

HIGH-THROUGHPUT SCREENING OF 75 EUPHORBACEAE AND MYRTACEAE PLANT EXTRACTS FOR IN-VITRO ANTITUMOR AND PRO-APOPTOTIC ACTIVITIES ON HUMAN TUMOR CELL LINES, AND LETHALITY TO BRINE SHRIMP

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ABSTRACT

Objective: Various approaches are used for discovering cytotoxic anticancer therapeutics from plants. In this study 75 extracts representing 47 plant species belonging to families Euphorbiaceae and Myrtaceae were screened by three in-vitro bioassays, to detect extracts with possible antitumor activity.

Methods: The methanol plant extracts were screened over three bioassays viz., cytotoxicity on HepG2 human hepatocellular carcinoma cell line assessed by MTT method, pro-apoptotic activity on HCT116 human colon carcinoma cell line measured by the increase of M30 neo-epitope apoptosis biomarker through sandwich ELISA, as well as brine shrimp lethality (BSL) bioassay. **Results:** 54 extracts were active in at least one bioassay. 43 extracts (57%) scored cytotoxic on HepG2, compared to 32 (43%) pro-apoptotic extracts in HCT116-M30 assay and 18 (24%) lethal extracts in BSL test. Out of the 43 cytotoxic extracts 15 (20%) possessed pro-apoptotic activity, which may be considered the ideal candidate agents. **Conclusion:** The relatively high number of active extracts detected in the 3 bioassays may be attributed to the selected families being rich in reported bioactivities and folk medicinal uses. The extracts of *Melaleuca leucadendron* L. and *Callistemon rigidus* R.Br. were considered the most promising ($\geq 90\%$ toxicity in HepG2 cytotoxicity and ≥ 3 fold increase in apoptotic background signal at 100 ppm); while the activity of *C. rigidus* is firstly to be recorded here and subsequent investigation for its active compounds is recommended. The three bioassay approach described could be useful in screening large numbers of extracts for detecting antitumor agents.

Keywords: Cytotoxicity, M30 apoptosis, HepG2 human hepatocellular carcinoma, HCT116 human colon carcinoma, Cancer therapeutics drug discovery

INTRODUCTION

Natural products have been considered as a valuable source of drug leads for many years [1]. They have been selected to interact with biological targets [2]. The high degree of their chemical diversity makes them attractive for discovery of new drugs [2]. The testing of their extracts has been widely practiced in the pharmaceutical industry [1].

Many species belonging to family Euphorbiaceae have been used as folk medicinal remedies to treat some skin disorders, diarrhea, dysentery, venereal diseases and as anthelmintic [3, 4]. Some of these species were reported to possess anti-proliferative, and antibacterial activities, as well as an effect against genital herpes (HSV-2) [5, 6]. Many of the species belonging to family Euphorbiaceae are known to be toxic and poisonous [5, 6] The main active principles found in this family are terpenoids [7], where specific diterpenes called phorboids are responsible for its toxicity [5, 6]. Family Myrtaceae also comprises several species that are used in folk medicine, mainly as an anti-diarrheal, antimicrobial, antioxidant, anti-rheumatic, anti-inflammatory and anti-hypercholesterolemic agents. This family is rich in essential oils [8].

Based on the above broad bioactivities of Euphorbiaceae and Myrtaceae, the present study aimed to screen some of their available species growing in Egypt for the following bioactivities: antitumor/cytotoxicity and pro-apoptotic effects on human tumor cell lines, in addition to lethality on brine shrimps. The general purpose is to test an approach for a systematic screening of large number of extracts in the search for therapeutics against tumors.

MATERIALS AND METHODS

Plant material

Specimens of the available and accessible wild and cultivated plants, belonging to families Euphorbiaceae and Myrtaceae, were collected from different localities in Egypt. Voucher specimens of these plants,

viz. 32 Euphorbiaceae species and 15 Myrtaceae (*cf.* Table 1) were prepared and deposited in the Herbarium of the Pharmacognosy Department, National Research Center, Egypt. The taxonomical nomenclature is in accordance with Boulos (1999, 2000, 2002, 2005) [9-12] and Huxley et al. (1992) [13].

Preparation of extracts

The 47 plant species under investigation were separated into their organs which yielded 75 parts (*cf.* Table 1); these were dried in solar ovens at 45°C, powdered and kept in labelled containers. Sufficient weight of each plant powder was exhaustively extracted with methanol. The methanol extracts were filtered, evaporated under vacuum at 35°C, freeze dried and stored at -20°C. The extraction and drying procedures during the preparation of the extracts were done cautiously to prevent de-naturation or artifact formation. The freeze dried plant extracts were deposited at the Extract Bank of the In-Vitro Bioassay Laboratory, National Research Center, Egypt.

In vitro antitumor bioassay on human hepatocellular carcinoma cell line (HepG2 cytotoxicity)

Cell culture—HepG2 cell line was maintained in RPMI-1640, 10% foetal bovine serum at 37°C in 5% CO₂ and 95% humidity. Cells were sub-cultured using trypsin versene 0.15%.

Viability test—After about 24h of seeding 10000 cells per well (in 96-well plates), when cells have reached 60-70% confluence, the medium was changed to serum-free medium containing a final concentration of the extracts of 100 ppm in triplicates. The cells were treated for 24h. 100ppm adriamycin was used as positive control and 0.5% DMSO was used as a negative control. Cell viability was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described by Mosmann 1983 [14].

The equation used for calculation of percentage cytotoxicity: $(1 - (av(x)/(av(NC)))) * 100$

Where: Av: average, X: absorbance of sample well measured at 495nm with reference 690nm, NC: absorbance of negative control measured at 495nm with reference 690nm

This method is feasible for high throughput screening

Pro-Apoptosis bioassay (HCT116-M30 pro-apoptosis) Cell culture—HCT116 colon carcinoma cell line was maintained in McCoy's 5A modified medium, 10% foetal calf serum at 37°C in 5% CO₂ and 95% humidity. Cells were sub-cultured using trypsin versene 0.15%. Cells were checked for mycoplasma using DAPI staining.

Apoptosis induction test—Pro-apoptotic activity was assessed using the M30-apoptosense® kit according to the instruction of the manufacturer (Peviva AB, Bromma, Sweden). Test: Day 1: 10000 cells per well were seeded in a 96-well plate. Day 2: The cells were exposed to 100 ppm extract for 24 h. 40 µM cisplatin was used as positive control. 10 µL of 10% NP 40 were added per well. Lysis of the cells was allowed to occur on a rotatory shaker for 5 minutes at room temperature. Gentle mixing was done by pipetting up and down, taking care not to create air bubbles, then 25 µL of the medium/lysate were added to the well of M30 Coated Microstrips [15, 16] 75 µL of the diluted M30 HRP (horseradish peroxidase) Conjugate solution were added to each well (within 20 min from the addition of the cell lysate). The plate was sealed and incubated on shaker (600rpm) for 4 h. The plate was then washed five times with 250 µL diluted wash solution. 200 µL of TMB (3,3',5,5'-Tetramethylbenzidine) were added to each well and incubation for 20 min in the dark. 50 µL of the stop solution (1.0 M sulphuric acid) were added. Shaking for 5-10 seconds was done to ensure complete mixing. The absorbance was determined after 5 minutes at 450 nm in a microplate reader within 30 min.

Results are expressed as fold increase of the negative control (FINC) background caused by spontaneous apoptosis according to the following formula: $FINC = OD_{450} \text{ (of test extract)} / OD_{450} \text{ (of negative control)}$, Where OD_{450} is the optical density obtained at 450 nm wavelength. 0.5% DMSO was used as negative control. This method is feasible for high throughput screening

Brine shrimp lethality bioassay (BSL)

A solution of instant ocean sea salt was prepared by dissolving 2.86g in 75ml distilled water. 50mg of *Artemia salina* eggs were added in a hatching chamber [17]. The hatching chamber was kept under a fluorescent bulb for 48h for the eggs to hatch into shrimp larvae. 20mg of each extract were dissolved in 2ml methanol, from which

50µl were transferred into vials corresponding to 100 ppm. The methanol was allowed to evaporate to dryness in about 24h at room temperature. 2.5ml of instant ocean solution containing one drop of DMSO were added to each vial and 10 nauplii of *Artemia salina* were added to each vial. The final volume of the solution was adjusted to 5ml with sea salt solution immediately after adding the nauplii. 24 h later, the number of surviving shrimps were counted and recorded [18]. The experiment was done in triplicates. 100ppm of *Annona cherimolia* leaves extract was used as a positive control. 0.5% DMSO was used as negative control.

The equation used for calculation of percentage lethality: Percentage lethality = $[10 - ((S1+S2+S3)/3)] * 100$, Where S1, S2, S3 are the number of viable nauplii in the sample vials.

Determination of LC values

In case of highly active extracts possessing $\geq 90\%$ cytotoxicity on HepG2 cells and those giving $\geq 90\%$ lethality on BSL, five descending concentrations were prepared and tested. The results were used to calculate the LC₅₀ values of each extract using Probit analysis and utilizing the SPSS computer program (SPSS for windows, statistical analysis software package / version 9 / 1989 SPSS Inc., Chicago, USA).

RESULTS

In this investigation an extract was counted as "active" when possessing (scoring) $>50\%$ in HepG2 cytotoxicity, $>50\%$ lethality in BSL, or inducing >1.5 fold increase in the apoptotic signal of the negative control in the HCT116-M30 pro-apoptosis.

The results obtained in the three bioassays are expressed in **Table (1)** and showed that 54 extracts were active in either one or more of the three bioassays. A summary of the distribution of the number of active extracts in the three bioassays is shown in **Figure (1)**.

43 extracts were cytotoxic to HepG2 compared to 32 pro-apoptotic extracts on HCT116-M30 pro-apoptosis and 18 lethal extracts in BSL assay. Only 2 extracts (*viz.* numbers 5 and 9) out of the 18 lethal extracts in BSL, were inactive in the other two bioassays. BSL assay detected 14 active extracts out of the 43 cytotoxic extracts on HepG2.

Highly active extracts, which gave $\geq 90\%$ cytotoxicity or lethality, possessed LC₅₀ values ranged between 10-40 and 41-91 ppm for HepG2 cytotoxicity and BSL, respectively (*cf.* **Tables 2, 3**). This might be interpreted by higher sensitivity of the HepG2 model compared to BSL bioassay.

Table 1: Activity of plant extracts (100 ppm) in three bioassays: HepG2 cytotoxicity, HCT116 pro-apoptosis and brine shrimp lethality

Ext No.	Plant name	Part used	HepG2 Cytotoxicity (%)	HCT116-M30 pro-apoptosis (FINC)	Brine Shrimp Lethality BSL (%)	Ref *
Family Euphorbiaceae						
1	<i>Acalypha wilkesiana</i> Müll. Arg. cv.	L	53.43	0.87	0	40
2	<i>Hoffmannii</i>	Br	3.88	0.82	0	40
3	<i>Andrachne telephioides</i> L.	H	54.80	1.5	10	
4	<i>Breynia nivosa</i> (W.G.Sm.) Small	L	38.73	0.77	17	
5		Br	17.77	0.9	80	
6	<i>Chrozophora oblongifolia</i> (Delile) A. Juss. ex Spreng.	H	40.87	0.98	0	
7	<i>Chrozophora plicata</i> (Vahl) A. Juss. ex Spreng.	H	71.97	1.39	7	
8	<i>Codiaeum variegatum</i> (L.) A.Juss.	L	42.60	0.62	43.3	41
9		Br	12.89	0.7	73.3	41
10	<i>Euphorbia paralias</i> L.	H	0	0.84	0	
11	<i>Euphorbia cotinifolia</i> L.	L, Br	7.42	1.39	13.4	
12	<i>Euphorbia dendroides</i> L.	H	78.70	2.32	13.33	21
13		H	81.27	3.03	54	21
14	<i>Euphorbia helioscopia</i> L.	H	96.43	0.97	64	22
15	<i>Euphorbia heterophylla</i> L.	H	57.67	6.21	0	23
16	<i>Euphorbia hierosolymitana</i> Boiss.	H	99.87	1.19	90	28
17	<i>Euphorbia hirta</i> L.	H	14.97	1.49	33.34	26
18	<i>Euphorbia ingens</i> E. Mey. ex Bioss.	B	0	1.32	10	
19		L, Br	26.23	4.86	67	
20	<i>Euphorbia lactea</i> Haw.	L, Br	95.23	1.25	20	

(Contd.)

Table 1 (Contd.): Activity of plant extracts (100 ppm) in three bioassays: HepG2 cytotoxicity, HCT116 pro-apoptosis and brine shrimp lethality

Ext No.	Plant name	Part used	HepG2 Cytotoxicity (%)	HCT116-M30 pro-apoptosis (FINC)	Brine Shrimp Lethality BSL (%)	Ref *
21	<i>Euphorbia nerifolia</i> L.	L, Br	89.25	2.1	63	42
22	<i>Euphorbia peplis</i> L.	W	39.53	1.55	10	28
23	<i>Euphorbia pseudocactus</i> A. Berger	Ap	97.30	1.8	60	
24	<i>Euphorbia pulcherrima</i> Willd. ex. Klotzsch.	L	52.37	2.16	10	43
25		Br	83.43	0.96	20	
26	<i>Euphorbia retusa</i> Forssk.	H	46.97	2	86	
27	<i>Euphorbia royleana</i> Boiss.	L, Br	59.47	3.68	70	
28	<i>Euphorbia terracina</i> L.	H	52.20	0.75	100	
29	<i>Euphorbia tirucalli</i> L.	Br	17.58	1.06	0	25
30		L, Br	93.17	0.48	37	25
31	<i>Euphorbia longan</i> (Lour.) Steud.	B	52.07	1.35	0	
32		Br	53.43	1.6	20	
33		L	38.37	1.2	10	
34	<i>Jatropha curcas</i> L.	L, Fl, Fr	55.27	0.72	23	27
35		Br	90.37	2.26	14	27
36	<i>Joannesia principes</i> Vell.	Br	9.48	1.68	47	
37		L	64.90	5	90	
38		B	86.65	1.9	90	
39	<i>Mercurialis annua</i> L.	H (female)	78.77	1.1	56.6	28
40	<i>Pedilanthus tithymaloides</i> (L.) Poit.	L, Br	83.73	0.97	83.3	29
41	<i>Drypetes roxburgii</i> (Wall.) Hurusawa	Br	9.60	1.08	10	30
42		L	5.70	1.02	0	30
43		B	93.47	2.67	54	30
44	<i>Ricinus communis</i> L.	Br, Fl, Fr	0	0.8	7	
45		L	16.60	0.92	13.3	
46	<i>Sapium sebiferum</i> (L.) Roxb.	Fl	40.70	2.29	37	31
47		L, Fr	92.27	1.52	44	31
48		Br	73.60	0.23	50	31
49	<i>Synadenium grantii</i> Hook. f.	L, Br	100.70	0.91	0	32
Family Myrtaceae						
50	<i>Callistemon citrinus</i> (Curtis) Skeels	Br	92.93	1.16	27	33
51		L	84.13	2.3	70	33
52	<i>Callistemon rigidus</i> R.Br.	L, Br, Fr	94.23	3.4	0	33
53	<i>Callistemon speciosus</i> (Bonpl.) Sweet	L, Br	79.82	2.56	20	37
54	<i>Callistemon viminalis</i> (Sol. ex Gaertn.)	L	85.02	3.46	13	33
55	Cheel	Br	57.26	2.25	0	33
56	<i>Eucalyptus rostratus</i> Cav.	B	47.03	2.95	34	37
57	<i>Eugenia aquea</i> Burm. f.	Fr	42.90	4.96	23.3	35
58		L, Br	23.27	1.5	0	35
59	<i>Eugenia javanica</i> Lam	Br	20.22	1.54	34	
60		L	83.75	0.85	17	
61	<i>Eugenia uniflora</i> L.	L	22.17	0.69	0	
62		Br	5.50	1.07	37	
63	<i>Heimia myrtifolia</i> Cham. & Schltld.	L, Br, Fl, Fr	29.17	1.12	23.3	
64	<i>Melaleuca ericifolia</i> Sm.	L, Br	66.65	1.98	3.3	36
65	<i>Melaleuca leucadendron</i> L.	B	98.47	2.39	4	37,24,36,44
66		Br	96.40	2.04	20	
67		L	91.43	3.98	40	
68	<i>Pimenta racemosa</i> (Mill.) J.W. Moore	B	68.03	3.23	0	
69		L	27.93	1.76	13.4	
70	<i>Psidium guajava</i> L.	L	83.33	0.73	10	38
71		Br	45.53	1.44	13.3	38
72	<i>Syzygium cuminii</i> (L.) Skeels	L, Br	51.67	1.06	0	39
73		Fr, S	13.50	0.78	7	39
74	<i>Syzygium jambos</i> (L.) Alston	L	81.13	0.68	86	39
75		Br	46.18	0.88	14	39
Positive control	Adriamycin		100.00	-----	-----	
Positive control	Cisplatin		-----	5.9	-----	
Positive control	<i>Annona cherimolia</i> L	L	-----	-----	100	
Negative control	DMSO		0.6	0.5	3.3	

Bold figures = activity of extracts possessing >50 % in HepG2 cytotoxicity, brine shrimp lethality, and/or >1.5 in pro-apoptosis bioassays.

FINC: fold increase of negative control.

Parts: Ap: Aerial part; B: Bark; Br: Branches; Fl: Flowers; Fr: Fruits; H: Herb; L: Leaves; R: Root system; S: Seeds; St: Stem; W: Weed.

*Reference to folk medicinal use or antitumor activity.

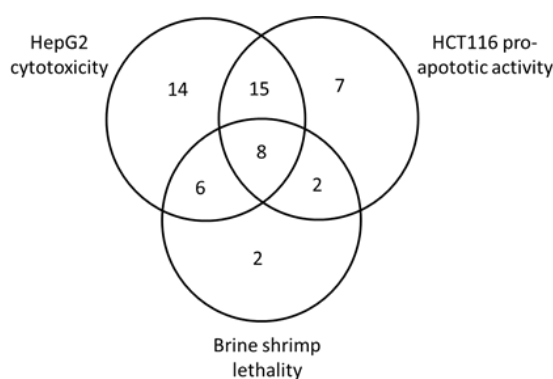


Fig. 1: Illustrative scheme showing the distribution of the numbers of active extracts in the three bioassays (cf. Table 1).

Table 2: LC₅₀ values of extracts inducing ≥ 90 percent in HepG2 cytotoxicity

Extract number	Plant name	Part used	*LC ₅₀ (ppm)
14	<i>Euphorbia helioscopia</i> L.	H	30.7 (± 2.25)
16	<i>Euphorbia hierosolymitana</i> Boiss.	H	26.8 (± 1.34)
20	<i>Euphorbia lactea</i> Haw.	L, Br	25 (± 2.26)
23	<i>Euphorbia pseudocactus</i> A. Berger	Ap	19.4 (± 1.06)
30	<i>Euphorbia tirucalli</i> L.	L, Br	23.4 (± 1.57)
35	<i>Jatropha curcas</i> L.	Br	35.2 (± 4.56)
43	<i>Drypetes roxburghii</i> (Wall.) Hurusawa	B	10 (± 1.91)
47	<i>Sapium sebiferum</i> Roxb.	L, Fr	33.4 (± 4.63)
49	<i>Synadenium grantii</i> Hook. F.	L, Br	40.3 (± 3.35)
50	<i>Callistemon citrinus</i> (Curtis) Skeels	Br	22.2 (± 0.52)
52	<i>Callistemon rigidus</i> R.Br.	L, Br, Fr	40 (± 2.6)
65	<i>Melaleuca leucadendron</i> L.	B	32 (± 4.56)
66		Br	27 (± 1.91)
67		L	14 (± 2.05)
Positive control	Adriamycin		21.6 (± 1.24)

Parts: Ap: Aerial part; B: Bark; Br: Branches, Fr: Fruits, H: Herb, L: Leaves, * Confidence limit = 95%

Table 3: LC₅₀ values of extracts inducing ≥ 90 percent in brine shrimp lethality

Extract number	Plant name	Part used	*LC ₅₀ (ppm)
16	<i>Euphorbia hierosolymitana</i> Boiss.	H	50 (± 3.48)
28	<i>Euphorbia terracina</i> L.	H	70.59 (± 6.78)
37	<i>Joannesia principes</i> Vell.	L	91.11 (± 3.73)
38		B	41.19 (± 1.21)
Positive control	<i>Annona cherimolia</i>	L	10 (± 3.73)

Parts: B: Bark; H: Herb, L: Leaves, * Confidence limit = 95%

23 extracts were active in both HepG2 cytotoxicity and HCT116-M30 pro-apoptosis assays, indicating that the cell death mode induced by these extracts is apoptosis. However, 20 extracts were cytotoxic on HepG2 but not inducing apoptosis in HCT116. This could be explainable by the death mode, induced by these extracts at the specified concentration, to be other than apoptosis and most likely necrosis. A similar observation was previously reported [19]. On the other hand, 9 extracts were pro-apoptotic, but not cytotoxic on HepG2. These extracts might be late acting, and if incubated for longer time (e.g. 72-96 hours) they might have shown cytotoxic activity [16].

DISCUSSION

The present study utilized three different in vitro bioassays to screen 75 plant extracts, representing 47 species belonging to families Euphorbiaceae and Myrtaceae, to predict their possible anticancer activity. The three bioassays used were: i- cytotoxicity on HepG2 human hepatocellular carcinoma cell line assessed by MTT method (*HepG2* cytotoxicity), ii- pro-apoptotic activity on HCT116 human colon carcinoma cell line, quantified by the M30-apoptosense® kit (HCT116-M30 pro-apoptosis), and iii- brine shrimp lethality assay (BSL). All extracts were screened at 100 ppm in the three bioassays and activity was assessed after 24 hours.

Comparing the results obtained (cf. Table 1) with the literature available on the species examined, it is apparent that many of the bio-active extracts detected were previously reported to possess related bio-activities, cf. references [20-44] (cf. Table 1). The most prominent genera relevant to the literature were *Euphorbia*, *Callistemon* and *Melaleuca*.

The results obtained in the BSL assay may lead to the conclusion that BSL is a useful simple tool that could be combined with other bioassays to detect cytotoxicity in large number of plant extracts [45]. However, false negative results can occur. Further studies with larger number of extracts and/or other human tumor cell lines may be recommended to reach a validated correlation.

Plant extracts which showed activity in both HepG2 cytotoxicity and HCT116-M30 pro-apoptosis bioassays are considered of particular interest. Drugs acting through induction of apoptosis are expected to act through a specific mechanism of action [27, 46]. It is generally accepted that many clinically used (and effective) anticancer agents exert their activity through induction of apoptosis [47]. The pro-apoptotic assay employed in this study has been recently developed [15] and only used once previously to screen plants for pro-apoptotic activity [16]. This assay is based on sandwich ELISA for detection of the M30 neo-epitope exposed during the cleavage of the

cytoskeleton component cytokeratin-18 by caspases during apoptosis [48].

Out of the 23 extracts which scored positive in both assays (HepG2 cytotoxicity and HCT116-M30 pro-apoptosis), the extracts of *Melaleuca leucadendron* L. and *Callistemon rigidus* R.Br. (cf. extract numbers: 52 and 67) were highly promising (possessing $\geq 90\%$ HepG2 cytotoxicity and ≥ 3 fold increase in apoptotic background signal).

CONCLUSIONS

C. rigidus extract is firstly to be reported here to possess in vitro anticancer and pro-apoptotic activities, and accordingly may be considered the most promising active in the present study and is recommended for further chemical and biological investigations. The three bioassay approach described in this study appears to be useful in screening large numbers of extracts for detecting antitumor candidate agents

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Note: Our Co-Author Prof. Dr. Gamila M. Wassel^{†1} was deceased in 25 Nov, 2012.

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