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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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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 botanist to be used as antibacterial and anti-cancer products; 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 anti-bacterial 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 extracted from Tuti Island-Khartoun State and purified since 2005. The major characteristic of this strain was given by Yousif. 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 four 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 100% inhibition of tumor on the potato discs. Only one out of the eight components separated from the oil black cumin crude extract in thin layer chromatography showed complete inhibition of tumors and was considered as the active anti-tumor component of the black cumin.

Keyword:

Cumin; Honey; Anti-tumor; *Agrobacterium tumefaciens*; *Nigella sativa*

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 [1,2].

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 tumorigenic 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. 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 [1]. 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 o 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 [3].

Materials and Methods

This study was conducted at the Microbiology Laboratory of the Faculty of Engineering and Technology in 2013-2014. 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 tumrfaciens* "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 tumrfaciens* strain SDB0012. The bacterium culture was maintained throughout the study by routine sub-culturing under aseptic microbiological methods.

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 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 of clay: sand) were injected with the prepared inoculums, at 1 ml of the inoculum per plant at stem base and allowed to continue growth. 21 days after injection plants were observed for the growth of tumors. Then plants were observed for the presence and absence of tumors. Then, 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 [1].

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 (chloramphenicol at concentration of 100 µl/L) was poured per Petri dish and was left to cool and solidify).

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, and chloramphenicol at the concentration of 0.1 mg /1, 0.2 g Magnesium sulphate hepta-hydrate and 0.1 g sodium chloride. This ingredient was being stirred between additions. The medium was then sterilized by autoclaving for 15 minutes, at 121°C and 15 psi.

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 [4].

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 replications. Each treatment was represented by 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" inoculate in NASA medium.

Preparation of potato discs

Red skinned potatoes tubers (*Solanum tuberousum*) 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 priory 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).

The potato disk bioassay protocol

Preparation of Potato discs: *Solanum uberosum* 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 aspect 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. Seal the edge of each Petri dish 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 count the tumors 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 [5].

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. 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 hour, 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 [6].

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.

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 $FeCl_3.6H_2O$, 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 [7].

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 Itd. spreader. Silica gel containing 13% CaSO4 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 running each plate was left in 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 [7].

Application of plant extracts and TLC development

In each experiment, the cumin solution was applied as a band 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 R*f* 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 [7].

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 [3]. 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. 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 [1]. The results showed the 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 octapine-type tumors mentioned.

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 in the size of inhibition zone. The bacterium grew normal in filter paper filled with bee honey, cumin oil and a mixture o 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.

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 O_2 and H_2O [1]. This result was in aline with Mandal and Mandal [8], 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 [9]. 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 [8]. 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, 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 [10]. 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 a line with Randhawa and Al- Ghamdi [11] 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 [12]. Since, Thymol is present in the methanol soluble portion of oil [13], 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 [14].

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 [15] 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. The 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 [5]. 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. 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 [16]. 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 and Figure 1). 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 [17].

Product	Inhibition of tumor metastasis (%)
Bee honey	40c ±3.2
Cumin oil	55b ±4.1
Mixture of Bee honey and Cumin oil (1:1)	100a ±0.13
Note: Results with different superscript letters with in columns are significantly different of $p \leq 0.05$.	

Table 1: In-vitro inhibition of tumor metastasis using honey, cumin oil and the mixture o them (1:1).

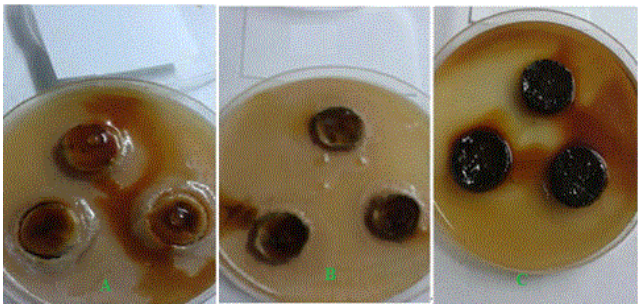

Figure 1: In-vitro inhibition of tumor growth and tumor metastasis using honey, cumin oil and the mixture o them (1:1). **A:** Bee honey **B:** Cumin oil **C:** Bee honey + cumin oil (1:1).

Fractionation of cumin oil

Fractionation of cumin oil using thin layer chromatography is common or studying its biological activities. For example, the study conducted by Ismail [18,19] on the neuro-protative 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. 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 and Figure 2). Yet, Fraction 6 larger prominent and prominent tumors compared to other fractions. Only the fraction 2 was resulted in 100% tumor metastasis inhibition and therefore considered the most antitumor active component in cumin oil. Further fractionation of fraction 2 was suggested to reach a stable fraction that could be subjected to chemical identification using the GC-MS (Figure 3).

	Fractions of cumin oil	Inhibition of tumor metastasis (%)*
Fraction number	**R*f***	
Fraction 1	0.118	5c ± 1.4

Fraction 2	0.147	100a ± 0.4
Fraction 3	0.294	5c ± 1.1
Fraction 4	0.426	5c ± 1.8
Fraction 5	0.603	10b ± 2.4
Fraction 6	0.735	5c ± 1.2
Fraction 7	0.897	0d ± 0.0
Fraction 8	0.926	0d ± 0.0

Note: Results with different superscript letters with in columns are significantly different of $p \leq 0.05$.

Table 2: Inhibition of tumor metastasis on potato discs treated with TLC products (fractions) of cumin oil.

Figure 2: 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.

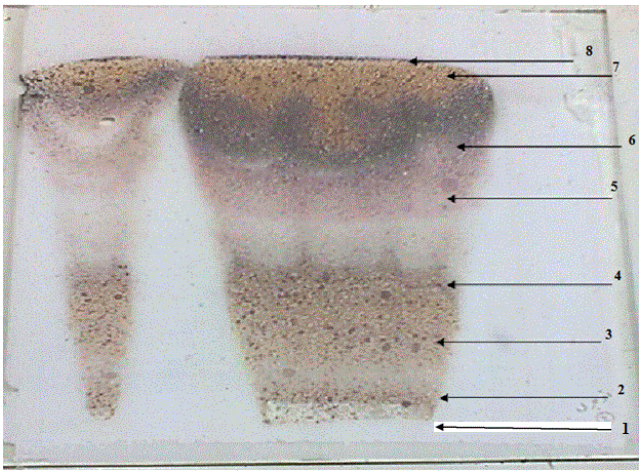

Figure 3: Fractionation of cumin oil on silica gel using the thin layer chromatography.

Conclusions

Both honey and cumin has no antibacterial activity. Honey, cumin oil and their mixture have antitumor activities of 40%, 55% and 100%, respectively. The fraction 3 and Fraction 2 of the cumin oil scored 60% and 100% inhibition of tumor growth, whereas, fraction 6 encouraged tumor growth compared to the control.

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