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Screening of Vietnamese Medicinal Plants for Cytotoxic Activity

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Abstract – Thirty-two methanol extracts of thirty-one Vietnamese medicinal plants were evaluated for the cytotoxic activity against five human cancer cell lines, including A549, MCF-7, HT 1080, Huh-7, and HepG2. Of these, the nine extracts of *Acanthopanax trifoliatus* (**4**), *Acanthopanax gracilistylus* (**5**), *Siegesbeckia orientalis* (**10**), *Betula alnoides* (**11**), *Passiflora edulis* (**18**), *Zanthoxylum simulans* (leaf, **23**), *Adenosma caeruleum* (**26**), *Solanum verbascifolium* (**29**), and *Alpinia malaccensis* (**31**), exhibited high potent cytotoxic activity showing a certain degree of selectivity against the different cell types, with IC_{50} values ranging from 2.1 to 3.8 μg/mL.
Keywords – Cytotoxicity, Vietnamese medicinal plants.

Introduction

Cancer is a generic term for a large group of diseases that can affect any part of the body. One defining feature of cancer is the rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs. This process is referred to as metastasis. Metastases are the major cause of death from cancer (WHO, 2006). According to the American Cancer Society, 7.6 million people died from cancer in the world during 2007 (American Cancer Society, 2007). Cytotoxicity screening models will provide important preliminary data to help select plant extracts for future work (Cardellina II *et al.*, 1999).

Nature has provided many effective anticancer agents in current use. Several plants-derived compounds are currently successfully employed in cancer treatment (da Rocha *et al.*, 2001). The most potent examples known as vinca alkaloid family isolated from *Catharanthus roseus*. The introduction of vincristine (vinca alkaloid) was responsible for reducing hodgkin's disease and leukemia (da Rocha *et al.*, 2001). The other highly active agents derived from natural products are vinblastine, irinotecan, topotecan, taxanes, and daunomycin, etc. (Newman *et al.*, 2003). In spite of growing study on flora, only ten percent of approximately 250,000 species of higher plants have been chemically and pharmacologically investigated. The search for new cytotoxic agents from natural-microbial, marine and plant-sources still has continued with the cooperation among scientists worldwide (Newman and Cragg, 2007).

Vietnamese, a Southeast Asian tropical country, has a rich plant biodiversity, with over 12,000 plants and no less than 2,500 species have been used in ethnomedicine (Chi, 1997; Loi, 2004). To date, few Vietnamese plants have been investigated for anticancer (Nam *et al.*, 2003). Thus, as part of a permanent screen program searching for Vietnamese medicinal plants and natural products, the aim of this study was to discover whether there was some scientific basis for the reputed efficacy of selected traditional medicinal plants from Vietnam in treatment of cancer based on cytotoxicity. Selection was based on literature research of traditional medicinal plant usage in Vietnam (Perry and Metzger, 1980; Loi, 2004).

Materials and methods

Plant materials – The medicinal plants were collected in the North area of Vietnam during spring and summer, 2008. The plants were botanically identified (Table 1) by Prof. Vu Van Chuyen, Department of Botany, Hanoi University of Pharmacy, Vietnam, where the voucher

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Table 1. Botanical names of plant species screened and percentage yield of the methanol extracts (w/w) from the selected Vietnamese medicinal plants

N°	Family	Scientific name	Part used	Yield (%)
1	Acanthaceae	*Phlogacanthus turgidus* Fua ex Hook. f.	Whole plant	5.8
2	Apiaceae	*Peucedanum terebinthaceum* Fischer et Turcz.	Root	8.4
3		*Bupleurum scorzononaefolium* Willd.	Rhizome	6.3
4	Araliaceae	*Acanthopanax trifoliatus* Seem	Stem bark	9.3
5		*Acanthopanax gracilistylus* W. W. Smith	Leaf	3.8
6		*Panax bipinnatifidus* Seem.	Root	13.2
7		*Panax stipuleanatus* H.T.Tsai & K.M.Feng	Root	9.7
8	Asteraceae	*Achillea millefolium* L.	Whole plant	6.2
9		*Matricaria chamomilla* L.	Flower	7.5
10		*Siegesbeckia orientalis*L.	Whole plant	8.2
11	Betulaceae	*Betula alnoides* Buch. Syn.	Whole plant	7.1
12	Eucomiaceae	*Eucomia ulmoides* Oliv.	Stem bark	9.4
13	Fabaceae	*Glycyrrhiza glabra* L.	Root	17.2
14		*Vigna radiata* L.	Cortex	8.8
15	Lamiaceae	*Elsholtzia cristata* Willd.	Whole plant	7.3
16	Lauraceae	*Lindera myrha* Lour.	Rhizome	4.8
17	Orchidaceae	*Dendrobium nobile* Lindl.	Whole plant	11.4
18	Passifloraceae	*Passiflora edulis* Sims	Cortex	9.3
19	Pinaceae	*Pinus merkusii* Jungh et De Vries	Leaf	3.6
20	Plantaginaceae	*Plantago asiatica* L.	Whole plant	9.5
21	Polygalaceae	*Polygala tenuiflorum* Willd.	Root	3.4
22	Rubiaceae	*Gardenia jasminoides* Ellis	Fruit	8.6
23	Rutaceae	*Zanthoxylum simulans* Hance	Leaf	4.3
24		*Zanthoxylum simulans* Hance	Seed	2.6
25	Schisandraceae	*Kadsura roxburghiana* Roxb.	Whole plant	3.7
26	Scrophulariaceae	*Adenosma caeruleum* R. Br	Whole plant	7.9
27		*Adenosma hirsuta* Miq.	Whole plant	9.1
28	Smilaceae	*Smilax glabra* Roxb.	Rhizome	8.4
29	Solanaceae	*Solanum verbascifolium* L.	Leaf	6.8
30	Zingiberaceae	*Zingiber zerumbet* Sm.	Rhizome	12.5
31		*Alpinia malaccensis* Burm. f.	Fruit	12.9
32		*Amomum cardamomum* L.	Rhizome	7.8

Extraction yielded: percentage extract yield (w/w) was calculated as (dry extract weight/dry starting material weight) × 100.

specimens were deposited. The plants were dried for 7 - 10 days in the shade at environmental temperatures. The dried plants were then ground and transfer to the laboratory for preparation of the plant extracts.

Extracts preparation – The air-dried and powdered parts of different amount of plants were extracted with methanol at room temperature (25 °C) by maceration process for 72 hr. The crude extracts were obtained after evaporation of solvent under reduced pressure at 40 °C. Percentage yields (w/w) were calculated (Table 1). All extracts were stored at –20 °C prior to screening.

Chemicals – Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, fetal bovine serum (FBS), and trypsin were purchased from GIBCO-BRL (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) (MTT) was purchased from Sigma Chemical Company (St Louis, MO, USA).

Cell culture – HepG2 (hepatocellular carcinoma cells), Huh-7 (human hepatoma cells), HT 1080 (Human fibrosarcoma cells) cells were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA), A549 cells were obtained from RIKEN Cell Bank (Ibaragi, Japan). The MCF-7 (human breast cancer) cells were obtained from Dr. Kenneth H. Cowan

(University of Nebraska Medical Center, Omaha, Nebraska). Hep G2 and MCF-7 cells were grown in DMEM containing 10% FBS. A549, Huh-7 and HT 1080 cells were grown in RPMI containing 10% FBS. These cell lines were cultured at 37 °C in a humidified CO_2 incubator (Mosmann, 1983) .

MTT assay – The cells were plated in 96-well plates. Following 24 hr of incubation at 37 °C, various concentrations (1 - 100 μg/mL) of extracts were added to each well. After 48 hr, cell viability was measured by MTT assay. The dark blue formazan crystals that formed in intact cells were solubilized with DMSO, and the absorbance at 550 nm was measured with a microplate reader. Percent cell viability was calculated based on the absorbance measured relative to the absorbance of cells exposed to the control vehicle (Mosmann, 1983).

Statistical analysis – The data were analyzed using the unpaired Student's t-test between the control and compounds; Data compiled from three independent experiments and values are expressed as mean ± SD.

Table 2. *In vitro* cytotoxic activity of the methanol extracts on the five tumor cell lines measured by the MTT assay[a]

Nº	Plants	IC_{50} (μg/mL)				
		A549	Huh -7	HT 1080	MCF-7	Hep G2
1	*Phlogacanthus turgidus*	4.5 ± 0.0	12.8 ± 0.2	4.3 ± 0.1	6.8 ± 0.0	9.4 ± 0.2
2	*Peucedanum terebinthaceum*	38.3 ± 4.2	9.8 ± 0.1	11.7 ± 0.0	5.9 ± 0.1	15.6 ± 0.2
3	*Bupleurum scorzononaefolium*	5.1 ± 0.2	15.3 ± 0.4	6.1 ± 0.1	18.5 ± 0.7	5.8 ± 0.1
4	*Acanthopanax trifoliatus*	12.4 ± 0.5	6.2 ± 0.9	3.7 ± 0.3	7.2 ± 0.2	7.9 ± 0.1
5	*Acanthopanax gracilistylus*	3.6 ± 0.2	7.2 ± 0.4	4.9 ± 0.2	7.9 ± 0.3	3.8 ± 0.1
6	*Panax bipinnatifidus*	7.3 ± 0.1	31.2 ± 3.5	9.4 ± 0.7	–	–
7	*Panax stipuleanatus*	–	40.3 ± 4.6	–	43.9 ± 5.7	45.1 ± 3.8
8	*Achillea millefolium*	5.2 ± 0.2	26.3 ± 1.5	5.6 ± 0.2	21.3 ± 0.5	8.3 ± 0.1
9	*Matricaria chamomilla*	5.1 ± 0.2	6.1 ± 0.1	4.5 ± 0.0	7.9 ± 0.3	7.1 ± 0.2
10	*Siegesbeckia orientalis*	7.2 ± 0.1	4.3 ± 0.2	3.7 ± 0.3	6.5 ± 0.0	5.8 ± 0.1
11	*Betula alnoides*	5.2 ± 0.1	5.1 ± 0.3	4.1 ± 0.4	3.7 ± 0.1	4.3 ± 0. 2
12	*Eucomia ulmoides*	27.2 ± 2.1	–	43.3 ± 3.1	–	45.6 ± 1.6
13	*Glycyrrhiza glabra*	6.6 ± 0.5	8.6 ± 0.1	9.8 ± 0.5	12.4 ± 0.2	15.4 ± 0.2
14	*Vigna radiata*	–	39.9 ± 5.9	47.5 ± 1.2	–	42.2 ± 4.7
15	*Elsholtzia cristata*	29.8 ± 2.3	27.4 ± 2.1	–	40.1 ± 2.5	37.4 ± 2.6
16	*Lindera myrha*	4.5 ± 0.1	25.3 ± 3.5	5.9 ± 0.8	6.8 ± 0.8	7.2 ± 1.1
17	*Dendrobium nobile*	4.9 ± 0.1	13.2 ± 0.3	4.4 ± 0.0	–	20.6 ± 1.2
18	*Passiflora edulis*	3.3 ± 0.1	6.1 ± 0.2	4.3 ± 0.0	3.7 ± 0.2	3.7 ± 0.0
19	*Pinus merkusii*	16 ± 0.5	9.5 ± 0.3	4.1 ± 0.0	4.5 ± 0.1	5.6 ± 0.1
20	*Plantago asiatica*	5.7 ± 0.1	19.8 ± 0.5	5.4 ± 0.2	28.9 ± 3.4	26.7 ± 1.5
21	*Polygala tenuiflorum*	4.1 ± 0.0	24.3 ± 2.6	6.1 ± 0.2	5.2 ± 0.2	4.6 ± 0.0
22	*Gardenia jasminoides*	5.0 ± 0.1	7.3 ± 0.1	5.1 ± 0.0	4.1 ± 0.0	4.4 ± 0.0
23	*Zanthoxylum simulans* (leaf)	2.7 ± 0.2	3.2 ± 0.0	2.8 ± 0.0	4.9 ± 0.1	7.1 ± 0.1
24	*Zanthoxylum simulans* (seed)	34.6 ± 2.3	28.6 ± 2.7	7.1 ± 0.1	26.9 ± 2.5	28.5 ± 4.3
25	*Kadsura roxburghiana*	5.5 ± 0.2	19.4 ± 1.2	5.6 ± 0.3	24.5 ± 2.6	20.3 ± 2.2
26	*Adenosma caeruleum*	2.1 ± 0.0	8.4 ± 0.2	3.3 ± 0.1	2.0 ± 0.1	2.1 ± 0.0
27	*Adenosma hirsuta*	6.3 ± 0.3	25.4 ± 1.4	5.1 ± 0.1	26.9 ± 1.5	25.7 ± 1.6
28	*Smilax glabra*	6.4 ± 0.1	–	6.9 ± 0.1	7.9 ± 0.4	5.8 ± 0.0
29	*Solanum verbascifolium*	3.3 ± 0.0	5.1 ± 0.1	3.5 ± 0.0	3.4 ± 0.1	4.4 ± 0.2
30	*Zingiber zerumbet*	6.5 ± 0.1	25.9 ± 2.5	7.1 ± 0.3	38.6 ± 2.8	–
31	*Alpinia malaccensis*	4.8 ± 0.2	5.6 ± 0.6	3.1 ± 0.1	6.1 ± 0.2	5.5 ± 0.5
32	*Amomum cardamomum*	–	39.4 ± 1.4	43.5 ± 1.7	–	–

–: Non-toxic

[a] Data compiled from three independent experiments and values are expressed as mean ± SD

Results

Current study, the plants are listed in alphabetical order of their family name, followed by the scientific name, part used, as well as percentage yield of extract (%) (Table 1). Thirty-one plant species which belonging to twenty-one families were selected. Total of thirty-two extracts of thirty-one Vietnamese medicinal plants were investigated for their cytotoxic activity against five human cancel cell lines, including A549, MCF-7, HT 1080, Huh-7, and HepG2. The results of screening for cytotoxicity of the extracts have been summarized in Table 2. In the US NCI plant screening program, a crude extract is generally considered to have *in vitro* cytotoxic activity if the IC_{50} value (concentration that causes a 50% cell kill) in human cancer cells, following incubation for 48 hr, is less than 20 mg/mL, while it is less than 10 mg/mL for pure compounds (Boik, 2001, p. 25). As shown in Table 2, five methanol extracts (**7**, **12**, **14**, **15**, and **32**) did not demonstrate any significant cytotoxic activity against the five human cancer cell lines. While eleven methanol extracts (**4**, **5**, **9 - 11**, **18**, **22**, **23**, **26**, **29**, and **31**) exhibited potent cytotoxic activity against the five human cancer cell lines with the IC_{50} values within 2.0 - 7.9 μg/mL; four methanol extracts (**16**, **19**, **21**, and **28**) displayed considerable inhibitory effect on the four cancer cell lines comprising A549, MCF-7, HT 1080, and HepG2 with the IC_{50} values ranging from 4.1 to 9.4 μg/mL; four extracts (**3**, **6**, **8**, and **13**) showed significant cytotoxicity in three cell lines with IC_{50} values within 5.1 to 9.8 μg/mL; six extracts (**2**, **17**, **20**, **25**, **27**, and **30**) exhibited considerable cytotoxicity in the two cancer cell lines with IC_{50} values ranging from 4.4 to 7.1 μg/mL; and only extract (**24**) from *Zanthoxylum simulans* (seed) showed high potent cytotoxicity in one cell line (HT 1080) with IC_{50} value of 7.1 μg/mL. The high potent cytotoxic activity was observed for the extracts of *Acanthopanax trifoliatus* (**4**) with IC_{50} values of 3.7 ± 0.3 μg/mL (in HT 1080 cells); *Acanthopanax gracilistylus* (**5**) with IC_{50} at 3.6 ± 0.2 μg/mL (in A549 cells) and 3.8 ± 0.1 μg/mL (in HepG2 cells); *Siegesbeckia orientalis* (**10**) with IC_{50} values of 4.3 ± 0.2 μg/mL (in Huh-7 cells) and 3.7 ± 0.3 μg/mL (in HT1080 cells); *Betula alnoides* (**11**) with IC_{50} value of 3.7 ± 0.1 μg/mL (in MFC-7 cells); *Passiflora edulis* (**18**) with IC_{50} value of 3.3 ± 0.1 μg/mL (in A549 cells), 3.7 ± 0.2 μg/mL (in MCF-7 cells), and 3.7 ± 0.5 μg/mL (in Hep G2 cells); *Zanthoxylum simulans* (leaf, **23**) with IC_{50} at 2.7 ± 0.2 μg/mL (in A549 cells), 3.2 ± 0.0 μg/mL (in Huh-7 cells), and 2.8 ± 0.0 μg/mL (in HT 1080 cells); *Adenosma caeruleum* (**26**) with IC_{50} values of 2.1 ± 0.0 μg/mL (in A549 cells), 3.3 ± 0.1 μg/mL (in HT 1080), 2.0 ± 0.1 μg/mL (in MCF-7), and 2.1 ± 0.0.0 μg/mL (in HepG2 cells); *Solanum verbascifolium* (**29**) played marked strong toxicity in all of cell lines with IC_{50} at 3.3 ± 0.5 μg/mL (in A549 cells), 5.1 ± 0.1 μg/mL (in Huh-7 cells), 3.5 ± 0.0 μg/mL (in HT 1080 cells), 3.4 ± 0.1 μg/mL (in MCF-7 cells), 4.4 ± 0.2 μg/mL (in HepG2 cells); *Alpinia malaccensis* (**31**) with IC_{50} value of 3.1 ± 0.1 μg/mL (in HT1080 cells). Further studies concerning the cytotoxic constituents of *Acanthopanax trifoliatus* (**4**), *Acanthopanax gracilistylus* (**5**), *Siegesbeckia orientalis* (**10**), *Betula alnoides* (**11**), *Passiflora edulis* (**18**), *Zanthoxylum simulans* (leaf, **23**), *Adenosma caeruleum* (**26**), *Solanum verbascifolium* (**29**), and *Alpinia malaccensis* (**31**) on which few or no phytochemical reports exist in the literatures, seem to be worthwhile.

Discussion and conclusions

Recently, there has been a global trend toward the use of natural phytochemical anticancer present in natural resources, such as herbs, vegetables, fruits and oilseeds (Walgren *et al.*, 2000; Mann, 2002; Yan *et al.*, 2006; Brandin *et al.*, 2007; Frederiksen *et al.*, 2007). Herbs have begun as raw materials for finding new drugs (Joy *et al.*, 2006; Lee *et al.*, 2006). Herbal medicines derived from plants are increasingly being utilized to treat a wide variety of clinical diseases, even though relatively little is known about their modes of action. Until now, numerous plants and plant constituents have already demonstrated cytotoxic activity (Newman and Cragg, 1997; Newman *et al.*, 2003; Kaileh *et al.*, 2007; Newman and Cragg, 2007; de Mesquita *et al.*, 2009), illustrating that there is still potential for novel innovative cytotoxic activities to be identified from natural plant resources. Vincristine, irinotecan, etoposide, and paclitaxel are examples of plant-derived compounds that are being used in cancer treatment (da Rocha *et al.*, 2001; Newman and Cragg, 2007). The taxanes and the camptothecins are presently approved for human use in various countries (da Rocha *et al.*, 2001).

This study provides high potent cytotoxic activities of *Acanthopanax trifoliatus* (**4**), *Acanthopanax gracilistylus* (**5**), *Siegesbeckia orientalis* (**10**), *Betula alnoides* (**11**), *Passiflora edulis* (**18**), *Zanthoxylum simulans* (leaf, **23**), *Adenosma caeruleum* (**26**), *Solanum verbascifolium* (**29**), and *Alpinia malaccensis* (**31**), indicating their ultimate potential for pharmaceutical use among the test samples. Of those, two plants (**4** and **5**) exhibited considerable anticancer activity (Kiem *et al.*, 2003a; Kiem *et al.*,

2003b; Kiem *et al.*, 2004). Phytochemical studies revealed the presence of lupane-type triterpenes, kaurane-type diterpenes, phenylpropanoid glycosides in *A. trifoliatus* (Kiem *et al.*, 2003a; Kiem *et al.*, 2003b; Kiem *et al.*, 2004) and in *A. gracilistylus* (Liu *et al.*, 2002; Yook *et al.*, 2002). The extract of *A. gracilistylus* markedly suppressed the proliferative responses of human peripheral blood lymphocytes stimulated with mitogens concanavalin A (Con A) and *Staphylococcus aureus* Cowan I (SAC) (Shan *et al.*, 1999). Previous studies on this plant also indicated the effects of its isolated saponins and diterpenes on the human platelet aggregation and platelet factor-4 liberation and anti tumor cell lines such as MT-2, Raji, HL-60, TMK-1 and HSC-2 (Chen *et al.*, 1996; Shan *et al.*, 2000; Shan *et al.*, 2005). Nevertheless, no report has been described for their effects on the five human cancer cell lines which were used in this study.

The other plants (**10**, **11**, **18**, and **23**) presented significantly immunosuppressive, anti-inflammatory, anti-platelet, and/or neuropharmacological effects (Xiang *et al.*, 2004; Giang *et al.*, 2005; Wang *et al.*, 2009). The number ent-pimarane-type diterpenoids which were recently isolated from *S. orientalis* exhibited immuno-suppressive activity (Xiang *et al.*, 2004; Giang *et al.*, 2005; Wang *et al.*, 2009). However no report related to cytotoxicity of this plant has been carried out (Hwang *et al.*, 2001). In 1995, Kamperdick et al. reported the presence of several triterpenoids, including lupeol, 3-*O*-acetoxyoleanolic acid, betulinic acid, and betulin in *B. alnoides* (Kamperdick *et al.*, 1995). Until now, no cytotoxicity and any phytochemical study have been reported to this plant, except for the significant antiinflammatory activity of *B. alnoides* extract was evaluated in acute and subacute inflammation models (Sur *et al.*, 2002). *P. edulis*, commonly known as "maracujá", belong to Passifloriaceae family. The species of Passiflora are popularly used as a sedative or tranquillizer, and also against intermittent fever and skin inflammation. Recently, the extraction and several isolated compounds, including isoorientin, vicenin-2 and spinosin from the BuOH fraction of *P. edulis* showed anti-inflammatory effect in the *in vivo* assay. Results also revealed that C-glucosylflavones isolated from *P. edulis* leaves can be responsible for the anti-inflammatory effect of *P. edulis* on the mouse model of pleurisy (Zucolotto *et al.*, 2009). Neuropharmacological activity of the pericarp of *P. edulis* flavicarpa degener: putative involvement of C-glycosylflavonoids (isoorientin, vicenin-2, spinosin, and 6,8-di-C-glycosylchrysin) (Sena *et al.*, 2009). In addition, the aqueous extracts of *P. alata* and *P. edulis* reduce anxiety-related behaviors without affecting memory process in rats (Barbosa *et al.*, 2008). Chemical investigation of *Z. simulans* showed the presence of alkaloids, lignans, monocyclic ã-pyrone in the root bark and root wood (Yang *et al.*, 2002). The isolated compounds have been evaluated for the anti-platelet aggregation activity *in vitro* and several compounds such as zanthopyranone, sinapic aldehyde, vanillic acid, sinigic acid, isofraxidin, and sepesteonol presented potent inhibitory effect on the aggregation of washed rabbit platelets induced by arachidonic acid, collagen, thrombin, and platelet activotins factor (Yang *et al.*, 2002). Recent phytochemical study reported that a new iridoid glycoside, adenosmoside and five known phenylpropanoids including crenatoside, verbascoside, cistanoside F, campneoside I, and campneoside II, and two known flavonoids, apigenin 7-O-β-D-glucuronopyranoside and apigenin 7-*O*-β-D-glucopyranoside, were isolated from the aerial parts of *A. caeruleum*. However no report toward the cytotoxic activity of the isolated compound to date (De Abreu *et al.*, 2009). Two other plants, *S. verbascifolium*, and *A. malaccensis*, have not yet been assessed for *in vitro* cytotoxicity and also phytochemical studies.

In conclusion, plants still remain a prime source of drugs for the treatment of cancer and can provide leads for the development of novel anticancer agents. Our screening of indigenous medicinal plants from Vietnam has led to the identification of the number of anticancer activity. Total thirty-two methanol extracts of thirty-one plant species were screened for *in vitro* anticancer activity against five human cancer cell lines. Results showed that nine methanol extracts exhibited high cytotoxic activity against one or many test cancer cell lines. Thus, the search for new drugs is imperative and the results of our screening call for future isolation and characterization of the active compounds by bio-guided assay.

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