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Identification of Antitumor Activity of Seed Extracts of Date Palm, Grape, Pomegranate and Olive, Using Potato Disc Bioassay Technique

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Abstract: ***This study aimed at determination of antitumor and anticancer activities of seed extracts of date palm, grape, pomegranate and olive using biological and biochemical techniques. The crude extracts of the four plant species, using hexane, methanol and ethyl acetate, were tested for antitumor activity. The indigenous strain of Agrobacterium tumefaciens SDB0012 was used to induce tumors on the potato discs in all experiments. The methanol crude extracts of all plant species were fractionated using thin layer chromatography (TLC) and the resulting fractions the different extracts were retested for antitumor activity. Fractions having antitumor activity of more than 70% were subjected to chemical analysis using Gas chromatography- mass spectrum (GC-MS) for identification of the chemical structure of compounds. Then, the activity of the compounds of all fractions was determined using computer surveys. 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, (except the date palm ethyl acetate extract which gave antitumor activity of 100%). The highest number of TLC- separated fractions of eleven was obtained by olive followed by pomegranates (10), and date palm and grape (9). GC-MS-analysis revealed the presence of seven different components common in all seeds with polymorphism in sixteen components among the different samples. Nevertheless, date palm exhibited the highest amount of diverse components of 15. Therefore, it was recommended to accumulate other components to date palm obtain the whole set of the 23 components.***

1. Introduction

Plants have a long history of use in the treatment of cancer (Hartwell, 1982; Cragg *et al*., 1994). Natural product synthesis is heavily integrated with medicinal and combinatorial chemistry as well as the traditional organic disciplines (Anne et al., 2009).

The potential of new drug discovery of natural products attract scientists of various disciplines (organic chemistry, bioorganic chemistry, pharmacology and biology etc) Koehn and Carter (2005). The medicinal value of plants lies in some chemical substances that produce a definite physiological action in human body. However, recent attention has been paid to biologically active components that isolated from plant species(Koehn and Carter, 2005). Olive, date palm, grape and pomegranate were proved to have antimicrobial and anti oxidant activities (Ghisalberti, 2008). Cancer cells can invade and damage tissues and organs near the tumor. Cancer cells also can break away from a malignant tumor and enter the lymphatic system or the bloodstream, which is how cancer can spread to other parts of the body (Burstein *et al*., 2011). The antitumor activity test using potato discs is crucial since the tumoregnic mechanisms are similar in plant and animals (Becker, 1975; Braun, 1972; Karpas, 1982). The bench-top test that has proven useful monitors the inhibition of crown gall tumor on potato discs (McLaughlin and Rogers, 1998).Recently techniques for detection of antitumor and natural products at the University of Gezira, such as the potato disc bioassay using the indigenous strain of *Agrobacterium tumefaciens* SDB0012 (Yousif *et al*., 2012; Jerry *et al.*, 1998) have been developed to encourage research on the promising natural products that could have the potential of being competitive drugs in the future. Objective of this study to determination of antitumor and anticancer activities of seed extracts of date palm, grape, pomegranate and olive using potato disk bioassay Technique.

2. Methodology

Equipments

2.1 Cold extraction: Cold extraction: A direct cold extraction procedure of finely ground material was developed for use in the phytomedicine programme (Eloff, 1998).

2.2 Bioassay techniques: the crudes extract obtained by cold extraction were evaluated for antibacterial and antitumor activity as described below.

(1) Antibacterial activity assay: Biological activity of the crude and the purified fraction were tested by using disc diffusion method.
(2) Antitumor activity: Using *Agrobacterium tumefaciens* strain SD0012 (local isolate) as biological tool for potato disk bioassay technique (McLaughlin and Rogres, 1998), and calculated as:

Inhibition% = 100 - (Average number tumor of sample ×100) \ Average number tumors of control

TLC-separated components: The isolation and separation of methanol extracts were done by using the procedure of Stahel (1964). The TLC solvent system Ethyl acetate / Methanol / glacial acetic acid (60:40: 0.5) and identified using Ultra Violet lamp of a short wave length of 254*nm*.

3. Results

Antibacterial activity of seed extracts

Table 1: Antibacterial activity (inhibition) of the fruits seeds crude extracts by disc diffusion method (The applied dose is 20 µl/disc)

Sample Solvent	Date palm	Grape	Pomegranates	Olive
Hexane	0.0	0.0	0.0	0.0
Methanol	0.0	0.0	0.0	0.0
Ethyl acetate	0.0	0.0	0.0	0.0

Results presented in Table (1) showed absence of an apparent inhibition zone on growth of the bacterium for the different extracts. No reports were available in the literature on antibacterial activity of seeds of date palm, grape, olive and pomegranates against *A. tumefaciens*. The importance of this test comes from the fact that sensitivity of *Agrobacterium* to crude extracts could render the antitumor test against these extracts since this bacterium, the indigenous strain SDB0012, was used as an inducing agent for tumors growth on potato discs. The initial step in the formation of *A. tumefaciens*-induced tumors involves attachment of the bacterium to a tumor-binding site (Glogowski and Galsky, 1978). The attachment of the bacterium to the tissue completed within 15min after inoculation (Glogowski and Galsky, 1978; McLaughlin *et al*.. 1993). Galsky *et al*. (1980) which necessitate conducting the antibacterial test prior to antitumor activity bioassay of the different extracts.

Antitumor activity

The highest antitumor activity was shown by methanol extract of date palm and grape seeds scoring100% inhibition for each (Table 2). The inhibition percentage of pomegranates and olive were 80% and 70%, respectively. Results suggested that the bioactive components of tumor inhibition in the four plant species may be attributed to presence of polar functional group. The antitumor activity test using potato discs is crucial since the tumoregnic mechanisms are similar in plant and animals (Becker, 1975; Braun, 1972; Karpas, 1982). The bench-top test that has proven useful monitors the inhibition of crown gall tumor on potato discs (McLaughlin and Rogers, 1999). To be virulent, the bacterium must contain a tumor-inducing plasmid (Ti plasmid or Tip), of 200 kb, which contains the T-DNA and all the genes necessary to transfer it to the plant cell (Berger et al., 1993). 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 (Tinland et al., 1995).

This bioassay is fairly accurate in predicting cyto-toxicity to the P388 cell line, giving some false-positives, but few false-negatives (McLaughlin and Rogers, 1999). The assay is not meant to replace the P388 assay, but it is particularly convenient for rapid screening of extracts or fractions and does not require expensive equipment or highly trained personnel. Crown gall is a neoplastic disease induced by the gram-negative bacterium Agrobacterium tumefaciens. During infection of the plant material with the bacterium, a large tumor-inducing (Ti)plasmid, found in the bacterial DNA, is incorporated into the plant's chromosomal DNA. The phenols released when the plant is wounded activate the Ti plasmid of the bacterium, which induces cell proliferation without the cells going through apoptosis, thus transforming normal, wounded cells into autonomous tumor cells. The induced tumor is similar in nucleic acid content and histology to human and animal cancers.

Table 2: Antitumor activity (inhibition %) of the fruits seeds crude extracts by potato disc bioassay technique (The applied dose is 50 µl/disc)

Sample Solvent	Date palm	Grape red	Grape green	Pomegranates	Olive black	Olive green
Hexane (non-polar)	10%	50%	50%	0%	10%	30%
Methanol (polar)	100%	100%	100%	80%	70%	70%
Ethyl acetate (semi polar)	100%	0%	0%	20%	50%	50%

* The inhibition% = Calculate of crown gall tumors as follows:
= 100 – (Average number tumor of sample / Average number tumors of control x 100).

3.2 Effect of the extraction methods

The antitumor activity of extracts of the all plant species using different polarity solvents (polar, non-polar and semi-polar) on potato discs treated with *A. tumefaciens* is given in Table (2) The previously conducted antibacterial test ensure that the action of the crudes extracts tested was on the formation of tumors themselves and not affected viability of the bacterium. Date palm and grape methanol extracts resulted in 100% inhibition followed by methanol extract of pomegranate (80%) and Olive (70%). Based on the literature precedence, a variety of procyanidins from grapes and their seeds have been shown to prevent the growth of cancer cells (Liviero and Poglisi, 1994). Another report (Tyagi *et al*., 2003) also showed that the grape seed extract has possible role in anti-proliferation and apoptosis of human prostate carcinoma. Awad and Fink (2000) the olives have some anticancer effect in colon, breast and prostate.Gil *et al* (1995), report the pomegranate proved to have high antioxidant activity and good potency for cancer preventation (Afag *et al*., 2003).

Hexane extracts (oils) of grape gave 50% inhibition followed by green olive (30%) and for date palm (10%). while pomegranates showed as zero% inhibition, as presented in Table (2) Whereas, ethyl acetate extract of date palm was found to be very promising as it gave 100% inhibition followed by olive (50%) and pomegranate (20%). Ethyl acetate extract of grape, olive and pomegranate showed zero inhibition. In addition, ethyl acetate extract (semi-polar) of date palm gave 100% inhibition. Variation among different extracts might be due to presence of different functional group (polar, semi-polar and non-polar) extracts by the different solvents. It was concluded that all polar extracts showed antitumor activity. On the other hand, non-polar extracts of all plant species gave poor result.

Figure 1: Photographs of antitumor activity of fruits extracts

TLC-separated components of the crude methanol extracts of all species

Results of TLC separation of methanol extract of all species are presented in (Figure 2). Whereas, the TLC solvent system Ethyl acetate / Methanol (60:40 v/v). The extracts of methanol showed no visual separated components even with the polar solvent system Ethyl acetate /Methanol meaning that isolated compounds were highly polar and fixed at the bottom of the plate. Application of 0.5 ml of glacial acetic acid helped at separating these components, which bound to silica gel to be identified using ultra violet lamp of a short wave length of 254*nm*.

The crude methanol extracts of date palm and grape gave the same number of nine TLC-separated compounds. The highest number of TLC- separated compounds of 11 was obtained by olive, followed by sample pomegranates (10 TLC-separated compounds). These compounds were numbered and calculated starting from solvent front of TLC-plate.

3.3 Determination of the active antitumor ingredients

Thin layer chromatographic fractionation of methanol extracts resulted in several components (Fig 2). The R_f values and inhibition percentage of the eluted TLC methanol extracts with chloroform/methanol (2:1, 1:1, 3:2, 9:1, 1:9). Previously, the crude extract of date palm was found to be promising as antitumor inhibitor, therefore efforts were exerted to fractionate it to identify the most effect fractions for antitumor activity using the bench top test. The highest tumor inhibition values was recorded by Fractions 6, 7 and 8 which scored 85%, follow by Fraction four (80%), Fractions two and nine(75%) and Fraction three (60%). The lowest inhibition value was recorded by Fraction one (30%). It was observed that none of these fractions gave inhibition value as high as the crude extract itself (100% inhibition), this result suggested presence of synergetic effects among the different fractions.

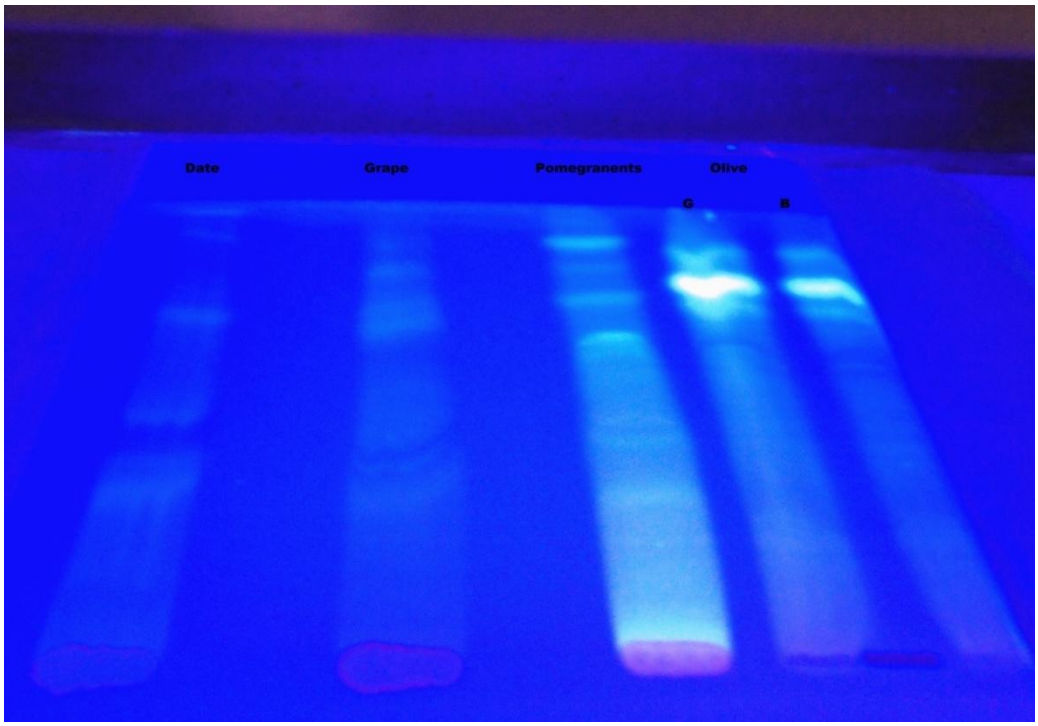

Figure 2: TLC separated Components of methanol extract of Seeds of fruits' date palm, grape, olive and pomegranate

Table 3: Components of fruits samples methanol extract identified by GC-MS

Compound	Date palm		Grape		Olive		Pomegranate
	Range	Means±SD	Range	Means±SD	Range	Means±SD	Value
Benzenedicarboxylic acid	44.36-58.46	52.21571±5.547458	55.02 -47.23	51.25± 3.901013	15.72 - 36.25	26.345± 8.426294	36.28
Phenol	0 – 22.83	6.902371±12.96714	5.85 - 2.55	3.963333± 1.525199	4.63 - 2.28	4.015 ± 1.157022	9.02
N – Dodecyl acetate	0 – 4.06	1.447143±1.548911	-		-		0.34
Heptanedioic acid	-	-	-		1.68	0.42 ± 0.84	1.04
Hexadecanoic acid	0 – 5.16	2.348571±2.17466	13.15 - 3.70	5.616667± 6.781286	13.99 - 4.87	8.18 ± 3.994221	8.99
7, 9 - di – tert – butyl	0 – 7.26	4.071429±2.420161	10.09 - 2.01	5.14± 4.336577	4.33 - 1.98	3.1625± 1.112606	2.09
Octadecaenoic acid	0 -10.22	4.721429±4.487458	5.57 -2.9	3.896667± 1.457955	22.28 - 1.63	6.8875 ± 10.30129	25.53
Oxiraneoctanoic acid	-	-	-		0.48	0.12 ± 0.24	0.74
Octadecenamide	2.55 -14.38	10.89286±3.91783	16.07 - 6.72	10.37± 5.00075	33.29 - 4.2	17.3125 ± 14.2278	15.98
Octadecenoic acid	0 -16.09	8.99 ±5.275535	23.73 - 9.11	17.95 ± 7.775526	51.05 - 18.84	30.975 ± 13.96715	-
Benzen ethanol	-		-		8.57 -2.37	2.735 ± 4.047258	-
Benzoic acid	-		-		0.77	0.1925 ± 0.385	-
9 - Eicosene	0 - 1.38	0.197143 ±0.521591	-		1.04	0.26 ± 0.52	-
Octadecanol	-		-	-	0.68	0.17 ±0.34	-
Nonanoic acid	0 - 0.58	0.082857± 0.219219	0.56	0.186667 ±0.323316	-		-
Tridecane	-		0.51	0.17 ± 0.294449	-		-
1-Pentadecanol	-		0 - 0.25	0.163333 ± 0.141539	-		-
Methyl ricinolate	2.40-4.39	1.444286 ±1.890889	0 - 1.82	0.99 ± 0.920489	-		-
Oxirane, hexadecyl	0 - 1.53	0.218571 ±0.578286	-		-		-
Nonadecene	1.65 -0.88	0.361429 ±0.65606	-		-		-
Dodecanol	0 - 0.57	0.081429 ±0.21544	-		-		-
1–Hexadecene	0 - 0.87	0.124286 ±0.328829	-		-		-
Cyclopropane carboxylic acid	0 - 0.97	0.138571 ±0.366626	-		-		-

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