PLANT CRUDE EXTRACTS COULD BE THE SOLUTION: EXTRACTS SHOWING *IN VIVO* ANTITUMORIGENIC ACTIVITY

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ABSTRACT

Screening active compounds from plants lead to discover new medicinal drugs which have efficient protection and treatment roles against various diseases including cancer. In our study, extracts from different plants represent seeds of: *Gossypium barbadense*, *Ricinus communis*, *Sesamum indicum, Nigella sativa, Vinca rosea* and *Melia azedarah*; fruits of: *Xanthium occidental*; flowers of: *Atriplex nummularia*; barks of: *Cinnamomum zeylanicum*; latex of: *Ficus carica* and rhizomes of: *Curcuma longa* and *Zingiber officinale* were tested *in vivo* using three subsequent bioassays: the BST (Brine Shrimp Toxicity bioassay), AWD (Agar well diffusion antimicrobial bioassay) and *At*PDT (*Agrobacterium tumefaciens* Potato Disc Tumor bioassay). AWD technique omitted any extracts have antimicrobial activities while BST omitted any extract did not has physiological activity and determined the various LC_{50} of each plant extract. For the first time, using a range of concentrations in the *At*PDT modified protocol allowed the detection of tumor promotion caused by extract represented by *A. nummularia*. Using cluster analysis leads to classifying the different plant extracts activities to six groups regarding to their toxicity, antitumor activities and both of them. The extracts from edible plants represent 50% of the first and the second group which have the highest antitumor activities represented in *F. caraica* (group 1) and *C. longa* (group 2) as well as the non-edible plant extracts of *Gossypium barbadense* and *Ricinus communis.* A comparison study between the edible and herbaceous plants different extracts for their antitumor activities was performed. We recommended using the modified protocols used in this study for investigating more plants and using crude plant extracts which have antitumor activities in cancer treatment. Edible plants, which show *in vivo* antitumor activities, are recommended as save sources for antitumor compounds.

Keywords: Potato disc, *Agrobacterium tumefaciens*, antitumor, brine shrimp, probit analysis.

INTRODUCTION

'Cancer' is a general term applied to malignant diseases characterized by rapid and uncontrolled abnormal cells formation which may mass together to form a growth or proliferate throughout the body, and it may progress until it causes death.

Scientists are interested in investigating medicinal plants which are commonly used by public and derived from folklore or anecdotal information (Helton, 1996; Mail *et al*., 1989).

Dubick 1986 reported that the medical use of herbs is deeply rooted in human history and folklore, and incorporated into the historical medicine of virtually all human cultures. He describe the history of Gineseng and Garlic as two famous plants widely used –till now- in traditional medicine and proved to have many active constituents (Dubick, 1986).

Some famous selected examples used to represent the importance of those plants based on human observation, trial and error, religious advices and from various generations' accumulated experiences, which should never neglected or classified as unscientifically based treatment. The medicinal plants derived from folklore are huge, *Vinca rosea* (Sun and Zeng, 2005), *Podophyllum peltatum* (Gordaliza *et al*., 1994) and *Taxus spp* (Wani *et al*., 1980) are selected examples. These plants and many others lead to discover important drugs including vincristine, vinblastine, podophyllotoxin, 10-hydroxy-camptothecin and Taxol (Wani *et al*., 1980; Coker *et al*., 2003).

For *in vivo* screening antitumor compounds expensive cell lines were used like the 3PS (P388) (methylcholanthrene-induced) leukemic mouse assay and the *in vitro* screening for 9KB (human nasopharyngeal carcinoma) cytotoxicity (Jackson *et al*., 1984; Wall and Wani, 1977).

Crown gall is a plant neoplastic disease induced by the gram-negative bacterium *A. tumefaciens* (Kahl and Schell, 1982; Lippincott and Lippincott, 1975). *A. tumefaciens* cause series plant infections with more than 60 dicotyledon families and many gymnosperms lead to great damages (Lippincott and Lippincott, 1975). The *A. tumefaciens* infection symptoms resemble tumor in mammalian cells. The tumor formation starts when

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Table 1: Effect of crude plant extract on Brine shrimp toxicity bioassay (expressed as % Mortality)

No.*	Plant species name	Family	Plant part	Type of extract	Yield (mg/ ml)	Color o the extract	Extract concentration (µg/ml)						
							5§	100	250	500	750§	1000	1500§
							% of Mortality++						
1	*Curcuma longa*	Zingibera-ceae	Rhizome	Hexane#	-ve	-							
2				Ethanol	57.5	Black brown	25	74	76	90			
3	*Zingiber officinala*	Zingibera-ceae	Rhizome	Hexane	37.5	Faint	50	85	90	100		100	
4				Ethanol	55.16	Faint brown		66	90	100		100	
5	*Sesamum indicum*	Pedaliaceae	Seed	Hexane**	421.8	Oily		0		11		8	9
6				Ethanol	8.75	Reddish yellow	30	53	71	80		90	
7	*Cinamomum zeylanicum*	Lauraceae	Bark	Hexane#	-ve	-							
8				Ethanol	37.5	Brown	8	17	35	54	60	68	
9	*Ficus carica*	Moraceae	Latex	Acetone	126.5	Reddish yellow		39	50	58		70	
10	*Atriplex nummularia*	Chenopo-diaceae	Flower	Hexane**	3.0	Yellowis	10	20	20	22		22	
11				Ethanol	60.0	Yellowis h green	24	50	77	98			
12	*Melia azedarah*	Meliaceae	Seed	Hexane**	34.78	Oily	9	12.5		11		16	
13				Ethanol**	20.0	Reddish yellow	27	45	77	11		16	
14	*Xanthium occidental*	Compositae	Fruit	Hexane**	86	Oily		13	8	12.5		10	
15				Ethanol	13.6	Green		30	50	58		70	
16	*Ricinus communis*	Euphor-biaceae	Seed	Hexane**	500	Oily	10	10		20		16.5	
17				Ethanol	34.9	Gray	12	28	46	61	77	79	
18	*Nigella sativa*	Ranuncu-laceae	Seed	Hexane**	350	Yellowis		0	0	0		8	
19				Ethanol	82.25	Yellowis h green		10	30	57		75	
20	*Gossypium barbadens*	Malvaceae	Seed	Petroleum ether**	177	Oily yellow	0	9	12		15	18	
21				Diethyl ether	33	Pink deep	9	27	46		68		
22	*Vinca rosea*	Apocyana-ceae	Seed	Hexane**	107.52	Yellowis		11	12	12		19	50
23				Ethanol	177.6	Black brown		10	22	40		51	

++ Thirteen plant extract showing LC_{50} ≥1000, ** Nine extract showing LC_{50} > 1000 µg/ml,* Total number of plant extracts used in this study are twenty-one extracts out of twelve plants., #Two plant did not give extract with those solvent., § Concentrations used with particular plants extracts to give more precise statistical profile.

bacterial cell transfer part of the Ti (tumor-inducing) plasmid to the infected plant cell genome (Lippicott and Lippicott 1975; Anand and Herberleim 1977).

The validity of potato disc bioassay predicted on the observation that certain tumorigenic mechanisms are similar in plants and animals like multiplying rapidly without apoptosis (Braun, 1972).

Several modifications on the original Potato disc antitumor protocol as described by Favus *et al*., (1977) have been performed by Galsky and Wilsey, (1980) and Galsky *et al*., (1981a,b) which show that crown gall tumor can be inhibited on potato (*Solanum tuberosum* L.) tubers discs with apparent correlation with compounds and extracts derived from plants origen known to be active with 3PS (*in vivo*, murine leukemia). *At*PDT has been used to evaluate the ethanol and hexane extracts of seeds of 41 Euphorbiaceae species and inhibition 20% or more of the tumors is considered significant (Ferrigni *et al*., 1982, Ferrigni and Mchaughlin 1984). Coker *et al*., (2003) proved that camptothecin, palitaxel, vinblastine, vincristine and podophyllin have a significant inhibitory effect on Crown gall induced tumour. Potato disc bioassay proved to be critical in investigating many new plants constituents (Das *et al*., 2007).

Brine Shrimp (*Artemia salina* L.) bioassay considered as a preliminary screening for the presence of antitumor compounds and used to determine the plant extract toxicity (Meyer *et al*., 1982). Using BS larvae, pharmacognosists and natural product chemists were able to detect and isolate plant constituents and active compounds with a variety of pharmaceutical activities

Table 2: Inhibition of *A. tumefaciens* exposed to various plant extracts using agar well diffusion technique at different concentrations dose

Plant Name	Solvent name	Concentration of plant extract (μg/ml)							
		100		250		500		1000	
		y	AU	y	AU	y	AU	y	AU
Curcuma longa	Ethanol	0	0	0	0	0	0	0	0
Zingiber officinala	Hexane	1	1.7	1.1	1.8	1.2	2	1.5	2.5
	Ethanol	0	0	0	0	0	0	0	0
Sesamum indicum	Ethanol	0	0	0	0	0	0	0	0
Atriplex nummularia	Ethanol	0	0	0	0	0	0	0	0
Melia azedarah	Ethanol	0	0	0	1.7	1.1	1.8	1.6	2.7
Xanthium occidental	Ethanol	0	0	0	0	0	0	0	0
Ficus carica	Acetone	0	0	0	0	0	0	0	0
Ricinus communis	Ethanol	0	0	0	0	0	0	0	0
Nigella sativa	Ethanol	0	0	0	0	0	0	0	1.7
Cinamomum zeylanicum	Ethanol	0	0	0	0	0	0	0	0
Gossypium barbadens	Diethyl ether	0	0	0	0	0	0	0	0
Vinca rosea	Ethanol	0	0	0	0	0	0	0	0

y = diameter of inhibition zone (cm); AU=y/x µl of plant extract where x =diameter of well (6mm)

(Alali *et al*., 2006; Kazmi *et al*., 1991). BS considered rapid, inexpensive, in-house bioassay for screening and fractionation monitoring of physiologically active plant extracts (Jayasuriya *et al*., 1989). Brine shrimp utilized previously in various bioassay systems (Meyer *et al*., 1982; Ratnayake *et al*., 1992). Among these applications, organophosphates (Venkatewara *et al*., 2007), mycotoxins (Harwing and Scott 1971), anaesthetics (Robinson *et al*., 1965), morphine-like compounds (Granada *et al*., 1976), antibacterial and antifungal (Sanchez *et al*., 1993; Chohan *et al*., 2005) and active compounds from marine environments (El-Masry *et al*., 1995). According to Meyer *et al*. (1982) several extracts derived from naturall products which had $LC_{50} \leq 1000$ µg/ml using BS bioassay were known to contain physiological active principles (Meyer *et al*., 1982).

BS bioassay can be used as preliminary *in vitro* antitumor assay while it has positive correlation with 9KB (human nasopharyngeal carcinoma) citotoxicity and 3PS (P388) (*in vivo* murine Leukemia) and can substitute both of them (Ferrigni *et al*., 1982; Ferrigni and Mchaughlin, 1984; McLaughlin and Roger, 1998).
AWD technique is another supported experiment which can detect any antimicrobial activity against *A.* tumefaciens (Tagg and Megiven, 1971; Stainer, *et al*., 1986).

Our study aims to screening plants different crud extracts for their antitumor activities, optimize the BS toxicity test and *At*PDT antitumor bioassay as well as the use of AWD test to improve the antitumor screening protocol and put them in correct subsequent steps to avoid any unexpected negative results. Keeping the main future of those bioassays as simple, inexpensive, and reliable is the main guideline in this study.

MATERIALS AND METHODS

Collection of plants materials

The plants different materials used in this investigation were collected from the agricultural station of Abeese, Alexandria, Egypt, except *M. azedarah, C. zeylanicum*, *C. longa* and *Z. officinale*, which were purchased from local commercial shop. The plants different materials were dried in fresh air then grounded to a powder using mechanical mortar. Plants different materials preserved routinely by storing at -20°C.

Preparation of extracts

Plants different crude extracts were prepared according to Ferrigni *et al*. 1982. Plant different parts were used in this study including: Seeds of *N. sativa* [Ranunculaceae]; (Rooney and Ryan, 2005) *S. indicum* [Pedaliaceae]; (Xu *et al*., 2003) *R. communis* [Euphorbiaceae] (Lin and Liu, 1986); *Catharanthus roseus* [*V. rosea*] [Apocynaceae] (Landini *et al*., 2001; Duffin, 2000); *M. azedarah* [Meliaceae] (Zhou *et al*., 2005) and G. *barbadense* [Malvaceae] (Chang *et al*., 1993); fruits of *X. occidental* [Compositae] (Khafagy and Metwally, 1971; Awney *et al*., 1997); Barks of *C. zeylanicum* [Lauraceae] (Aweny *et al*., 1997); rhizomes of *Z. officinale* [Zingiberaceae] (Shukla and Singh, 2007; Miyoshi *et al*., 2003) and *C. longa* [Zingiberaceae] (Surh and Chun 2007; Ammon and Wahi 1991); flowers of *A. nummularia* [Chenopodiaceae] (Christensen and Omar 1985) and latex of *F. carica* [Moraceae] (Wang and Ma, 2005).

Table 3: Screening of different plant extracts for their activity to inhibit tumor formation using potato disc bioassay; result are represented as a number of inhibited tumor.

Plant Name[§]	Type of solvent	Extract concentration (µg/ml)											
		100			250			500			1000		
		Exp.1	Exp2	Exp3	Exp.1	Exp2	Exp3	Exp.1	Exp2	Exp3	Exp.1	Exp2	Exp3
Curcuma longa	Ethanol	16± 1.69	12± 1.1	12± 1.49	16± 1.37	11± 1.41	12± 2.62	16± 2.33	14± 1.6	13± 1.24	19± 2.16	15± 0.94	13± 1.97
Zingiber officinala	Hexane	11± 3.35*	12± 3.28*	10± 2.82*	14± 1.34*	14± 1.34*	14± 1.34*	21± 1.37*	20± 1.6*	21± 1.46*	24± 0.47*	24± 0.47	24± 0.47*
	Ethanol	19± 2.26	21± 1.7	20± 2.26	21± 2.16	22± 0.68	21± 0.76	22± 1.73	23± 0.76	21± 1.67	19± 2.92	23± 0.76	23± 0.76
Sesamum indicum	Ethanol	4± 1.57	3± 2.35	4± 1.21	7± 2.7	5± 2.42	7± 2.92	17± 1.46	18± 1.41	18± 1.41	18± 1.1	18± 1.06	18± 1.06
Cinamomum zeylanicum	Ethanol	2± 2.3	2± 2.3	1± 1.86	9± 1.57	8± 2.92	9± 1.5	13± 1.77	13± 2.08	13± 1.77	13± 2.4	12± 1.59	13± 1.77
Ficus carica	Acetone	13± 1.24	10± 1.49	9± 2.69	15± 1.82	12± 1.14	13± 1.06	18± 2.74	20± 0.81	20± 0.94	21± 1.95	21± 2.06	22± 0.89
Atriplex nummularia	Ethanol	11± 1.1	10± 1.37	10± 1.24	7± 2.13	5± 1.5	4± 1.06	5± 1.77	3± 1.06	3± 1.06	2± 3.54	2± 3.54	3± 2.03
Melia azedarah	Ethanol	5± 2.11	7± 2.05	5± 2.11	9± 2.49*	9± 2.29*	9± 2.49*	10± 1.89*	10± 1.89*	10± 2.11*	12± 2.91*	13± 2.03*	13± 3.03*
Xanthium occidental	Ethanol	8± 1.89	7± 1.06	8± 1.89	14± 0.74	11± 2.29	13± 1.7	15± 0.74	14± 0.47	15± 1.41	16± 1	15± 1.67	15± 1.58
Ricinus communis	Ethanol	10± 5.45	10± 4.18	8± 4.22	10± 3.38	11± 3.57	13± .78	12± 4.67	10± 4.6	13± 4.48	19± 2.05	21± 0.57	20± 1.59
Nigella sativa	Ethanol	10± 2.4	10± 2.13	9± 2.05	9± 2.42	13± 1.57	11± 3.49	13± 1.77	13± 2.79	12± 1.67	13± 3.59*	18± 2.74*	13± 3.4*
Gossypium barbadens	Diethyl ether	19± 0.94	19± 0.68	19± 1.1	20± 1.1	20± 1.1	19± 1.1	20± 0.94	22± 0.94	22± 0.94	22± 1.11	23± 1.5	22± 1.97
Vinca rosea	Ethanol	10± 3.49	10± 3.4	8± 2.28	12± 1.49	12± 1.49	13.6± 1.5	13± 0.94	12± 1.69	13± 0.94	17± 1	14± 1.52	14± 1.49

[§]Control estimated to be 25 Crown galls/Potato disc [inhibition of 5 Crown galls= 20% inhibition and consider being significant Ferrigni *et al*., (1982) and Ferrigni and Mchaughlin (1984)

*Results have been ignored due to the presence of antimicrobial activity at these concentrations.

Extraction process

One hundred gram of each plant dried material (after complete grinding to fine powdered particles) was weighted and defatted using 250 ml hexane in a 1L Erlenmeyer flask then agitated for 2 hr, at 25°C using a reciprocal shaker at 250 rpm (Wrist Action shaker Model 75, Burrel Corporation Pittsburgh, PA, USA). This step was repeated several times till the extraction was complete (by test one drop of the extract on watch glass; left the solvent to evaporate and test the transparency of the glass surface). The total extracted volume of each plant subjected to filtration using Whatman No.1 filter paper.

The final unextracted portion was air dried in an exposed 20 cm glass Petri dish under aseptic condition. After complete drying, the same process was repeated -as above- using absolute ethanol. *F. carica* (Moraceae) latex was extracted using acetone and *G. barbadense* was defatted using petroleum ether followed by diethyl ether extraction.

Different extracts were concentrated using rotatory evaporator (labo-ratoriums-Technic AGCH-9230 Flawil/ Schweiz) at 40°C. The concentrated extracts were collected and dried using vacuum drier. Each extract was then transferred to an l0 ml weighed, small and clean glass vial and the crude extract weight was determined. All the extracted materials were preserved at -20°C.

Bioassays

Three subsequent bioassays were carried out to determine the biological activity of the plants different extracts.

1-BST assay

Artemia saline Letch., encysted eggs (10 mg) [collected freshly from Port Said station, Port Said, Egypt] were incubated in 5 ml distilled water for 5 min to activate hatching and then transferred in 300 ml of seawater under artificial light at 28°C, pH 7-8. After incubation for 24 hr, nauplii were collected with Pasteur pipette and kept for an additional 24 hr under the same conditions to reach the metanauplii stage.

Preparation of extracts for toxicity bioassay was carried out following the procedure of Meyer *et al*., 1982 with modification. The percentage of mortality for each concentration was determined from the number of dead and live nauplii. In case where death occurred in the control, the data were corrected using the following formula:

% death = [(dead test- dead control)/10] X 100

One ml stock solutions representing 6.66, 16.66, 33.33

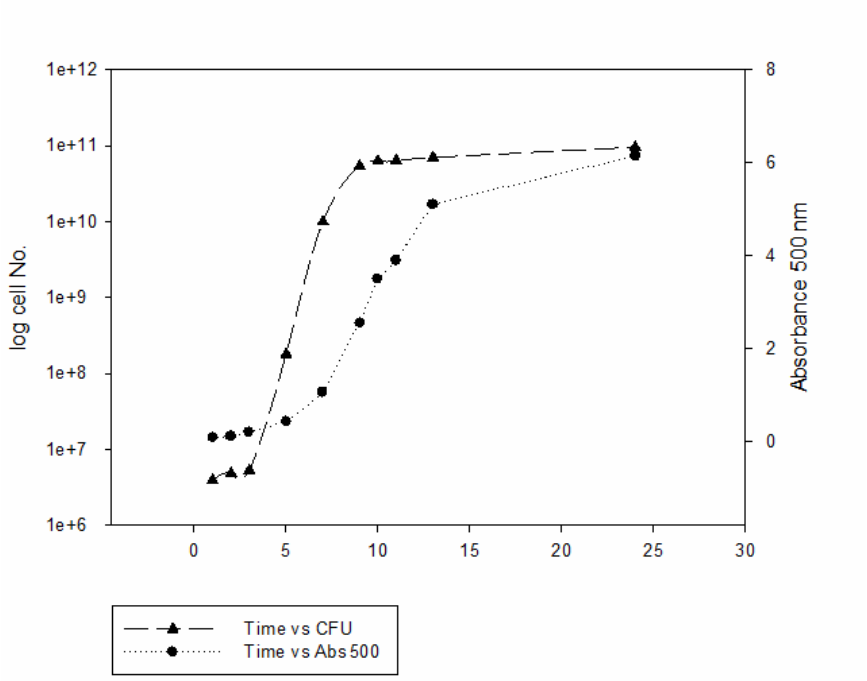


Fig. 1: *A. tumefaciens* growth curve represented as CFU and OD_{500}.

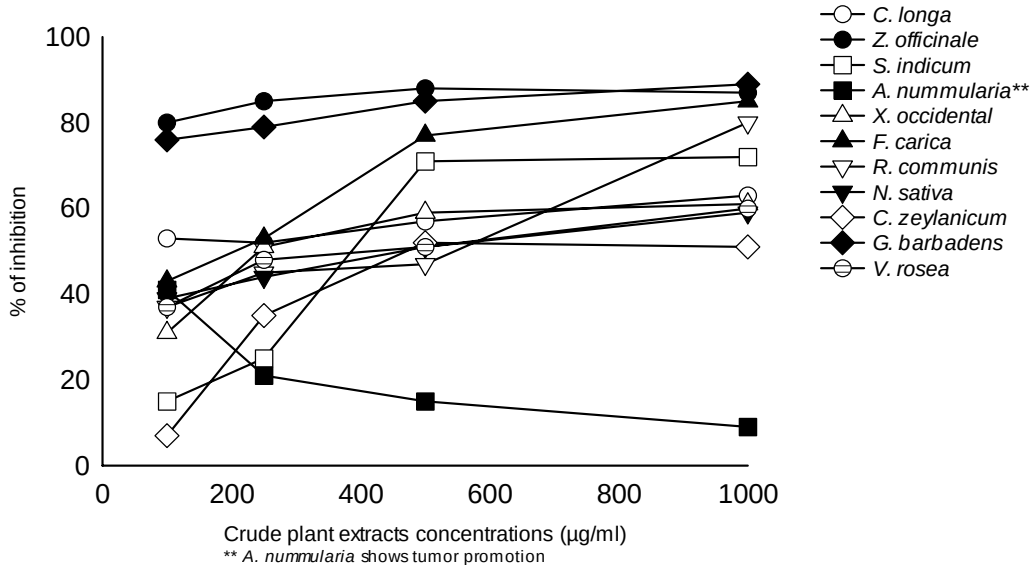


Fig. 2: Crown galls inhibition % for different crude plant extracts using *At*PDT bioassay.

and 66.66 $mg_{plant\ extract}/ml_{DMSO}$ were prepared for each plant extract and sterilized using bacterial membrane filter (0.22 µm diameter). Seventy five µl from each stock solution completed to five ml sterilized sea water (using bacterial membrane filter 0.22 µm) -in suitable glass container- to represent concentrations 100, 250, 500 and 1000 $\mu g_{plant\ extract}/ml_{sea\ water}$ for each plant extract then mixed well using vortex. Three replicas of each concentration were used. The blank vials were prepared using filter sterilized seawater. The control vials were prepared using 25 µl DMSO. Ten of 48 hr old nauplii were transferred to each vial, and left for 24 hr incubation period at 28°C. The treated nauplii were counted and recorded as live or dead.

A. tumefaciens culture

A. tumefaciens strain was routinely cultured on high nutrient ager (NA) media, consisting of 5 g/l peptone, 3 g/l beef extract, 5 g/l glucose, 15 g/l agar (pH 7.0). Culture for experimental work was grown in the

equivalent liquid medium (NB) at 28 °C on a rotary shaker at 150 rpm.

A. tumefaciens growth curve

Counts of viable bacteria was determined as CFU according to Stainer *et al*. (1986). An absorbance calibration curve was determined during the growth of the bacterial cells using a Perkin-Elmer UV/VS spectro-photometer.

AWD technique for screening antimicrobial activity

Antimicrobial activity was tested by the AWD technique for determination the antimicrobial effect of plants different extracts on *A. tumefaciens*. In this method, Petri dishes were supplied with a deep NB medium inoculated previously with 1% of test bacterium. Four wells were punched out of the agar, by using a clean sterile cork borer (6 mm in diameter). The base of each hole was sealed with a drop of melted sterile water agar (15 g agar per liter H_2O) using sterile Pasteur pipette. For each plant

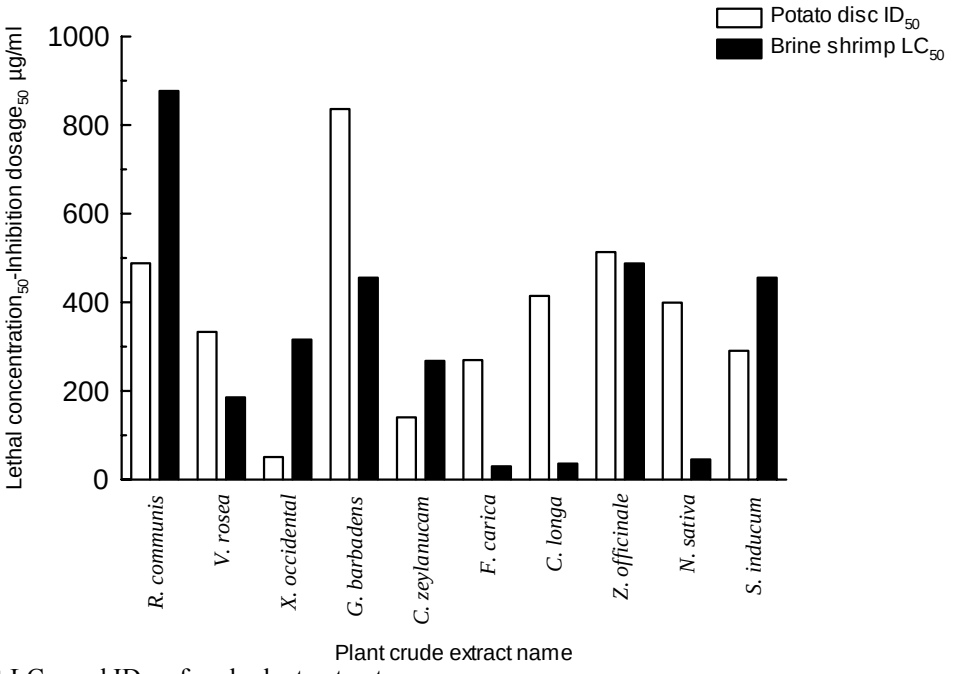


Fig. 3: Different LC_{50} and ID_{50} of each plant extract

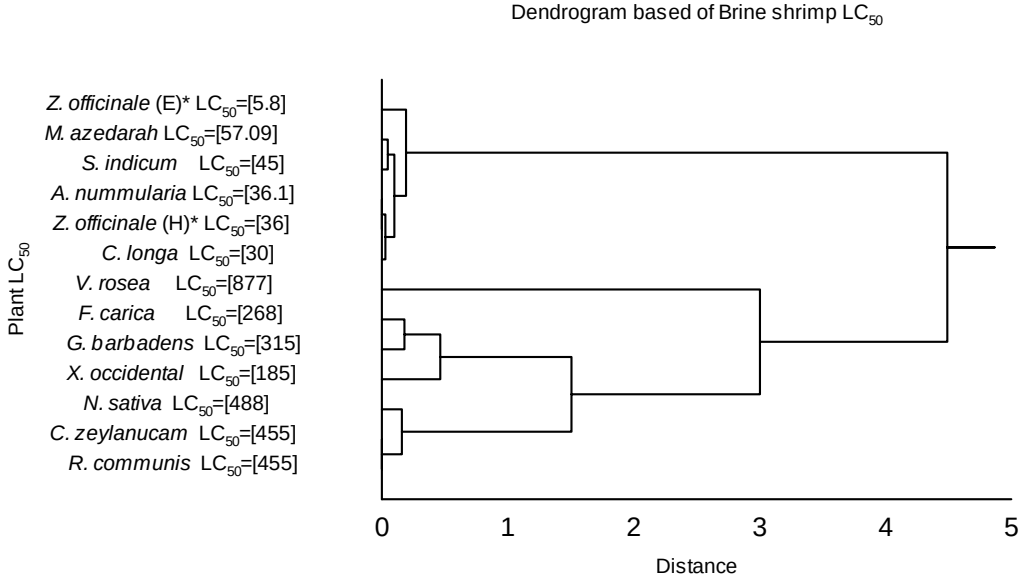


Fig. 4: Cluster analysis of different crude plant extracts has $LC_{50} < 1000$

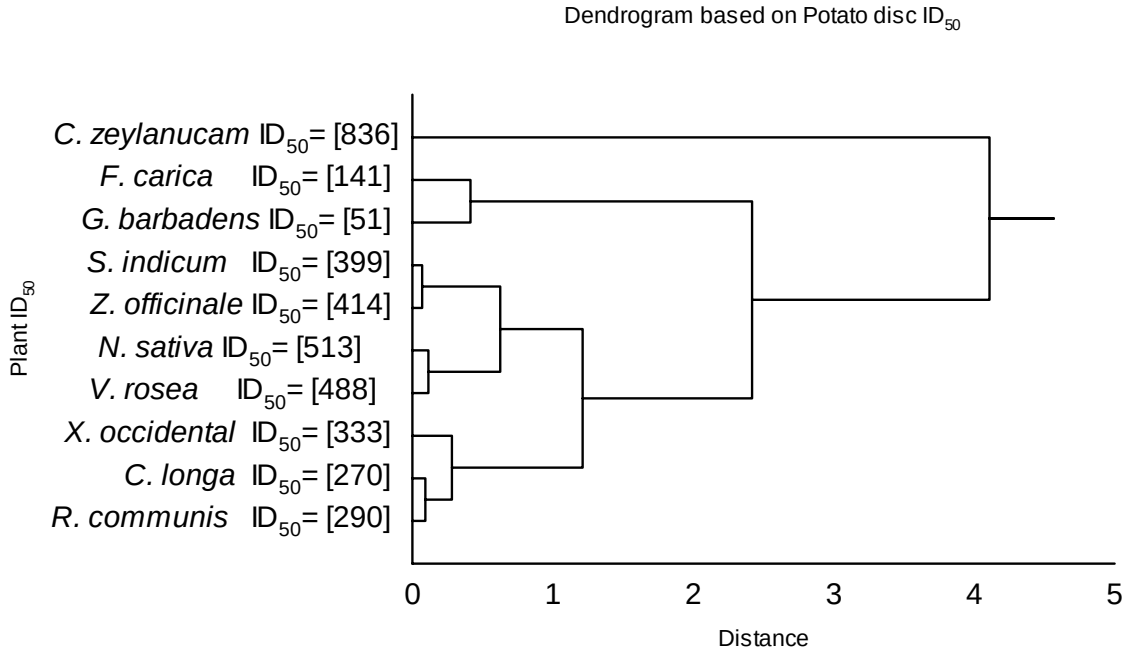


Fig. 5: Cluster analysis of different crude plant extracts has $ID_{50} < 1000$

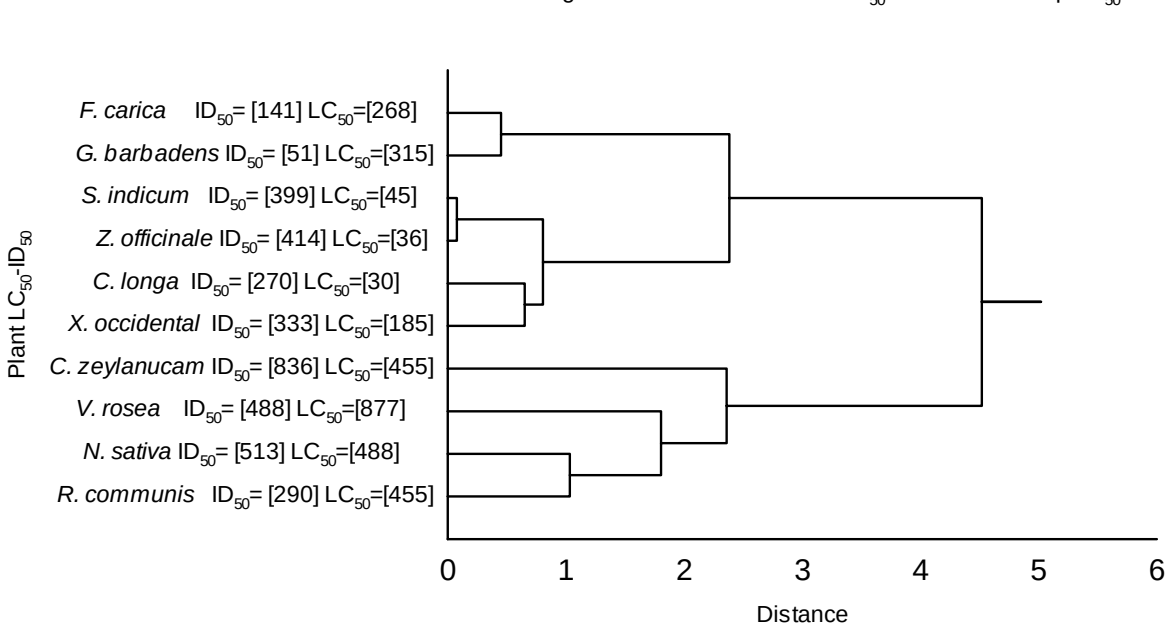


Fig. 6: Cluster analysis of different crude plant extracts has LC_{50} and $ID_{50} < 1000$

extract four concentrations representing 100, 250, 500 and 1000 µg/ml were used to determine their antimicrobial effect on the *A. tumefaciens*. Each concentration was pipetted into one well, then the plates were incubated at 4°C for 4 hr to allow the extracts to diffuse into the medium. The plates were then incubated at 28°C for 24 hr after the incubation period, the square radius of the clear zone around each well (y^2) were measured and divided over the square well radii (x^2) to obtain absolute unit (AU) for the inhibition zone (Tagg and Mcgiven 1971).

3-AtPDT bioassay

This technique was carried out according to the original method used by Favus *et al*. (1977) and modified by Galsky and Wilsey, (1980); Ferrigni *et al*. (1982) and Ferrigni and Mchaughlin (1984) with further

modifications performed in our study.

***A. tumefaciens*-crude extract mixture preparation**

Stock solutions representing 15.625 mg of each plant extract were dissolved in 1 ml DMSO and then filtered through bacterial membrane sterile Millipore filter (0.22 µm), and received into a 2 ml sterile Ependorf tube. Volumes of 6.4, 16, 32 and 64 µl from each stock solution were added to 1.5 sterile Ependorf tubes respectively and then adjusted to a volume of 500 µl using sterile distilled water and 500 µl of *A. tumefaciens* broth culture (10^9 cells/ml) were transferred to each of the previous tubes, and then mixed well by gentle vortexing. The different extracts at final concentration represent 100, 250, 500 and 1000 µg/ml and calculated by the general formula:

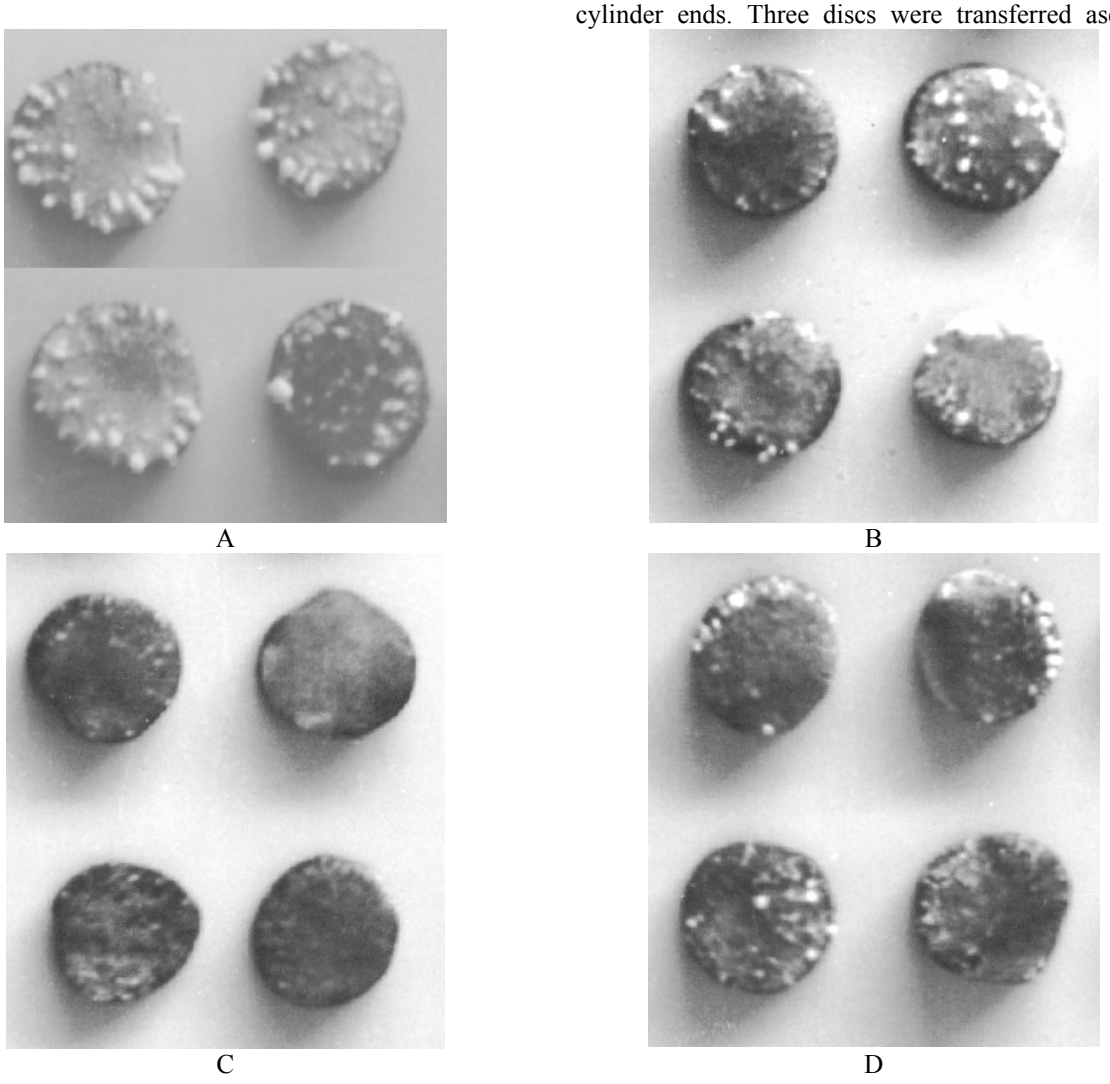
Plant final concentration (µg/µl) = [Used stock (µl) X Stock concentration (µg)]/ Final volume (µl)

The DMSO in final concentration was not more than 12.5%.

Control was made by adding 50 µl of filtered DMSO to 450 µl of sterile distilled water, and then mixed with 500 µl *A. tumefaciens* broth culture (10^9 cells/ml).

Preparation of potato discs

Whole tubers of potato of moderate size were surface disinfected by immersing in 0.5% sodium dichloro-isocyannurate solution for 30 min, washed twice in sterilized distilled water and left to air dry for further 20 min, under aseptic conditions. The two ends of the potato tuber were cut using a sterilized sharp cutter. Potato cylinder of 1.5 cm in diameter was obtained using a sterilized cork borer and then the cylinder was cut into discs of 0.5 cm thickness, after discarding 1 cm from both cylinder ends. Three discs were transferred aseptically



A B C D

Fig. 7: Different Crown galls formed during *At*PDT bioassay A: Control B: Moderate inhibition C: High inhibition D: Incorrect mixing and spreading *A. tumefaciens* on the surface of Potato disc leads to inhibition in the middle and tumor formation on the edge of the Potato discs.

onto the surface of autoclaved water agar in 9 cm sterilized Petri dish, where the discs have to be inoculated within 30 min, with the plant extract-bacterium mixture. Fifty µl of each plant extract-bacterium mixture of each concentration was used to inoculate each potato disc. Each experiment included three plates, each plate contained three potato discs, and the nine discs were inoculated with one concentration of the previous mixture and placed onto the surface of sterilized water agar. Each experiment repeated three times.

The Petri dishes were incubated at 25°C for 15-20 days. After the incubation period, the tumors were counted with the aid of binocular after staining the surface of the discs with Lygol's solution (I_2 3 g/l + KI 30g/l).

STATISTICAL ANALYSIS

Data were collected and entered to the personal computer and the LC_{50} and ED_{50} were determined for BS and *At*PDT bioassays respectively, using Probit analysis method which generated by Priprobit ver., 1.63 software. Probit analysis estimates the log doses by applying a distribution model to the observed quantal data, so a median effective dose LC_{50} or ID_{50} where 50% of organisms in population respond can estimate relative potencies (Rho), between preparations.

Hierarchical cluster analysis methods performed using Kyplot (version 2.0 beta) statistical package software to analyze LC_{50} and ID_{50} derived from the result of BS assay, *At*PDT assay, and both together. The Probit analysis for LC_{50} and ID_{50} were further analyses with two way ANOVA statistical method using Kyplot statistical package to determine the significant difference between the treatments within each of experiment and between both of them.

RESULTS AND DISCUSSION

BST assay

BS eggs are readily available at low cost and used as a general toxicity test. Out of twenty-one extracts tested by BS bioassay (table 1) and applied to Probit analysis statistical method to determine their LC_{50}; thirteen extracts showing $LC_{50} \leq 1000$ µg/ml. The LC_{50} general range of those thirteen extracts was between 5.8 and 877 µg/ml. The extracts under investigation showed different toxicity levels increased proportionally with the elevation of concentration levels (table 1). The other extracts which did not show $LC_{50} \leq 1000$ µg/ml were considered being insignificant as described by Ferrigni and Mchaughlin, (1984) and was omitted from any further investigations.

Screening for antimicrobial activity

The thirteen extracts (showing $LC_{50} \leq 1000$ µg/ml with BST bioassay) were further investigated for their antimicrobial activities against the growth of *A. tumeficians*. It was found that those extracts at all doses used in this study have no antibacterial inhibitory effect against the growth of *A. tumefaciens*, except with *Z. officinale* hexane extract at all doses; *M. azedarah* ethanol extract at 250, 500 and 1000 µg/ml and *N. sativa* ethanol extract at 1000 µg/ml (table 2).

Identification of plants different extracts for their antitumorigenic activity.

Results showed that the thirteen extracts under investigation exhibited variable antitumoregenic effect at different screening doses (table 3 and figs. 2 and 3).

Ten extracts out of the tested thirteen extracts, displayed ≥ 20% (25 Crown galls considered as 100%; the inhibition of 5 galls considered as 20% inhibition) inhibition for Crown gall tumors (table 3).

A. nummularia for the first time identified as tumor promotion when the concentration was > 250 µg/ml (table 3 and fig. 2).

Extracts which show antitumor activity at concentrations proved to have antimicrobial activity against *A. tumefaciens* (Table 1) were considered as negative results (table 2).

Using bacterial cells growing at loge phase (10^9 cells/ml-Ffg. 1) proved to be efficient in Crown galls induction (fig. 7). The Crown galls can be easily distinguished from normal tissue on potato discs (fig. 7).

For evaluating the antitumorgenic effect of crude plant extracts on Potato disc, Probit analysis was used to determine the ID_{50} of each plant extract (table 4).

Table 4: The LC_{50} and ID_{50} of BST and *At*PDT bioassays with different plant extracts

Plant Name	Probit Analysis	
	ID_{50}	LC_{50}
G. barbadens	51	315.8
F. carica	140.6	268.119
C. longa	269.5	30.16
R. communis	290.2	455.35
X. occidental	333.24	185.27
S. inducum	399	45.4
Z. officinale	414.37	36.12
V. rosea	488.12	877
N. sativa	513.3	487.8
C. zeylanucam	836.1	455.3

Plants having $ID_{50} \leq 1000$ µg/ml considered being significant which agree with Ferrigni *et al*. (1982) and Ferrigni and Mchaughlin (1984) statement consider that

Table 5: ANOVA table

Factor	SS	Df	Ms	F(cal)		P(F<= F(cal))	F(0.05)
A (Columns)	50256.16	1	50256.16	9600.821	*** (P<=0.001)	2.87E-49	4.084746
B (Rows)	2100297	9	233366.3	44581.76	*** (P<=0.001)	4.18E-77	2.124029
AxB (Interaction)	1085968	9	120663.2	23051.21	*** (P<=0.001)	2.23E-71	2.124029
ABR (Total)	3236731	59	-	-	-	-	-

*** Highly significant

plant extract ≥ 20% inhibition being significant (Ferrigni *et al*., 1982; Ferrigni and Mchaughlin 1984).

BS which was reported early for their significant effect on 3PS (Mayer *et al*., 1982) could not detect the presence of tumorginic compounds such as in case of *A. nummularia*. Meanwhile BS is necessary to omitting any extracts have no activity. BS used as an indicator for the presence or absence of active compounds as well as a preliminary toxicity test gives a suitable evaluation for the toxicity level of each crude plant extract.

Applying potato disc bioassay directly will give some wrong results (as proved in this study) based on the antimicrobial activity of extracts against the bacterial strain used in this assay (table 2).

Z. officinale hexane extract has been omitted totally but the data from *M. azedarah* ethanol extract and *N. sativa* were taken in consideration as extracts containing antitumor compounds, while they are inhibited crown gall ≥ 20% (Ferrigni *et al*., 1982; Ferrigni and Mchaughlin 1984) and have LC_{50} ≤ 1000 µg/ml at concentrations (Mayer *et al*., 1982) did not shows antimicrobial activities and shows antitumor activities (tables 2 and 3).

The steps in each bioassay were optimized separately and together in the same time. Part of the optimization based on the date parameter recommended from the previous studies such as the recommendation of using plant extracts showing LC_{50} ≤ 1000 with BS (Mayer *et al*., 1982) and considering that ≥ 20% inhibition using potato disc bioassay as significant result (Ferrigni *et al*., 1982; Ferrigni and Mchaughlin 1984).

Compounds from edible and edible like plant extracts clearly proved very important when they used in their original concentrations as agreed with Awnay *et al*. (1997) and the results in this study (Awnay *et al*., 1997).

In contrast using herbaceous plant extract put some risk factors because of the presence of some harmful compounds like what we proved in this study with *A. nummularia* (Which give performance to tumor at concentrations > 250 µg/ml). Scientists should focus on using edible plant extract for treatment diseases especially cancer. Treating patients have cancer with plant extracts or compounds derived from edible or edible-like plants will decrease the extra-toxicity as well as any risk factors when using non-edible plants, which could kill one type of cancer cells to elevate another.

Using edible plants different extracts is the best way for treatment especially for those plants have been well studied and used regularly by public a fact support a lot and can save many lives annually.

In case of *Z. officinala* hexane extract which has been completely omitted due to their antimicrobial activities at all concentrations as well as the other crude extracts shows the same activities as in table 2, these plants extracts can be used in *At*PDA bioassay after determining the antimicrobial LC_{50} and other related calculations to omitted their antimicrobial effect on *A. tumefaciens* as reported by Zakaria and Amara, (2006) and Amara and Zakaria, (2006) a work can be done in future studies (Zakaria and Amara, 2006; Amara and Zakaria, 2006).

Using Probit analysis programs for calculating the LC_{50} of BS is considered to be tricky because it is the first step to evaluate the plant extract by evaluating it's toxicity, we advice the researcher to use manual Probit paper during experiments which will give early strong indicator about the importance of using more concentrations as in table 1 for manual fit the toxicity line to determine the LC_{50} [data not shown]. For more accurate and unbiased Probit analysis results we applied the computer program Priprobit ver., 1.63.

While the ideal plant extract will be that one which have high LC_{50} (less toxic) and low ID_{50} (high antitumor activity), we use Hierarchical cluster analysis (Nearest Neighbor method) for each of LC_{50} and ID_{50} alone and together to investigate the linkage between different

extracts performance. From ID_{50} cluster analysis extracts are classified to six groups based on their ID_{50} value. The most efficient and highly active crude extracts against tumor located in two groups: group 1 and 2 where, group 1 includes *F. carica* and *G. barbadens* and group 2 include *C. longa* and *R. communis*. The lowest efficient and less active in group 6 include one plant *C. zeylanucam* which has the high ID_{50} value (less active against tumor). Group 3, 4 and 5 present at the middle with close value where Group 3 contain *S. indicum* and *Z. officinale*, Group 4 contain *N. sativa* and *V. rosea* and Group 5 contain *X. occidental* (fig. 5). Fig. 4 represent the cluster analysis of BST LC_{50} data. The cluster analysis of BST group the extracts in groups different from that of *At*PDT which agree with ANOVA result and with the fact that both of toxicity and antitumor activity different in their biological mechanisms. Clustering the data of both of BST and *At*PDT classify the extracts to another group's based on the combination between both of LC_{50} and ID_{50} (fig. 6). By clustering both of LC_{50} and ID_{50} *F. carica* and *G. barbadense* again located in one group as well *S. indicum* and *Z. officinale*. The same thing happened in clustering BST LC_{50} where, *F. carica* and *G. barbadense* located in one group as well *S. indicum* and *Z. officinale* plus *M. azedarah* which are the great evidence about the biological activity and stability of these plants which could be suitable for *in vivo* cancer treatment.

Three edible plant extracts located in group 1 and 2 (When clustering both of ID_{50} and LC_{50}) which is high evidence about the suitability of using extracts from different edible plants in cancer treatment. In other hand *A. nummularia* which is an herbaceous plant show tumor promotion which is a significant indication about the risk in using crude plant extracts of herbaceous plants in tumor treatment without investigation. Table 5 show the results of the two way ANOVA statistical analysis where the data from table 4 were tested using kyplot program to compare both of the BS LC_{50} and *At*PDT ID_{50} to evaluate the data from each experiment alone and in combination. The result show significant differences between BST and *At*PDT values. The ANOVA analysis of the LC_{50} and ID_{50} value of table 4 give significant differences between both of LC_{50} and ID_{50} experiment as well as between plants used in each of them, a clear and a logic result where both experiment are different in their biological aspects as well as each plant crude extract is different from the other in their active compounds constituent (table 5). The ANOVA results agree with the cluster analysis results as well as the logical partition of the data.

Our study concerning the optimization of potato disc bioassay practically to be more accurate for screening antitumorgenic compounds as well as using BS and AWD techniques as a sieve for omitting extracts did not have active compounds or have antimicrobial activities. Using practical methods is more useful for optimizing any experiment instead of using other kind of optimization especially in biological experiments where case-by-case study is important. In our case study *A. nummularia* is clear example about the important of using more than one concentration to investigate the behavior of the tested crude extracts. We recommend using *At*PDT, BST, AWD and Probit analysis to investigate more crude extracts especially in developmental countries where the cost of mammalian tissue culture or experimental animals is high and to support the research focused on discovery of new natural compounds which could help a lot for investigating or discovery new medicinal drugs.

Using edible or edible-like plants have active constituent in our daily life and it could be the best inexpensive choice for protecting us from cancer.

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