Biological Activity of Black Cumin (Bunium bulbocastarum L.) Seed Extracts Against Tumor Caused by Agrobacterium tumefaciens "SDB0012" Using Potato Disc Bioassay

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Date of Examination: 3.11.2016
DEDICATION

To my dear family members and friends, with grateful thanks for their support and patience during my study
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PRAISE AND THANKS ARE DUE TO ALLAH WHO HAD GIVEN ME THE POWER AND PATIENCE TO COMPLETE THIS STUDY. AFTER THAT, I AM DEEPLY INDEBTED TO MY SUPERVISOR PROF. MOHAMED TAHAYOUSIF WHOSE SUPERVISION, PATIENCE, VALUABLE DATA ANALYSIS AND CONSTRUCTIVE CRITICISM WERE PARAMOUNT TO THE MATERIALIZATION OF MY EFFORTS.

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I WOULD FURTHER EXTENDED MY THANKS TO MY FAMILY AND COLLEAGUES WHO HAD PUT UP WITH FRUSTRATION DURING THE STUDY.
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**ABSTRACT**

Black cumin (*nigella sativa* L.), family Ranunculaceae, is a widely distributed annual herbaceous plant, *Nigella sativa* has been used as traditional medicine for centuries. The present study was investigating the biological activity of the black cumin oil extracts and essential oil against the tumor using potato disc bioassay. Samples of black cumin seeds were obtained from the local market in Wad Medani City, Gezira State, Sudan. Five cumin products were used; the cumin essential oil which was extracted by distillation, the cumin oil which was extracted by cold extract (using petroleum ether), methanol and ethyl acetate extracts, in addition to a commercial sample of cumin oil which was brought from the local market of Wad Medani City. The indigenous strain of *Agrobacterium tumefaciens* "SDB0012" was brought from University of Gezira. At first, the oils and their extracts were tested against *A. tumefaciens* using potato disc bioassay. The results showed that, ethyl acetate extract inhibited the bacterium as 50%, meanwhile, 10% inhibition zone was observed for petroleum ether extract and the commercial oil sample and 0% inhibition was recorded for methanol extract and essential oil. Any extracts have anti bacterial effect (including ethyl acetate, Petroleum ether and Commercial sample) did not used in anti tumor activity. methanol and essential oil extracts were used as anti tumor effect and showed the highest inhibition percentage of 100% and 87% respectively. These records was obtained after fourteen day of incubation. After 21 days, methanol extract gave 100% inhibition followed by essential oil (87%). Data was collected based on inhibition in reagent which turns • tumor development (zone) caused by *A. tumefaciens*, using lugols tumergenic tissues into dark brown color. *Agrobacterium* infection led to development of tumergenic tissues on the surface of the potato discs 2 cm in rise, as comparing with the discs used as control (complete coverage of the disc surface). It was concluded that methanol extract of black seed was very effective in tumor inhibition and hence, it can be recommended to further studies in this area.
المستخلصات النباتية لمستخلصات بذرة الحبة السوداء (Bunium bulbocastarum L.) ضد الأورام المسببة بواسطة بكتريا (Agrobacterium tumefaciens).

تستخدم تكنولوجيا تقنية التقييم الإحصائي لأقراص البطاطس مجدى صديق الحاج منير ملخص الدراسة

الحبة السوداء تنتمي إلى عائلة Ranunculaceae وهي نباتات عشبية حولية واسعة الانتشار.

الحبة السوداء الطيار، والزيت الطيار، ومستخلصات الميثانول وخلاطات الأثيل لمكثفة تم استخدامها في الطب التقليدي منذ قرون مضت.

هدف هذه الدراسة هو تحديد النشاط البيولوجي لمستخلصات بذرة الحبة السوداء، حيث تم الحصول على عينات من بذرة الحبة السوداء من السوق المحلية لمدينة ود مدني، ولاية الجزيرة، السودان. تم استخدام خمسة مستخلصات للحبة السوداء، حيث تم استخدام تقنيات التقطير، زيت الحبة السوداء (الثابت) والثاني، مستخلصات الميثانول وخلاطات الأثيل، بالإضافة إلى عينة تجارية من زيت الحبة السوداء، حيث تم الحصول عليها من السوق المحلي لمدينة ود مدني.

تم استخدام Agrobacterium tumefaciens سلالة "SDB0012" لفحص مستخلصات الحبة السوداء، باستخدام تقنية التقييم الإحصائي لأقراص البطاطس.

أظهرت النتائج أن مستخلصات العينات التجارية (مثل الميثانول، وخلاطات الأثيل، الزيت الطيار) كانت أكثر كفاءة في تثبيط النبات، حيث تمكنت العينة التجارية من تثبيط النبات بنسبة 100%، وorable (87%)، بينما تم استخدام عينة السائل (الثابت) بنسبة 50% في حين تمت استخلاص الطيور (الثاني) بنسبة 10% فقط.

تم استخدام عينة النبات التجارية (خلات الأثيل، العينة التجارية)، حيث تم استخدامها كمصمم للأورام وأعطيت النتائج (87%) على النتائج، حيث تم استخدام العينة التجارية (خلات الأثيل، العينة التجارية) بأكثر من 100% من النتائج.

تم استخدام العينة التجارية (خلات الأثيل، العينة التجارية) بأكثر من 100% من النتائج من قبل السائل (الثابت) بنسبة 87%.

استخدام المعلومات المتاحة على مستويات التثبيط الذي يحدث في الورم الذي اكتسبته البكتريا يعد إضافة قوية في مجال البحث، حيث تم استخدام تقنيات التقييم الإحصائي في تصميم الأورام واعتمادها كлим على النتائج. الإصابات ادت تجربة الخلايا الورمية في سطح أورام البطاطس بارتفاع 2 سم (التي تغذي السطح بكاملها) مقارنة بالأورام المستخدمة كشاملة. هذا يعني أن مستخلص الميثانول للحبة السوداء فعال جدا في تثبيط الأورام ويدعى نموذج من الدراسات في هذا المجال.
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ABBREVIATION

SSMO : Sudanese standard and metrology organization.
NASA : Nutrient agar selective for *Agrobacterium tumefaciens*.
min :/oscillations

min :/OSC
Nigella sativa (Black cumin) has been used as traditional medicine for centuries. It belonged to the family Ranunculaceae; Commonly Known as black cumin, nigella, fennel flower, nutmeg flower, roman coriander or Habbatul Barakah. Seed of the plant have long been used in folk medicine in the Arabian Gulf region, far east Asia and Europe (Phillips, 1992). The prophet Mohammad has described the healing powers of the black seed against a variety of diseases. According to common Islamic and Arabic belief ("Habbatul Barakah") is a remedy for all ailment (universal healer). Black seed were also mentioned as the curative in the Holly Bible and described as Melanthion by hippocrates and dioscorides and as dith by pliny. In the traditional system of medicine practiced in the Arabian Gulf region (Dehkordi and Kamkhah, 2008). The active ingredients of N. sativa have beneficial effects against many diseases including cancers (Sayed, 1980). For example, it is effective in diminishing the risk of atherosclerosis by decreasing the serum low density lipoprotein cholesterol level and increasing the serum high density lipoprotein cholesterol levels (Nader et al., 2010); it exerts therapeutic and protective effect in diabetes by decreasing morphological changes and preserving pancreatic beta-cell integrity (Kanter et al., 2009) and by beneficially changing the hepatic enzyme activities (Pari and Sankaranarayanan, 2009). It is effective against hypertension, has a potent antihistaminic effect on airways of asthmatic patients (Boskabady et al., 2010), its components are promising agents to complement schistosomiasis specific treatment (El Shenawy et al., 2008); its oil protects kidney tissue against oxygen free radicals and preventing renal dysfunction and morphological abnormalities. For thousands of year, the seeds, oils and extracts of N. sativa have been used as an anticancer agent by unani, ayurveda and the chinese system of medicine that have originated from the Arab, Ind-Bangla and China, respectively (Ragheb et al., 2009). The modern scientific research with the investigation of anticancer activity of N. sativa is a comparatively recent affair (for the last 2-3 decades). There are not so many research works done in this field and very few review articles exist in this area. Recently, an indigenous strain of Agrobacterium tumefaciens “SDB0012” was identified at the National Institute for Promotion of Horticultural Export-University of
Gezira, Sudan (Yousif et al., 2011). The potato disc bioassay technique using *Agrobacterium tumefaciens* strains 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 purposes of the standardization or quality control for bioactive components in such heterogenous botanical (Jerry et al., 1998).

The history of *A. tumefaciens*’s nomenclature is quite interesting. In the initial period since its discovery, it was known by various names based on phenotypic and genetic evidences including 16S rDNA analyses (Shah, 2013).

**OBJECTIVE**

The main objective of the current study was to evaluate antitumor activity of different extracts of Nigella sativa using the potato disc bio assay technique.
CHAPTER TWO
LITERATURE REVIEW

2.1 Black seed

2.1.1 Plant description

*Nigella sativa* is an annual flowering plant. It grows to 20–30 cm (7.9–11.8 inch) tall and has linear lanceolate leaves. The delicate flowers have 5-10 petals and the colors are usually yellow, white, pink, pale blue or pale purple. The fruit of plant is large and inflated capsule composed of 3-7 united follicles, that each of them has numerous seeds. The black colored seeds are flattened, oblong and angular, funnel shaped, with the length of 0.2 cm and 0.1 cm wide (Goreja and New York, 2003).

This plant is known by numerous names, for example black cumin (English), black caraway seeds (USA), shonaiz (Persian) and kalajira as Bangali name (Khan, 1999).

2.1.2 Nigella seeds

The seeds of *N. sativa* are used as a spice in Indian and Middle Eastern cuisines. The black seeds taste like a combination of onions, black pepper and oregano. They have a pungent bitter taste and smell (Bharat and Aggarwal, 2015).

The dry-roasted Nigella seeds flavor curries, vegetables and pulses. It can be used as a "pepper" in recipes with pod fruit, vegetables, salads and poultry. In some cultures, the black seeds are used to flavor bread products. It is also used as part of the spice mixture panch phoron (meaning a mixture of five spices) and by itself in many recipes in Bengali cuisine and most recognizably in naan bread. Nigella is also used in Armenian string cheese, a braided string cheese called majdouleh or majdouli in the Middle East (Bramen, 2015).

2.1.3 History

According to Zohary and Hopf, archaeological evidence about the earliest cultivation of *N. sativa* is still scanty", but they report supposed *N. sativa* seeds have been found in several sites from ancient Egypt, including Tutankhamun's tomb. (Zohary and Hopf, 2002). Although its exact role in Egyptian culture is unknown, it is known that items entombed with a pharaoh were carefully selected to assist him in the
afterlife. The earliest written reference to N. sativa is thought to be in the book of Isaiah in the Old Testament, where the reaping of Nigella and wheat is contrasted (Isaiah 28: 25, 27). Easton’s Bible dictionary states the Hebrew word ketsah refers to N. sativa without doubt (although not all translations are in agreement). According to Zohary and Hopf, N. sativa was another traditional condiment of the Old World during classical times, and its black seeds were extensively used to flavor food. Seeds were found in a Hittite flask in Turkey from the second millennium BCE (Saliha et al., 2009).

2.1.4 Chemical constituents

Extensive studies were done to identify the composition of the black cumin seed, the ingredients of N. sativa seed includes: fixed oil, proteins, alkaloid, saponin and essential oil. The fixed oil (32-40 \%) contains: unsaturated fatty acids which includes: arachidonic, eicosadienoic, linoleic, linolenic, oleic, almitoleic, palmitic, stearic and myristic acid as well as beta-sitosterol, cycloeucalenol, cycloartenol, sterol esters and sterol glucosides (Tembhrune et al., 2014). The volatile oil (0.4-0.45 \%) contains saturated fatty acids which includes: nigellone that is the only component of the carbonyl fraction of the oil, Thymoquinone (TQ), thymohydroquinone (THQ), dithymoquinone, thymol, carvacrol, α and β-pinene, d-limonene, d-citronellol, p-cymene volatile oil of the seed also contains: p-cymene, carvacrol, t-anethole, 4-terpineol and longifoline (Enomoto et al., 2001).

Black cumin seed have two different forms of alkaloids: isoquinoline alkaloid that includes: nigellicimine, nigellicimine n-oxide and pyrazol alkaloid that includes: niggellidine and nigellicine (Ahmad et al., 2013). The nutritional compositions of N. sativa are vitamins, carbohydrates, mineral elements, fats and proteins that include eight or nine essential amino acids. By sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) the fractionation of whole N. sativa seeds was done which shows the bands ranged from 94 to 100 kDa molecular mass (Haq et al., 1999). Black cumin seeds also have saponin and alpha hederine and in trace amount has carvone, limonene and citronellol, as well as provide relatively good amounts of different vitamins and minerals such as Fe, Ca, K, Zn, P, Cu (Tembhurne et al., 2014). Most of the pharmacological effects are due to quinine constituent, of which TQ is the mainly abundant. TQ possess anticonvulsant activity, antioxidant, anti-
inflammatory anti-cancer, antibacterial and antifungal activit (Abdel Azeiz et al., 2013).

2.1.5 Traditional uses of folk remedies

*N. sativa* seeds have been used traditionally in middle eastern folk medicine as a treatment for various diseases for more than 2000 years ago (Phillips, 1992). The seeds were used as pungent appetizer, aromatic, thermogenic, diuretic, expectorant, purgative, stimulant, sudoriferous, sedative and carminative .Black cumin seeds have a history of use in traditional Arabic herbal medicine to treat many diseases such as skin diseases, jaundice, gastrointestinal problems, anorexia, conjunctivitis, dyspepsia, rheumatism, diabetes, hypertension, intrinsic hemorrhage, paralysis, amenorrhea, asthma, cough, bronchitis, headache, fever, influenza and eczema (Sayed, 1980).

2.1.6 Preliminary human research

Mainly for its seed oil extract, thymoquinone, *N. sativa* is under research for its potential to affect human diseases, such as cancer or dyspepsia, although studies to date remain insufficient to understand its potential for clinical efficacy (Abdel Azeiz, 2013).

2.1.7 Pharmacological properties

In recent years huge number of studies have been carried out, acclaimed medicinal properties emphasized on different pharmacological effects of *N. sativa* seeds such as antioxidant, anti-tussive, gastroprotective ,anti-anxiety, anti-ulcer, antiasthmatic, anti-cancer, anti-inflammatory, immunomodulatory and anti-tumor properties, hepatoprotective effect, also gastric ulcer healing, tumor growth suppression , men infertility improvement, cardiovascular disorders, memory improvement, stimulate milk production, protective effects on lipid peroxidation, antibacterial activity, anti dermatophyte, antiviral activity against cytomegalovirus, have been reported for this medicinal plant.

2.1.8 Antibacterial activity

The antimicrobial properties of herbal plants and their extracts have been recognized since ancient times, while attempts to illustrate these qualities in the laboratory date
back to the early 1900s (Dorman et al., 2000). Now, the development of resistance via a pathogen to several of the usually used antibiotics provides a drive for additional attempts to find new antimicrobial agents to eradicate the infection and defeat the problems of resistance and side effects of the antimicrobial drugs that are of that the key processes of the organisms are affected. The mechanism of the antimicrobial effect of N. sativa seeds has not been reported, its antimicrobial property could be attributed to the active constituents particularly TQ and melanin. Their broad spectrum of activity may be the reason of that the key processes of the organisms are affected (Monika et al., 2013).

Cancer is one of the major threats of modern life, which is considered as the second cause of death after myocardial infarction. Millions of people die every year in different types of cancer despite tremendous efforts to find methods of control and cure. In the last century, great advances were made in modern medical science to control disease. But many diseases like cancers are not yet curable fully. To find out new and authentic therapies, scientists are working with traditional or folk medicines in parallel of modern medicine. N. sativa has been used for medicinal purposes for centuries. It originated from Southeastern Asia and also used in ancient Egypt, Greece, Middle East and Africa. In Islam, it is regarded as one of the greatest forms of healing medicine available. It is a flowering plant, of which seed is used as a spice. The seed is called black cumin in English, while in old Latin it was called ‘Panacea’ meaning ‘cure all’; in Arabic it is termed as ‘Habbah Sawda’ or ‘Habbat el Baraka’ translated as ‘Seeds of blessing’. It is also known as ‘Kalo jeera’ (in Bangladesh), ‘Kalonji’ (in India) and ‘Hak Jung Chou’ in (China) (Aggarwal et al., 2008). Both seeds and oil extracted from this plant are used in medicinal purposes.

The active ingredients of N. sativa have beneficial effects against many diseases, including cancers. For example, it is effective in the diminishing the risk of atherosclerosis by decreasing the serum low density lipoprotein cholesterol level and increasing the serum high density lipoprotein cholesterol levels (Nader et al., 2010); it exerts therapeutic and protective effect in diabetes by decreasing morphological changes and preserving pancreatic beta-cell integrity (Kanter et al., 2009) and by beneficially changing the hepatic enzyme activities (Pari and Sankaranarayanan, 2009); it is effective against hypertension (Dehkordi and Kamkhah, 2008); it has a potent antihistaminic effect on airways of asthmatic patients (Boskabady et al., 2010); its components are promising agents to complement schistosomiasis specific
treatment (El Shenawy et al., 2008); its oil protects kidney tissue against oxygen free radicals, preventing renal dysfunction and morphological abnormalities (Ragheb et al., 2009). For thousands of year, the seeds, oils and extracts of *N. sativa* have been used as an anticancer agent by Unani, Ayurveda and the Chinese system of medicine that have originated from the Arab, Ind-Bangla and China, respectively. The modern scientific research with the investigation of anticancer activity of *N. sativa* is a comparatively recent affair (for the last 2-3 decades). There are not so many research works done in this field and very few review articles exist in this area. We have searched the scientific databases like Pubmed, Web of Science and Google scholar and summarized the current scientific information about the anticancer activities of *N. sativa* with mechanisms of action.

### 2.1.9 Role of *N. sativa* as an anticancer agent

Many active ingredients have been found in the seeds of *N. sativa*. The seeds contain both fixed and essential oils, proteins, alkaloids and saponin. Described the quantification of four pharmacologically important components: thymoquinone (TQ), dithymoquinone (DTQ), thymohydroquinone (THQ) and thymol (THY), in the oil of *N. sativa* seed by HPLC. Much of the biological activities of the seeds have been shown to be due to thymoquinone, the major component of the essential oil, which is also present in the fixed oil. TQ is considered as potent anti-oxidant, anti-carcinogenic and anti-mutagenic agent (structure of thymoquinone is shown in Figure 1a). Moreover, TQ is a relatively safe compound, particularly when given orally to experimental animals (Al-Ali et al., 2008). Alpha (α)-hederin, a pentacyclic triterpene saponin (structure: Figure 1b) isolated from the seeds of *N. sativa*, was also reported to have potent in vivo antitumor activity (Swamy and Huat, 2003).

*N. sativa* seeds or oils or its active ingredients like TQ are effective against different cancers:

### 2.2 Agrobacterium tumefaciens

*A. tumefaciens* is a member of the family *Rizobiaceae*. These bacteria are Gram-negative and grow aerobically, without forming end spore. The cells are rod-shaped and motile, having one to six peritrichous flagella. cells are 0.6-1µm 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 (Schaad et al., 2001). An Agrobacterium isolate was extracted from the root of a mulaita plant (*Sonchus cronutus*) grown in Tutti Island in Khartoum State- Sudan 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 is now used effectively in identification of natural products having antitumor activity at University of Gezira (Yousif et al., 2011). A tumor 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 can benign, pre-malignant, or malignant, or can represent a lesion without any cancerous potential. 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).

(updated scientific name *Rhizobium radiobacter*, synonym Agrobacterium radiobacter) is the causal agent of crown gall disease (the formation of tumours) in over 140 species of eudicots. It is a rod-shaped, Gram-negative soil bacterium. Symptoms are caused by the insertion of a small segment of DNA (known as the T-DNA, for 'transfer DNA’), from a plasmid, into the plant cell, which is incorporated at a semi-random location into the plant genome (Young et al., 2001).

Economically, *A. tumefaciens* is a serious pathogen of walnuts, grape vines, stone fruits, nut trees, sugar beets, horse radish, and rhubarb (Moore et al., 1997).

### 2.2.1 Scientific classification

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<td>Species</td>
<td><em>A. tumefaciens</em></td>
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</table>
Binomial name

*Agrobacterium tumefaciens* (Smith and Townsend, 1907).

Synonyms

*Bacterium tumefaciens* (Smith and Townsend, 1907).

*Pseudomonas tumefaciens* (Smith and Townsend, 1907).

*Phytomonas tumefaciens* (Smith and Townsend, 1907).

*Polymonas tumefaciens* (Smith and Townsend, 1907).

2.2.2 Method of infection

*A. tumefaciens* infects the plant through its Ti plasmid. The Ti plasmid integrates a segment of its DNA, known as T-DNA, into the chromosomal DNA of its host plant cells. *A. tumefaciens* has flagella that allow it to swim through the soil towards photoassimilates that accumulate in the rhizosphere around roots. Some strains may chemotactically move towards chemical exudates from plants, such as acetosyringone and sugars. The former is recognized by the VirA protein, a transmembrane protein encoded in the virA gene on the Ti plasmid. Sugars are recognised by the chvE protein, a chromosomal gene-encoded protein located in the periplasmic space (Stanton and Gelvin, 1932).

2.3 Cancer and tumor

2.3.1 Benign tumors and cancer

Not all tumors are cancerous. Tumors that aren’t cancer are called benign. Benign tumors can cause problems – they can grow very large and press on healthy organs and tissues. But they cannot grow into (invade) other tissues. Because they can’t invade, they also can’t spread to other parts of the body (metastasize). These tumors are almost never life threatening. They usually can be removed and, in most cases, they do not come back. Most important, cells from benign tumors do not spread to other parts of the body. Cells from benign tumors stay together and often they are surrounded by a containing membrane. Benign tumors are not usually a threat to life (Santen and Mansel, 2005).
2.3.2 Genetic transformation using *A. tumefaciens*

*A. 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 cytokines and responsible for tumor formation; and the genes encoding for the synthesis of opines. The latter compounds are excreted by the crown gall cells and consumed by *A. tumefaciens* as carbon and nitrogen source. 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 the plant cell and the genes involved in bacterium-bacterium plasmid conjugative transfer (Zupan and Zambrsky, 1995). Ti plasmid 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 (Zupan and Zambrsky, 1995). The Ti plasmid also contain the genes for opine catabolism produced by the crown gall cells, and regions for conjugative transfer and for its own integrity and stability. (Jeon et al., 1998).
CHAPTER THREE
MATERIALS AND METHODS

This study was conducted at the Food Laboratory of the Faculty of Engineering and Technology in 2015 and the Laboratory of Microbiology of the Sudanese Standards and Metrology Organization (SSMO) at Medani. The potato disc bioassay was used to estimate antitumor activity of *N. sativa* using the indigenous strain of *Agrobacterium tumefaciens* “SDB0012”. Experiments were conducted in a septic conditions using the Randomized Complete Design with three replications.

3.1 Sample collection

*Nigella sativa* (Black cumin) were purchased from a local supermarket in Wad Medani city, Sudan. Morphological description of seeds of the purchased *Nigella sativa* is presented in Figure 3.1.

3.2 Extraction methods

Different extraction methods of the collected samples were carried out using steam extraction looking for volatile and cold extraction for fixed components.

3.2.1 Steam distillation

Steam distillation is done by placing the plant parts – such as whole flowers, small twigs, stems or in the case of bark (chopped or ground into smaller pieces first) into a vat typically these parts are resting on a type of screen, next these large vats have steam forced from beneath through the plant parts. Essential oils are released into the steam which continues to rise as the steam cools through the cooling tubes the essential oils float on top of the cooler water component that collect in the ‘collection tank’. The oils are removed and readied for sale. The distillation water is recovered too and is known as a hydrosol or floral water. (Janardan, 2008).

Hundred grams of ground sample were subjected to steam distillation using assembled unit. The condensate was received in a separating funnel and further purified by using dichloromethane and evaporated at room temperature. The residue (extract) was kept in a refrigerator until use.
3.2.2 Cold extraction

Three hundred grams of seeds were weighted and ground in a mortar. The seeds powder was extracted with 500 ml of each solvent petroleum ether, methanol, and ethyl acetate and allowed to shake overnight in speed 400 OSC/min at room temperature by use shaker instrument, the mixtures were filtered by filter paper, the filtrates were concentrated by natural evaporation at room temperature in a dark place. (Uquiche et al, 2012).
Plate (1): Morphological characteristics of black seeds.
3.3 Preparation of NASA Media

It is the selective media for growth of *Agrobacterium*. It contains nutrient agar and sucrose. Nutrient agar ingredient 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 20g sucrose and 28g nutrient agar to 1000 ml of distilled 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. 15ml sterilized media solution which contain the antibiotic Chloroamphenicol at concentration of 100µl/L was poured per petri dish and was left to cool and solidify.

Note: Chloroamphenicol is one of the antibiotic to which the indigenous train of *Agrobacterium* SDB0012 is very sensitive.

3.4 Anti-bacterial activity test of black seed extracts

Anti-bacterial activity of black seed extracts was investigated by disc diffusion method (Cole, 1994) A. tumefaciens strain SDB0012 was obtained from stock culture in nutrient agar. 100 ml of warm agar and 0.5 ml of the bacterial suspension mixed. 10 ml volumes of mixture were distributed in sterile Petri dishes and allowed to solidity. Three discs were made by using sterile filter paper, then loaded with 50 µl of black seed extracts. Distilled water was used as negative control and floxacin was used as positive control. All discs were incubated at 37°C for 24 hour. Anti-bacterial activity was evaluated by measuring zone inhibition around disc in ml, or percentage less than 9 mm, in activity ; 9-12 mm, partially active ; 13-18 mm, active ; more than 18 mm, very active.

3.5 The potato disc bioassay

This test was conducted to study the level of antitumor potency of *Nigella sativa* extract on the growth of the bacterium expressed in the size of tumor, as illustrated in experiment were conducted to estimate anti-tumor activity of Blak seed different extracts. Potato discs 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. Cylinders were cut from the
disinfested section using a sterile cork borer (10-15 mm) discs (0.5 cm thick) were cut aseptically from the cylinders. These disks were placed in Petri dishes containing solidified (NASA) medium. 3 discs per Petri dish were placed by gently pushing the discs into the agar using aspect technique. Three Petri dishes per sample were use and one as control for each experiment. Preparation of bacterial suspension done by taking a loop full of *A. tumefaciens* of 24 hr old culture from storage culture on slant agar, was added to 10 ml sterile distilled water to make concentration of (106-107 cell /ml) which then overlaid on each disk of potato disc.

Each treatment was represented by three Petri-dishes, each containing three potato disc. One drop (50 µl) of the prepared suspension of the *A.tumefaciens* was added onto each potato disc then crude cumin oil extracts (50 µl) were conducted. The potato discs which were used as negative control were treated with sterilized water. Seal the edge of each Petri-dish with paraffin stripes to prevent moisture loss during the incubation period, then incubated in the dark at the 37°C for 24 hr. Data was collected based on inhibition in tumor development (zone) caused by *A.tumefaciens*, using Lugols reagent which turns tumorigenic tissues into dark brown color. Agrobacterium infection led to development of tumorigenic tissues on the surface of the potato disc (2 cm in diameter), as detected on surface of disc used as control (complete coverage of the disc surface), 21 days after infection.
CHAPTER FOUR
RESULTS AND DISCUSSION

Inhibitory effect (%) of different extracts of black cumin against *A. tumefaciens* were presented in Table (4.1) and Figure (4.1), (4.2). Results showed that, methanol and essential oil extracts have not anti bacterial effect (0%), in other hand, ethyl acetate, Petroleum ether and Commercial sample have bacterial effect (50%, 10%, 10% respectively). Any extracts have anti bacterial effect (including ethyl acetate, Petroleum ether and Commercial sample) did not used in anti tumor activity. Methanol and essential oil extracts were used as anti tumor effect and showed the highest inhibition percentage of 100% and 87% respectively. Different among incubation periods were found to be not significantly but have positive effect due to inhibitory effect stability. The best incubation at (14 day) from application gave the best inhibition percentage in methanolic (Table 4.2) and (Figure 4.3). Results indicated that the methanol extract gave darker color obtained on disc treated with Lugols solution which in turn showed the highest inhibitory effect of the extract Figure (4.3). Addition of Lugols solution to potato disc used as control showed yellowish color indicating presence of tumor Figure (4.4). The disks were stained with Lugol's reagent 5% (the 5% solution consist 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 stain 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 Rogres, 1998).

This agree with Ashwag (2016) reported that, Methanol extracts of all samples gave the highest antitumor activity of 100% for grape and date palm, 80% for pomegranate and 70% for olive; while ethyl acetate and hexane extracts gave poor results. The only exception was the date palm ethyl acetate extract which gave antitumor activity of 100%, In this study we presented that Methanol extracts of all samples above gave the highest antitumor activity of 100%.

Mean while Suhair (2016) reported that application of the crude extract of Tepary bean lectin to potato discs on tumor formation produced by *A. tumefaciens* at a concentration of 50 μl/disc gave significantly at 0.01% level, the highest inhibition
percentage of 99% on average followed by 37.5 μl/disc (80%) and 25 μl/disc (50%). Differences among incubation periods were found to be not significantly different at 5% level. The best combination of 50 μl/disc at 7 days from application gave the best inhibition (99%). Showed phenotypic results of ant-tumor activity of the three levels of lectin used 100%, 75% and 50% dilution rates. Results indicated that the highest concentration of the crude extract gave darker color obtained on discs treated with lugols solution which in turn showed the highest inhibitory effect of the extract.
Table 4.1. Antibacterial activity of different black cumin extracts against *A. tumefaciens*

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<td>Commercial sample</td>
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<tr>
<td>Ethyl acetate</td>
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<td>Essential oil</td>
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Figure 4.1 Antibacterial activity of Ethyl acetate extract against Agrobacterium tumefaciens "SDB0012"
Figure 4.2 Antibacterial activity of Methanol extract against Agrobacterium tumefaciens "SDB0012"
Figure 4.3. Anti-tumor activity of Methanol extract against tumor caused by Agrobacterium tumefaciens "SDB0012"
Figure 4.4. Patoto disk with Agrobacterium tumefaciens and sterilized distil water (control sample)
Table (4.2): Anti-tumor activity of different black cumin extract against *A. tumefaciens*

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<thead>
<tr>
<th>Extract</th>
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CHAPTER FIVE
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

- Methanolic extract of black seed inhibited tumor produced by *A. tumefaciens*.
- The time of incubation don’t gave significantly results.
- Different among incubation periods were found to be positive effect due to inhibitory effect stability.

5.2 Recommendations

- The crude extract of black seed should be used in further experimentation.
- Fractionation of methanol extract using thin layer chromatography and recognition of active components with the potato disc bioassay.
- Determination of active components using specific instrument (GC-MS).
- Additional studies are required to evaluate and explore the specific cellular and molecular mechanisms of the antitumor effects of *N. sativa*, alone or in combination with other drugs.
REFERENCES


## APPENDICES

### Appendix (1): ANOVA table

ANOVA: Two-Factor Without Replication

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