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An In-Vitro Assessment of Anti-Tumor Activity of Some Plant Extract and Natural Products, Using Potato Discs Bioassay Technique

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Abstract:

Objectives: This study aimed to examine some plant extracts and natural products for anti-tumor activity.

Methodology: Potato disc bioassay was used to estimate anti-tumor activity of some plant extracts and natural products. Five plant extracts and natural products recommended in a questionnaire filled by local botanists in addition to an introduced fermented food "Manda Koso" were used in this study.

Study design: A questionnaire was designed and filled by 60 botanists looking for plant extracts and natural products having anti-cancer activity. In addition, computer surveys were conducted for gathering information on promising natural compounds act as anticancer agents. The potato disc bioassay was conducted using the Complete Randomized Design with three replicates. Each treatment in each replicate was represented by two Petri dishes contained five potato discs each. Petri dishes treated with sterilized water were used as control. Four separate experiments were carried out using TLC technique.

Results & Discussion: Results showed highly significant differences among treatments with respect to total number of tumors and inhibition average percentage (%). The inhibition average percentage (IAP) for the different products ranged between 80.95-100% for the different products and equal zero for the control. It was 100% for Manda Kosa and bees honey followed by 90.23% for olive oil. Other treatments showed moderate anti-tumor effect of 80.95% IAP.

Conclusions: Results concluded that Manda Koso, bees honey and olive oil have anti-tumor activity. Great association was found between anti-tumor activity obtained in this study and those published for anticancer activity of the promising material.

المخلص:

أجريت الدراسة بمعامل الاحياء الدقيقة بكلية الهندسة والتكنولوجيا بجامعة الجزيرة في الفترة من 3 ابريل وحتى 21 سبتمبر (2008) وذلك بغرض دراسة مقدرة بعض المستخلصات النباتية على تثبيط الاورام المستحثة باستخدام سلالة محلية من البكتيريا الزراعية *Agrobacterium tumefaciens* تسمى "SDB0012" وذلك باستخدام تقنية قرص البطاطس وتقنية الفصل الكروماتوجرافي للتعرف على الجزيئات الفعالة للمستخلصات الطبيعية المستخدمة في هذه الدراسة. اوضحت النتائج فروقات معنوية عالية ما بين المستخلصات المستخدمة في العدد الكلي للاورام واحجامها. اعطي المستخلص الياباني ماندا كوسو "Manda Koso" فعالية عالية على التثبيط

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الكلية للأورام المستحثة , تليها عينة محلية لعسل النحل ثم زيت الزيتون ثم زيت الحبة السوداء (الكمون) ثم زيت الثوم واقل نسبة رصدت في زيت الحلبة. أوصت الدراسة لاستخدام كل من ماندا كوسو, العسل و زيت الحبة السوداء كمثبطات نمو الاورام. اتضح من خلال هذه الدراسة فعالية استخدام تقنية اقراص البطاطس في تحديد مثبطات لنمو الاورام في كل من البطاطس والانسان وذلك من خلال تقارب النتائج بين هذا الاختبار في هذه التجربة و النتائج العالمية في هذه المستخلصات. هذا وقد اقترحت الدراسة إجراء استخدام تقنيات الفصل الكروماتوجرافي و كروماتوجرافيا الغاز GC و تقنية HPLC بالاضافة لتقنية الهجرة الكهربائية gel electrophoresis لفصل البروتينات.

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 podophylloids. 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 (Gordon and David, 2005). Sudan is rich in medicinal plants with a wide biodiversity. The use of these natural products is still lacking behind due to a limited scientific handling of such products.

The crown gall tumor assay (CGTA) is one of several bench top bioassays recommended for the rapid screening of plants for 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 Galasky *et al.* (1980). 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 (Srirama *et al.*, 2007).

Several plant derived compounds are successfully used in cancer treatment such as 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. It is also active against small cell lung carcinoma. It is a topoisomerase II inhibitor stabilizing enzyme –DNA cleavable complexes leading to DNA breaks. Moreover, the plant derived anticancer agents vineristine is used against leukaemia's, breast, lymphoma, germ-cell and renal cancers. Paclitaxel is used against ovary, breast, lung, bladder, head and neck cancer. Topotecan is used against ovarian and lung cancer and docetaxel is used against breast and lung cancer (Adriana *et al.*, 2001).

The main objective of this study is to examine local plant species and natural products for anti-tumor activity.

Materials and Methods:

Methodology: This study was conducted at the Food Technology Laboratory of Faculty of Engineering and Technology, University of Gezira in the period 2008-2009. It was established by a questionnaire filled by botanists and traditional folk medicine practitioners who believe in some natural products having potential use as anti-tumor agents. Moreover, computer surveys were conducted for gathering information on promising natural compounds as anticancer agents. Based on results obtained by the questionnaire and

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computer surveys; some indigenous natural products were collected and subjected to potato disc bioassay. The indigenous strain of *Agrobacterium tumefaciens* "SDB0012" which was identified at the National Institute for Promotion of Horticultural Exports since 2005 was used to induce tumors in the potato disc bioassay (El Skeikh, 2007).

Collection of samples: The questionnaire was filled by 60 botanists and traditional folk medicine practitioners in both Wad Medani and El Hasahisa areas. Based on the questionnaire results, six natural products were proposed by botanists and were subjected to potato disc bioassays and thin layer chromatography (Table 1). Extracts from each of fenugreek and garlic were prepared following the A.O.C.S. official method (1993).

The potato disc bioassay: The potato disc bioassay was conducted in aseptic conditions using the Complete Randomized Design with three replicates. Each treatment in each replicate was represented by two Petri dishes containing five potato disc each. Petri dishes treated with sterilized water were used as negative control (-ve control). Bacterial suspension of *A. tumefaciens* was prepared from two of the selected subcultures of the original *A. tumefaciens* slant of the indigenous strain SDB0012. Subcultures three (3) was used in the first experiment (exp. No. 1) while the subculture five (5) was used in the second experiment (exp. No. 2). The growth medium Yeast Mannitol Broth (YMB) was prepared following Galasky *et al.* (1980). The medium was allowed to cool and a loop of *Agrobacterium tumefaciens* subcultures (3) or (5) from storage cultures on agar slant was added to the media. The flasks were then placed on an orbital shaker at speed of 150 rpm for 48 hrs at 30 °C for the growth of the bacterium (Jerry *et al.*, 1988). The potato disc bioassay was performed following Elsheikh (2007). The number of tumors per potato disc was calculated after 7 days as a parameter to determine anti-tumor activity of each treatment. Then, inhibition average percentage was calculated following Jerry *et al.*, (1982); where:

$$\text{Inhibition percentage} = \frac{100 \times \text{Average number tumors of the treatment}}{\text{Average number tumors of the control}}$$

Results and Discussion:

The questionnaire guide lines:

Sixty botanists responded to fill the questionnaire in both Wad-Medani and El Hasahisa areas. Among them 54% mentioned; bees honey, 48% with olive oil, 49% a mixture of camoon (black cumin) and bees honey, 52% camoon oil, 43% fenugreek oil and 42% garlic oil as natural products with anticancer activity. Other natural products believed to have anticancer activities were royal jelly 28%, pollen grains 25% and sunflower oil 2%; in addition to a recently introduced fermented food Manda Koso from Manda Fermentation Co Ltd-Japan. The anticancer activity of Manda Koso was high-lightened by Higa and Wididana (1991). Results of the questionnaire are shown in Table 2. These results were in line with Apitherapy News, (2011) for honey, Mendez *et al.* (2005) and Reuters (2005) for olive oil, Paresh (2010) for black cumin, Sumiyoshi (1997) for garlic, Apitherapy (2011) for royal jelly and pollen grains. Similarity of questionnaire findings with the international findings pointed out the capabilities of local botanists and the role of complementary and alternative medicine in Sudan. Our interest to study anti-tumor effects of these products was encouraged by findings obtained by Srirama *et al.* (2007), who concluded that plants with anti-cancer activity have higher properties of their species resistant to crown

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gall formation. Potato disc as a bench top bioassay was adapted to aid "drug discovery" work with botanicals. This method was used over the past 15 years for standardization and quality control of bioactive components in such heterogenous botanicals (Jerry, *et al.*, 1998).

Number of tumors and inhibition average percentage (%) of treatments:

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 (El Shiekh, 2007). 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. The surface of the tumors was smooth with no shoot growth which recognizes the octapine-type tumors. This bacterial strain was further used in this study to be applied on potato discs treated with plant extracts and natural products to assess their anti-tumor activity.

Differences among treatments were highly significant for number of tumors/disc ($P=0.000$). Number of tumors of treatments ranged between 4.3 in the control to zero in Manda Koso and virgin bees honey (Figure 2). Table 3 shows number of tumors and inhibition average percentage in the two experiments, 21 days after infection. No tumor growth was found on potato discs treated with bees honey and Manda Koso in the two experiments with inhibition percentage of 100%. These results were in a line with those published for the anticancer effects of the two products. Honey Thai protis has an in-vitro proliferative activity of partially purified *Trigona laeviceps* propolis from Thailand on human cancer cell lines (Apitherapy News, May 09, 2011). On the other hand, royal jelly has significant anti-cancer properties as it drastically reduced spread of cancer cells injected to mice (Apitherapy News, May 03, 2011).

Moreover, honey itself has been found to have anti-mutagenic, anti-inflammatory, anti-microbial, anti-atherogenic and anti-irradiation effects. It has anti-proliferative effects on colon cancer cells. Honey samples with higher phenolic content were found to have more significant anti-proliferative effect. It has also been shown to inhibit the growth of bladder cancer cell lines in-vitro and in-vivo and murine leukemia cells (Apitherapy News, May 03, 2011). Furthermore, Orsoli *et al.* in 2004 stated that honey bee products given orally or systemically may have an important role in the control of tumors growth and tumor metastasizing ability. On the other hand, the fermented food Manda Koso inhibited tumors growth in lung carcinoma (Yashiyki *et al.*, 1999) and has a pronounced anti-oxidizing capability (Higa, 1996).

Olive oil ranked the second with respect to number of tumors induced by *A. tumefaciens*, it showed 0.2 and 0.6 tumors on average with inhibition average percentage of 94.74 and 85.71% in the first and the second experiments, respectively. Olive oil has an anticancer effect. Oleic acid has effects on human protection against breast cancer. It blocks the action of a cancer-causing oncogene called *HER-2/neu*, which is found in about 30% of breast cancer patients (Mendez *et al.*, 2005). It has also synergistic effect as it enhances cancer drug effectiveness for example it improves the effectiveness of Herceptin, a breast cancer drug (using targeted therapy) works against the *HER-2/neu* gene. Moreover, the effect of oleic acid on the efficacy of trastuzumab (HerceptinTM), a humanized monoclonal antibody binding with high affinity to the ecto-domain of the *HER-2/neu* called P185 Her-2/neu *onco*-protein was investigated (Mendez *et al.*, 2005). Other extracts scored 0.8 tumors per disc with inhibition average percentage of 78.94% and 80.95% in the first and second experiments, respectively. Number of tumors in the control was found to be 3.8 tumor/disc in the first experiment and 4.2 tumor/disc in the second experiment.

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Although inhibition average percentage of black cumin and garlic was considered as moderate, they were recommended to have anti-cancer activities. The former inhibits DNA synthesis and mostly it inhibits HDAC enzyme in the chromosome (Pareesh, 2010). Whereas, the latter has a wide use as anti-bacterial, anti-fungal, anti-inflammatory, anti-microbial, anthelmintic, anti-septic, anti-viral, hypotensive-vasodilator, chlagogue, antioxidant, hypoglycemic, expectorant, PAF anagonist, anti-tumor, anti-neoplastic, anti-mutagenic, diuretic, carminative...*etc* (Pizzorno and Murray, 1995). Experimental studies with animals and some human cell lines *In-vitro* have shown garlic extracts to have potent chemo-preventative and anticancer effects. Selenium is known to be protective against tumorigenesis and high selenium garlic extracts showed inhibitory activity in animal mammary carcinogenesis (Sumiyoshi (1997). Concerning *Malva saylevestris* no information are available with respect to its use as anti-tumor product.

The observed tumor growth rate was too weak and the size was small in olive oil. This result was supported by reports of Seeram *et al.* (2004) who stated that efficacy of the natural products as anti-tumor activity can be determined by reduction of size and number of tumors per unit area. It was concluded that both Manda Koso and honey have the highest anti-tumor activity followed by olive oil. The anti-tumor activity of black cumin and garlic was considered as moderate.

Crude potential extracts:

Cancer growth inhibitors are related to the use of chemical substances which could retard or inhibit the growth of cancer cells (Higginson and Oettle, 1953). Physical analytical methods such as chromatography are useful for determining sensitivity to the chemical complexities found in crude botanical extracts. 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 (Rijken *et al.*, 1999). Moreover, results obtained in this study also suggested development or introduction of cancer cell lines to be used in further research activities. Results also encourage collection, conservation and utilization of germplasm of natural products of medicinal uses.

Conclusions:

Results concluded that Manda Koso, bees honey and olive oil have anti- tumor activity. Great association was found between anti-tumor activity obtained in this study and those published for anticancer activity of the promising material.

Table (1): Plant extracts and natural products used in potato disc bioassays and thin layer chromatography

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Entry	Source
Crude olive oil (virgin)	Shelf commodity-Wad Medani market
Black cumin (<i>Nigella sativa</i>)	Shelf commodity-Wad Medani market
Manda Koso	Manda Fermented Co Ltd.-Japan and kindly provided by AlZahra for Agricultural Trade and Commodities-Khartoum
Garlic (<i>Allium sativum</i>)	Wad Medani market
Bee honey	EL-nahalaa Co Ltd. from the local market in Wad Medani.
Fenugreek (<i>Trigonella foenum- graecaum</i> L.)	Wad Medani market

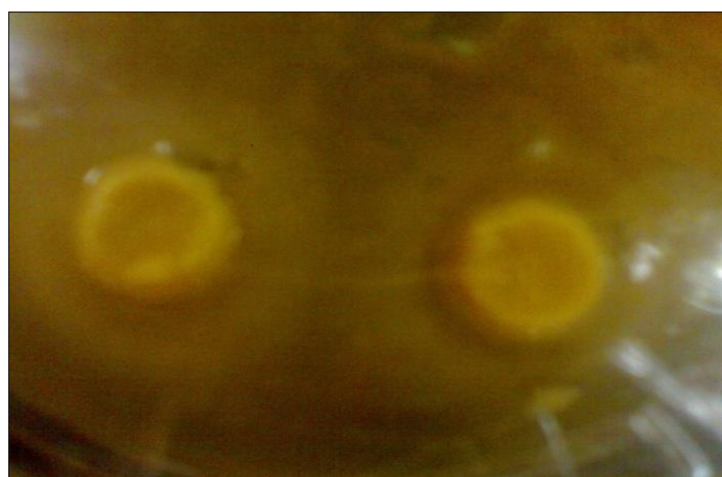


Figure (1): Size and number of tumors grown on potato discs inoculated with 0.005ml of *Agrobacterium tumefaciens* SDB0012

Table (2) Results of the questionnaire on beliefs of botanists regarding some natural products with anti-cancer activity

Sample name (0.2 ml/disc)	Positive result percentage (%)
Bees Honey	54
Black cumin oil	52
Olive oil	48
Mixture of black cumin & bees	49

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**Table (3):
numbers of
21 days after
treatment with
products/plants**

honey	
Fenugreek oil	43
Garlic oil	42
Royal jelly	28
Pollen grains	25

**Average
induced tumors
application of
natural**

extracts using two different Agrobacterium tumefaciens sub-cultures 3 and 5

Treatment (0.2 ml/disc)	Experiment one		Experiment two	
	Average No. of tumors/disc	Inhibition average (%)	Average No. of tumors/disc	Inhibition average (%)
Bacterial suspension (-ve control)	3.80	0.00	4.20	0.00
<i>Alullum sativum sp</i>	0.80	78.94	0.80	80.95
<i>Nigella sativa sp</i>	0.80	78.94	0.80	80.95
<i>Olive oil</i>	0.20	94.74	0.60	85.71
Manda Koso	0.00	100	0.00	100
Honey	0.00	100	0.00	100
<i>Trigonella foenum- graecaum L</i>	0.80	78.94	0.80	80.95
Prob.	0.00		0.00	
S.E. (+)	0.05		0.05	
C.V.	32.08%		19.44%	
LSD.	1.689		0.3229	

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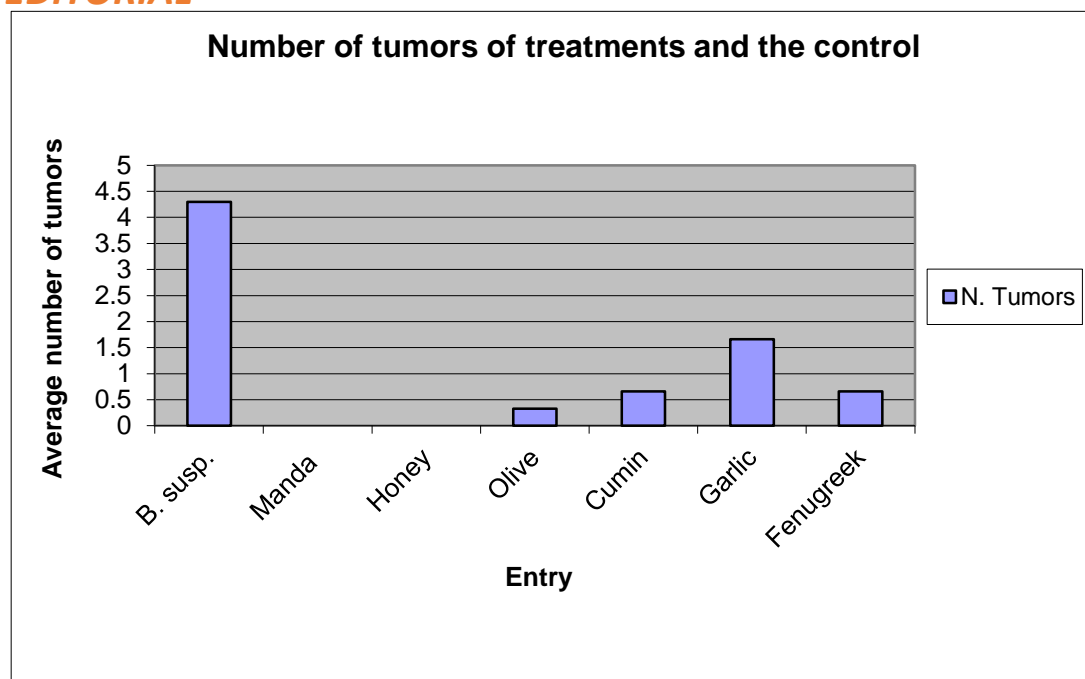


Figure (2): A histogram showing average number of tumors of treatments and the control on potato discs inoculated with *Agrobacterium tumefaciens* SDB0012

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