato discs for bioassays. The selected potato tubers were washed using distilled water and surface sterilized by immersing in 10% commercial bleach (Clorox) for 20 minutes. Excess Clorox was washed off by rinsing in five changes of sterilized water and the tubers were then transferred into a laminar flow hood where the working surface was priorly cleaned with ethanol and sterilized with ultra violet light for 15 minutes. In the laminar flow hood the ends of potato tubers were cut away and the tubers were cut by a sterilized cork borer into small discs were briefly dipped into bleach and were then placed in Petri plates (2 discs per Petri plate). The discs were placed by gently pushing them onto the nutrient agar media which scores (28 g Nutrient Agar and 20 g Sucrose).

**3.5.2. The potato disk bioassay protocol**

**Preparation of potato discs:** *Solanum tuberosum* L were disinfested by scrubbing under running water with a brush, then immersing in 10% Clorox for 20 minutes. Potatoes were removed from the Clorox, blotted on sterile paper towels, and placed in sterile distilled water. Each side removed allowing for a flat surface without skin. Cylinder were cut from the disinfested section using a sterile cork borer (10-15 mm). Disks (0.5 cm thick) were cut aseptically from the cylinders. These disks were placed in a 24-well culture plate containing (NASA) media and place 5 discs per Petri dish by gently pushing the discs into the agar using aseptic technique. Three Petri dishes per sample and control were used in each experiment. One drop (10 μl) of the prepared bacterial suspension (1 loop of *A. tumefaciens* new subculture from storage culture on agar slant, was added to 10 ml sterile distilled water) overlaid each disk of potato. For each natural product or plant extract appropriate doses was added to top of the potato disk. The edge of each Petri dish was sealed with paraffin stripes to prevent moisture loss during the incubation period. Keep the dish level at all times to keep the inoculum on the tops of the discs. Incubated in the dark at 37°C and were screened or tumor growth after 12 to 21 days.

On the day 12, the disks were stained with Lugol's Reagent 5% (The 5% solution consists of 5% "w/v" iodine (I₂) and 10% "w/v" potassium iodide "KI" mixed in distilled water and has a total iodine content of 126.5 mg/mL) plus 10% KI in distilled water. Lugol's reagent stains the starch in the potato
tissue a dark blue to dark brown color, but the tumor produced by *A. tumefaciens* will not take up the stain, and appear creamy to orange (McLaughlin and Rogers, 1998).

### 3.6. Determination of the inhibition zone:

A bacterial strain of interest is grown in pure culture. Using a sterile swab, a suspension of the pure culture is spread evenly over the face of a sterile plate. The antimicrobial agent is applied to the center of the agar plate (in a fashion such that the antimicrobial doesn't spread out from the center). The agar plate is incubated for 24-48 hours, at a temperature suitable for the test microorganism. If antimicrobial agent leaches from the object into the agar and then exerts a growth-inhibiting effect, then a clear zone (the zone of inhibition) appears around the test product. The size of the zone of inhibition is usually related to the level of antimicrobial activity present in the sample or product - a larger zone of inhibition usually means that the antimicrobial is more potent (Antimicrobial Test Laboratories, 2013).

### 3.7. Thin layer chromatography (TLC)

Thin layer chromatography (TLC) was used for fractionation of cumin crude extract to investigating their anti-tumor potentialities using the potato disc bioassay.

#### 3.7.1. TLC solvent system and detection reagent

TLC chromatography was performed using a solvent mixture of: hexane, diethyl ether, and acetic acid “or formic” (80:20:2). Freshly prepared FeCl$_3$.6H$_2$O, 50 mg of water, 90 ml of acetic acid, 5 ml of sulphuric acid, were used to spray dried TLC plates after completion of a chromatographic run to reveal the separated spots. Each TLC plate was sprayed with about 10 ml of the freshly prepared reagent and was then heated at 100°C for 2-4 minutes until the developing colors were observed. The developed spots were documented by photography (William, 1973).

#### 3.7.2. Preparation of silica gel plates

TLC silica gel plates used in this study were of 0.25 mm thickness. They were prepared using a Shandon Scientific Instruments Ltd spreader. Silica gel containing 13% CaSO$_4$ was shaken vigorously for about one minute with a volume of distilled water equivalent to twice the weight of gel “w/v” and applied to the 20×20 cm glass plates to the required thickness. The plates were heated in an oven for half an hour at 110 °C before cooling in desiccators. The samples were applied to a base line at one end of the plate and were then equilibrated with the running solvent developing solvent. After
the chromatographic run, each plate was left at room temperature in a fume chamber and was then developed to reveal the separation of spots. Separated spots (bands) were visualized using UV lamp or by spraying (as was described in 3.7.1). Preparation of the silica gel plate was carried out following William (1973).

3.7.3. Application of plant extracts and TLC development

In each experiment, the cumin solution was applied as a spot onto a TLC plate coated with silica gel (0.25 mm thickness), using a micro-syringe. The plate was developed in a tank containing the solvent system of hexane: diethyl ether: acetic acid (80:20:2) for 45 minutes at room temperature after which the plates were taken out of the chromatography tank and were dried in a fume cupboard. The dried TLC plates were then sprayed to visualize and detect any developing spots for detection and comparison between specimens. The Rf values for separated spots for each specimens were determined to give a solid base for making comparisons between the constituents of the different used specimens following William (1973).
CHAPTER FOUR
Results and Discussion

*Agrobacterium tumefaciens* is a widespread naturally occurring soil bacterium that causes crown gall, and has the ability to introduce new genetic material to plant cells. The genetic material that is introduced is called T DNA (transferred DNA) which is located on a Ti plasmid. A Ti plasmid is a circular piece of DNA found in almost all bacteria. *Agrobacterium*-mediated transformation is the most commonly used method for plant genetic engineering because of relatively high efficiency. Initially, it was believed that this *Agrobacterium* only infects dicotyledonous plants, but it was later established that it can also be used for transformation of monocotyledonous plants such as rice. The indigenous strain of *A. tumefaceins* SDB0012 has the ability to induce tumors growth on roots of some plant species such as pigeon pea, melon, sorghum, tomatoes and on potato discs (Elseedig, 2007). In this study, addition of the bacterial suspension to small discs of potato (1.5 cm in diameter) used as control resulted in a high tumors growth, 21 days after inoculation. The overall mean of tumors was found to be four large tumors /disc as presented in Figure 1. This bacterial strain facilitated use of the potato disc bioassay to study antitumor activity of bee honey, cumin extract and a combination of both at a rate of 1:1. The origin of the bacterial suspension used in this study was an old culture of the bacterium preserved at the Microbiology Laboratory of Faculty of Engineering and Technology. This was proved to be a pure culture of *A. tumefaciens* strain SDB0012 in this study following the description given by Yousif *et al.* (2011a). Figure 1 shows tumors expressed on potato discs treated with *Agrobacterium tumefaciens*, 21 days after inoculation. The surface of the tumors on potato discs was smooth with no shoot growth which recognizes the noplume-type tumors mentioned.

4.1. Inhibition zone test

This test was conducted to study the level of antibacterial potency of honey and cumin oil on the growth of the bacterium expressed as the size of the inhibition zone. The bacterium demonstrated normal in filter paper filled with bee honey, cumin oil and a mixture of them at a rate of 1:1. Results indicated that honey, cumin and their mixture has no anti-bacterial effects against the indigenous strain SDB0012. This strain resisted application of honey despite its antimicrobial property as well as wound-healing activity, as presented in Figure (2).
Figure (1): Tumors expressed in potato discs treated with *Agrobacterium tumefaciens*
Figure (2): Inhibition of *Agrobacterium tumefaciens* SDB0012 growth in filter paper treated with honey, cumin and the mixture of them (1:1)

(A): Bee honey
(B): Cumin oil
(C): Bee honey + cumin oil (1:1)
In general, the antimicrobial activity in most honeys is due to the enzymatic production of hydrogen peroxide. Resistance of this strain to honey application might be due to production of catalase which is known to detoxify hydrogen peroxide by catalyzing its decomposition to O₂ and H₂O (Yousif et al., 2011a). This result was in line with Mandal and Mandal (2011), who mentioned that most bacteria appear to express one or more catalases in response to peroxide stress, and the different types of catalases are regulated independently. Two types of structurally unrelated catalases are common in bacteria: a bifunctional catalase-peroxidase (HPI) and a monofunctional catalase (HPII). Both of these catalases contain heme as the prosthetic group. In addition, a nonheme manganese-containing catalase is present in some bacteria as well (Chelikani et al., 2004). However, another kind of honey, called non-peroxide honey (viz., manuka honey), displays significant antibacterial effects even when the hydrogen peroxide activity is blocked. Its mechanism may be related to the low pH level of honey and its high sugar content (high osmolarity) that is enough to hinder the growth of microbes. The medical grade honeys have potent in vitro bactericidal activity against antibiotic-resistant bacteria causing several life-threatening infections to humans (Mandal and Mandal, 2011). Nevertheless, this strain was described as multi-resistant or superbug bacterium since it resisted application of penicillin (30 mg), Chloroamphenicol (30 mg) and Ciprofloxacin (30 mg).

Results also indicated that this strain survived well on potato discs treated with cumin, as presented in Fig 2, in spite of the fact that cumin seed may be useful either alone or when combined with antimicrobial agents, to treat bacterial infections. The antibacterial properties of cumin essential oil are mostly attributable to the cumin aldehyde (Derakhshan et al., 2010). It is the biologically active constituent of Cuminum cyminum seed oil. C. cyminum seed-derived materials and have an inhibitory effect in vitro against rat lens aldose reductase and alpha-glucosidase. Cuminaldehyde is a volatile compound representative of cumin aroma present in trace amounts in the blood and milk of ewes fed with cumin seed. Resistance of the indigenous strain to cumin oil was not in line with Randhawa and Al- Ghamdi, (2002) who stated that the Nigella sativa oil as well as methanolic extract has been found to possess remarkable antibacterial activity against multidrug resistant such as the Coagulase negative staphylococci, Moreover, Cumin has thymol which acts as antibacterial. Thymol is a phenolic alcohol present in the essential oil that has been reported to possess antibacterial activity (Karapinar and Aktug, 1987). Since, Thymol is present in the methanol soluble portion of oil (Enomoto et al., 2001), it will also be extracted in the methanolic extract. The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly
through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Mason and Wasserman, 1987).

4.2. Potato disc bioassay

The importance of this technique for identification of natural products having antitumor and anticancer activity comes from the fact that antitumor results obtained in this technique were found to be highly correlated with results obtained on the same products in treating animals and human beings. In his evaluation of seventeen samples consisting of purified compounds and various ethanol extracts from plant sources (Galsky and Wiley, 1980) concluded that results demonstrated definite correlation between the ability of these samples to inhibit the formation of crown gall tumors and their activity on the P388 leukemia system in mice. Samples showing only cytotoxic effects in KB cell cultures did not affect tumor initiation in our system. Bioassay is the preliminary step in drug discovery which allow the screening of biological and synthetic bioactive compounds. In this study, both honey and cumin extracts were not affect the bacterial viability or its ability to attach to a tumor binding site and therefore were recognized to have not antibacterial effect on this bacterium. Figure 3 presents differences in color and light intensity between tumors and the potato disc matrix after addition of the Lugol's reagent stains, 21 days after inoculation, as recommended by McLaughlin and Rogres (1998). The creamy to light brown colors evident presence of tumors; whereas dark brown to dark color showed not tumor growth because they took the stain. Uses of this reagent added to the efficiency of tumors detection on the potato discs (Fig., 3). Generally, metastasis of cancer is due to spread from the part of the body where it started (the primary site) to other parts of the body is well known in human beings (Chiang and Massague, 2008). Screening for antitumor activity resulted in 40%, 55% and 100% inhibition from the total surface area of the potato disc due to application of honey, cumin oil and the mixture, respectively (Table 1; Fig., 4). This result suggested the use of honey and cumin oil (1:1) for inhibition of tumor growth and further elucidation of this mixture in animal experimentation (in-vivo). Since results obtained from potato disc bioassay were considered 100% similar as if in-vivo treatment of human cancer (Trigui et al., 2013).
**Fig. (3):** Metastasis of tumors in a Petri dish treated with the bacterium suspension, detected by using the Lugol's reagent
**Table 1.** In-vitro inhibition of tumor metastasis using honey, cumin oil and the mixture of them (1:1)

<table>
<thead>
<tr>
<th>Product</th>
<th>Inhibition of tumor metastasis (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bee honey</td>
<td>40</td>
</tr>
<tr>
<td>Cumin oil</td>
<td>55</td>
</tr>
<tr>
<td>Mixture of Bee honey and Cumin oil (1:1)</td>
<td>100</td>
</tr>
</tbody>
</table>

* Inhibition of tumor metastasis (in percentage) out of the total surface area of the potato disc.
Figure (4): In-vitro inhibition of tumor growth and tumor metastasis using honey, cumin oil and the mixture of them (1:1)

(A): Bee honey
(B): Cumin oil
(C): Bee honey + cumin oil (1:1)
4.3. Fractionation of cumin oil

Fractionation of cumin oil using thin layer chromatography is a common practice for studying its biological activities. For example, the study conducted by Ismail et al. (2008) on the neuro-protectative effects of cumin oil and its fractions. In this study, addition of cumin oil extract to silica gel in TLC resulted in eight different fractions, as presented in Fig. (5). Application of the different fractions to the potato discs treated with A. tumefaciens “SDB0012” indicated that the fractions, starting from the bottom, including Fraction 3, Fraction 4, Fraction 5, Fraction 7, Fraction 8 has no antitumor activity since tumor grew normally in potato discs treated with these fractions (Table 2). Yet, Fraction 6 larger prominent and prominent tumors compared to other fractions. Only the fraction 2 resulted in 100% tumor metastasis inhibition and therefore considered the most antitumor active component in cumin oil (Fig., 6). Further fractionation of fraction 2 suggested to reach a stable fraction that could be subjected to chemical identification using the GC-MS.
Figure (5): Fractionation of cumin oil on silica gel using thin layer chromatography
Table 2. Inhibition of tumor metastasis on potato discs treated with TLC products (fractions) of cumin oil

<table>
<thead>
<tr>
<th>Fracions of cumin oil</th>
<th>Inhibition of tumor metastasis (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction number</td>
<td>$R_f$</td>
</tr>
<tr>
<td>Fraction 1</td>
<td>0.118</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>0.147</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>0.294</td>
</tr>
<tr>
<td>Fraction 4</td>
<td>0.426</td>
</tr>
<tr>
<td>Fraction 5</td>
<td>0.603</td>
</tr>
<tr>
<td>Fraction 6</td>
<td>0.735</td>
</tr>
<tr>
<td>Fraction 7</td>
<td>0.897</td>
</tr>
<tr>
<td>Fraction 8</td>
<td>0.926</td>
</tr>
</tbody>
</table>

* Inhibition of tumor metastasis (in percentage) out of the total surface area of the potato disc.

** Fraction showed large tumors.
Figure (6) : Application of the different fractions crushed from silica gel in the first fractionation

(A): fraction 1    (B): fraction 2    (C): fraction 3    (D): fraction 4
(E): fraction 5    (F): fraction 6    (G): fraction 7    (H): fraction 8
CHAPTER FIVE
Conclusions and Recommendations

5.1 Conclusions
- Both honey and cumin has no antibacterial activity.
- Honey, cumin oil and their mixture have antitumor activities of 40%, 55% and 100%, respectively
- Fraction 2 of the cumin oil scored 100% inhibition of tumor growth, whereas, fraction 6 encouraged tumor growth compared to the control.

5.2 Recommendations:
- Further fractionation of fraction 2 of cumin oil should be run to reach a stable homogenous component after successive fractions followed by bioassay.
- Animal experimentations (in mice having induced cancer) should be conducted to study the effect of the most promising fractions (fraction 2 and 3) end product in-vivo.
REFERENCES


Hall, L. A.; Krauthauser, C. M.; Wexler, R. S.; Hollingshead, M. G.; Slee, A. M. and Kerr,


Kinghorn, A. D.; Farnsworth, N. R.; Soejarto, D. D.; Cordell, G. A.; Swanson, S. M.;
Pezzuto, J. M.; Wani, M. C.; Wall, M. E.; Oberlies, N. C.; Kroll, D. J.; Kramer, R.
Novel strategies for the discovery of plant-derived anticancer agents. Pharm Biol., 41


native and oxidized phenolic compounds. Phytochemistry, 26:2197-220215.


McKibben, J. and Engeseth, N. J. (2002). Honey as a protective agent against lipid oxidation


Food Chem. 91: 571–577.

Ltd.p.301-40.

Newman, D. J. and Cragg, G. M. (2007). Natural products as sources of new drugs over the


