Original Article

TIPS Trends in Pharmaceutical Sciences 2022: 8(1): 57-64.

Cytotoxicity Analysis of Nineteen Medicinal Plants Extracts on Breast Adenocarcinoma (MCF-7) and Rhabdomyosarcoma (RD) Cancer Cell Lines

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Abstract

The burden of cancer is alarmingly great on humans. However, the relevance of plants in combating cancer cannot be undermined. We joined the global search for anticancer agents from plants by screening nineteen (19) medicinal plant extracts on human breast carcinoma (MCF-7) and rhabdomyosarcoma (RD) cell lines using the 3- [4, 5-dimethylthiazol–2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Vincristine was used as the positive reference. Results were presented as mean IC_{50}±standard error of the mean using GraphPad Prism (6.0). One-way ANOVA was used to establish the level of statistical significance. The results showed that twelve of the extracts were moderately cytotoxic on MCF-7 while seven were moderately active on RD (IC_{50}, 30-100 µg/mL). Two of the extracts, *Afzelia africana* stem and *Anogeissus leiocarpus* root, showed significant activity on MCF-7 while seven had significant activity on RD (IC_{50}<30 µg/mL) (P<0.05). *Calliandra portoricensis* root had the highest activity on RD (2.57±0.88 µg/mL). *Anogeissus leiocarpus* root and *Olax subscorpioidea* root showed significant activity on both cancer cell lines (RD: 13.82±2.05; MCF-7: 25.64±7.58 µg/mL and RD: 6.89±2.10; MCF-7: 36.47±1.57 µg/mL, respectively). Most of the extracts also demonstrated selectivity for the cancer cell lines. Bio-guided fractionation of four active extracts revealed that activity lies in the hexane and dichloromethane regions, inhibiting between 60-82% proliferation of the cell lines, at 50 µg/mL. This study demonstrated that the selected plants possess cytotoxic properties. Further investigations are underway to isolate and characterize the active fractions' cytotoxic molecules.

Keywords: Cytotoxicity, Medicinal Plants, Breast Cancer, Rhabdomyosarcoma.

Please cite this article as: Yemi A. Adekunle, Babatunde B. Samuel*, Toluwanimi E Akinleye, Adekunle J. Adeniji, Omonike O. Ogbole. Cytotoxicity Analysis of Nineteen Medicinal Plants Extracts on Breast Adenocarcinoma (MCF-7) and Rhabdomyosarcoma (RD) Cancer Cell Lines. Trends in Pharmaceutical Sciences. 2022;8(1):57-64 .doi: 10.30476/TIPS.2022.94313.1136

1. Introduction

Cancer is a term used to describe a group of malign diseases characterized by abnormal proliferation of tumor cells, which could invade and affect almost all parts of the body; this spread of tumor cells from one region of the body to another is called metastasis. Cancer is a serious human problem and it continues to be a global leading cause of death with about 10 million deaths every year (1). Cancer has been designated the second cause of death worldwide since 2013. About 19, 292, 789 cancer incidences and 9, 958, 133 cancer deaths were reported in 2020 (2). The survival

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Received: 08/12/2021; Accepted: 01/03/2022

rates for all cancer types in Africa are lower than that of developed countries (3). Despite advances in cancer biology –oncogenic molecular targets– the associated mortality rates and economic burden of cancer are grievous (4). Breast cancer is one of the most common cancers in Africa region, a top cause of death globally among women and about 2.2 million cases of breast cancer were reported in 2020 (1). Also, rhabdomyosarcoma is the most common soft tissue sarcoma of children and a leading cause of death among children in Nigeria (5, 6).

Research interest is currently drawing its attention to medicinal plants for the discovery of anticancer agents. This is primarily due to poor selectivity, unbearable side effects and resistance associated with clinically available anticancer agents (7). Natural products from medicinal plants are bioactive principles which have contributed tremendously to cancer management (8). Medicinal plants and the secondary metabolites isolated from them have long history of being used for cancer. Many plant-derived molecules have been clinically established for the treatment of different types of cancer including Vincristine and Vinblastine from *Catharanthus roseus*, Taxol from *Taxus brevifolia*, Camptothecin from *Camptotheca acuminata*, Combretastatin A-4 phosphate from *Combretum caffrum*, Pomiferin from *Maclura pomifera* and Epipodophyllotoxin from P*odophyllum peltatum* (9). Because of their availability, affordability and reliability over the years, medicinal plants are major part of traditional medicine all over the world. So far, only a few research efforts have focused on cytotoxic drug discovery from *African flora* (4); hence, African botanicals have been enormously unstudied for their cytotoxicity against human cancer cell lines (10). There is constant demand on medicinal plants for anticancer drugs as naturally-derived compounds are believed to have lesser adverse effects compared to chemotherapy.

Limited scientific studies exist in literature regarding prominent findings on anticancer plants from Africa despite Africa's biodiversity (3). In this study, *in vitro* cytotoxicity data for nineteen (19) selected medicinal plant extracts in Nigeria were collected against MCF-7, RD and VERO cell lines using the 3-[4, 5-dimethylthiazol–2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Efforts are underway to isolate and characterize bioactive cytotoxic compounds responsible for the activity of the experimentally identified active extracts and fractions.

2. Materials and methods

2.1. Materials

Biohazard safety cabinet, incubator, oven, centrifuge, binocular microscope, 70% alcohol, cryo vials, powder-free nitrile gloves, tissue culture treated plates, sealers, micropipette, UV spectrophotometer, fetal bovine serum (FBS), trypsin, human rhabdomyosarcoma (RD) and MCF-7 cell lines, phosphate buffered-saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), vincristine.

2.2. Plant Collection and Authentication

Sixteen medicinal plants were used in this study (Table 1). The plants collected are from different families with about 56% from Combretaceae family. The parts used include leaves, stem bark, root bark and seed. The plants were collected from the University of Ibadan Botanical Garden and environment between the months of December 2019 and January 2020. Voucher specimens were deposited at the University of Ibadan Herbarium (UIH), Botany department, University of Ibadan and Forestry Herbarium Ibadan (FHI), Forestry Research Institute of Nigeria, Ibadan.

2.3. Extraction

The plant materials were air-dried for about three (3) weeks. The dried plant materials were then pulverized. About 50-100 g of each of the powdered samples was extracted with methanol using soxhlet extraction method. Each extracted fraction was concentrated using rotary evaporator at <40 °C (Heidolph HB Digital, Germany). The concentrated extracts were allowed to dry under laboratory temperature and stored until used. Nineteen medicinal plant extracts were obtained based on the number of the plant parts collected.

2.4. Solvent Fractionation of Active Extracts

Being guided by their biological activities, four (4) of the active extracts –*Anogeissus leio-*

Table 1. List of Plant Species Analyzed for Their Cytotoxic Properties.

Name of Plants	Plant Parts	Extract Code	Voucher Number	Family
Afzelia africana	Stem	AAS		Fabaceae
Axonopus compressus	Leaves	ACL	UIH:23035	Poaceae
Anogeissus leiocarpus	Root	ALR		Combretaceae
Aristolochia ringens	Leaves	ARL	UIH:23017	Aristolochiaceae
Byrsocarpus coccineus	Root	BCR		Connaraceae
Calliandra portoricensis	Root	CPR	UIH:23036	Leguminosae
Combretum constrictum	Leaves	CCL		Combretaceae
Combretum dolichopetalum	Leaves	CDL	UIH:101219	Combretaceae
Combretum hispidum	Leaves	CHL	UIH:22819	Combretaceae
Combretum platypterum	Leaves	CPL	UIH:23034	Combretaceae
Combretum smeathmanii	Leaves	CSmL	UIH:23018	Combretaceae
Combretum sordidum	Leaves	CSL	FHI:109923	Combretaceae
Olax subscorpioidea	Root	OSR	FHI:113182	Olacaceae
Olax subscorpioidea	Stem	OSS	FHI:113182	Olacaceae
Sphenocentrum jollyanum	Leaves	SJL		Menispermaceae
Sphenocentrum jollyanum	Stem	SJS		Menispermaceae
Terminalia ivorensis	Leaves	TIL	FHI:110382	Combretaceae
Terminalia mantaly	Stem	TMS	UIH:22715	Combretaceae
Terminalia mantaly	Seed	TMSee	UIH:22715	Combretaceae

carpus root (ALR), *Byrsocarpus coccineus* root (BCR), *Olax subscorpioidea* root (OSR) and *Olax subscorpioidea* stem (OSS)– were further partitioned into four (solvents): n-Hexane (01), Dichloromethane (02), Ethyl acetate (03) and Methanol/Water (04). The fractionated extracts yielded sixteen (16) fractions and the fractions were further subjected to cytotoxic analysis.

2.5. Preliminary Cytotoxicity Screening of the Plants Extracts

2.5.1. Sample Preparation

For each of the extracts tested, 20 mg was weighed into 2 ml cryovial bottles. The extracts were dissolved in 2 mL of dimethyl sulphoxide (DMSO) to produce the final concentration of 10 mg/mL. Complete homogeneity was ensured, when necessary, by sonication (Eppendorf Centrifuge 5417C).

2.5.2. Cell Lines and Culture

The human rhabdomyosarcoma (RD), human breast carcinoma (MCF-7) and normal African green monkey kidney (VERO) cell lines were obtained from the WHO Reference Polio Laboratory, University College Hospital, Ibadan, Nigeria. The cells were grown in T75 flask containing Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin suspension, 2 mM L-glutamine, 1% non-essential amino acids and vitamin solution, and 0.07% $NaHCO_3$. The cells were maintained at 37ºC under a humidified atmosphere containing 5% CO_2 and passaged bi-weekly.

2.5.3. In vitro cytotoxicity assay

Cytotoxicity evaluation of the extracts was done using the in vitro spectrophotometric MTT assay method described by Mossmann (1983) (11). The assay examined the ability of metabolically viable cells to reduce yellow tetrazolium salt (MTT) to purple formazan by their mitochondrial enzymes. From the stock solutions prepared, 10 mg/mL, six (6) concentrations (1,000 µg/mL -0.01 µg/mL) were made for each extract by serial dilutions in 2% FBS in MEM. MCF-7, RD and VERO cells were grown for 24 hours at 37 °C. After 24 hours, the old medium was aspirated from the 96-well microtitre plate and each concentration of the test extracts, positive control vincristine in DMSO

was added to triplicate wells. The plates were incubated at 37 °C/5% CO_2 for 72 hours. Maintenance medium (2% FBS in MEM) was used as cell control (CC). After this period, 25 µL of 2 mg/mL MTT was added to all wells and incubated for another 2 to 4 hours. DMSO was added to solubilize the purple formazan that was formed by the activity of viable cells. Absorbance was taken at 490 nm on a spectrophotometer (Multiskan, Thermo Fisher Scientific, Waltham, MA).

2.6. Data Analysis

2.6.1. Percentage (%) Cell Inhibition

The % inhibition of the cell growth was determined using the formula (Equation 1):

$$\%\ Inhibition = \frac{Cell\ \ Control\ \ OD\ \ -\ \ Test\ \ OD}{Cell\ \ Control\ \ OD} \times 100 \quad \text{(Eq. 1)}$$

2.6.2. Selectivity Index Determination

Most active extracts were taken further to investigate their selectivity on non-cancerous cell. Normal African Green Monkey Kidney cell (VERO) was used as the normal cell. The selectivity index (SI) was calculated using the formula (Equation 2):

$$SI = \frac{IC_{50}of\ \ Vero\ \ cell\ \ line}{IC_{50}of\ \ cancer\ \ cell\ \ line} \quad \text{(Eq. 2)}$$

2.6.3. Cytotoxicity and IC_{50} Analysis of Solvent Fractions

A concentration of 10 mg/mL was prepared for each of the solvent fractions. The fractions were screened against both RD and MCF-7 at 50 µg/mL as outlined above.

2.7. Statistical Analysis

All experiments were carried out in triplicates and data presented as mean±standard error of mean (SEM). GraphPad Prism (6.0) was used to calculate the IC_{50}. One-was ANOVA was used to determine the level of statistical significance and *P* values <0.05 were considered significant.

3. Results

The result of the cell killing potentials of the nineteen (19) tested medicinal plant extracts is

Table 2. *In vitro* Cytotoxicity Bioassay of Methanol Extracts of Selected Plants on Human MCF-7 and RD Cells (mean IC_{50}±SEM µg/mL).

S/N	Extracts	MCF-7	RD
1	AAS	18.71±6.53*	102.06±2.35
2	ACL	67.90±14.12	23.95±3.26*
3	ALR	25.64±7.58*	13.82±2.05*
4	ARL	71.303±22.72	18.85±1.48*
5	BCR	95.247±4.84	6.62±2.63*
6	CCL	54.557±5.94	55.11±19.65
7	CDL	41.683±6.58*	176.9±1.65
8	CHL	30.173±6.22*	156.87±23.93
9	CPL	54.107±6.70	37.243±7.71*
10	CPR	62.173±8.74	2.57±0.88*
11	CSmL	40.973±25.91*	134.7667±2.72
12	CSL	116.433±7.46	118.47±10.11
13	OSR	36.47±1.57*	6.89±2.10*
14	OSS	176.16±1.12	4.79±0.43*
15	SJL	181.867±4.31	48.97±11.54
16	SJS	95.37±6.77	31.5±8.99*
17	TIL	30.99±4.19*	40.19±3.56
18	TMS	132.06±12.51	59.42±9.15
19	TMSee	163.267±8.51	57.58±2.76
20	VINC	3.972±1.25	0.633±0.43

*activity not statistically different significantly when compared with positive control vincristine.

Table 3. Results of Selectivity Index (SI) for Active Extracts.

Extracts	Cytotoxicity (IC50 µg/mL)			Selectivity Index (SI)	
	MCF-7	RD	VERO	MCF-7	RD
ALR	25.64±7.58	13.82±2.05	57.74±1.62	2.25	4.18
BCR	95.247±4.84	6.62±2.626	86.15±1.58	0.90	13.01
CPR	62.173±8.74	2.57±0.88	67.35±15.81	1.08	26.21
OSR	36.47±1.57	6.89±2.10	13.21±0.43	0.36	1.92
OSS	176.16±1.12	4.79±0.43	93.59±3.47	0.53	19.54
AAS	18.71±6.53	102.06±2.35	109.33±9.22	5.84	1.07

shown in Table 2. From the results obtained, only five extracts had IC_{50} greater than 100µg/mL on MCF-F and five extracts on RD cell lines. These extracts had low cytotoxic effects on the cancer cell lines. Twelve extracts were moderately cytotoxic on MCF-F while seven extracts were moderately cytotoxic on RD with IC_{50} between 30 and 100µg/mL. Two of the plant extracts – A*nogeissus leiocarpus* and *Afzelia africana* – had significant cytotoxicity on MCF-7 and seven extracts – *Calliandra portoricensis*, *Anogeissus leiocarpus*, *Axonopus compressus*, *Aristolochia ringes*, *Byrsocarpus coccineus*, *Olax subscorpioidea* root and *Olax subscorpioidea* stem – had significant activities (P<0.05) on RD with IC_{50}<30 µg/mL. *Anogeissus leiocarpus* root (RD: 13.82±2.05, MCF-7: 25.64±7.58 µg/mL) and *Olax subscorpioidea* root (RD: 6.89±2.10, MCF-7: 36.47±1.57 µg/mL) extracts showed significant activities on both cancer cell lines. *Calliandra portoricensis* had the highest cytotoxicity on RD cell line (IC_{50} 2.57±0.88 µg/mL) while *Afzelia africana* had the highest activity on MCF-7 (IC_{50} 18.71±6.53 µg/mL).

Anogeissus leiocarpus (ALR), *Byrsocarpus coccineus* (BCR) and *Olax subscorpioidea* (OSR and OSS) were chosen for further studies based on their cytotoxic activities. The four extracts, as well as *Calliandra portoricensis* (CPR) and *Afzelia africana* (AAS), were screened on Normal African Green Monkey Kidney (VERO) cell line for selectivity index (SI) analysis (Table 3). Some of the extracts with strong cytotoxic properties also have good selectivity for the cancer cells (SI >2) (12). ALR and AAS were active on MCF-7 and were also selective on it. ALR, BCR, CPR and OSS had strong cytotoxic effects on RD and were also significantly selective on it. However, OSR has low selectivity on both cell lines.

ALR, BCR, OSR and OSS were further partitioned into n-hexane (01), dichloromethane (02), ethyl acetate (03) and water/methanol (04). At 50 µg/mL, all hexane and dichloromethane fractions of the four extracts (ALR-01; ALR-02; BCR-01; BCR-02; OSR-01; OSR-02; OSS-01; OSS-02) inhibited between 60-82% of RD cell line while three of the solvent fractions (OSR-01, OSR-02 and OSS-02) displayed significant inhibition (>75%) of MCF-7 cell line (Table 4). The fractions which had >60% inhibition on both cell lines were good for isolation of bioactive molecules.

4. Discussion

Nineteen selected medicinal plant extracts were investigated for their cytotoxicity potentials against both human breast adenocarcinoma (MCF-7) and human rhabdomyosarcoma (RD) cells. Calliandra portoricensis had the highest cytotoxicity on RD among the extracts, and *Afzelia africana* had the highest cytotoxicity on MCF-7 with the IC50 values of 2.57±0.8840 and 18.71±6.525 µg/mL respectively. *Anogeissus leiocarpus* (RD: 13.82±2.0521; MCF-7: 25.64±7.5811 µg/mL) and *Olax subscorpioidea* root (RD: 6.89±2.0968; MCF-7: 36.47±1.5653 µg/mL) were observed to have significant activity on both cell lines.

MTT assay is a colorimetric bioassay developed by Mossmann. (1983) (11), and it has since then received a wide application in the screening of anticancer agents. The assay is believed to be the gold standard for cytotoxicity testing (13), and it provides rapid and precise results (11). Metabolically active cells cleave the tetrazolium ring of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tet-

Table 4. Percentage cytotoxicity inhibition of MCF-7 and RD by Solvent Fractions at 50 µg/mL.

Fractions	Concentrations (µg/mL)	% Inhibition	
		MCF-7	RD
ALR-01	50	24.10551	80.48946
ALR-02	50	36.04238	73.73215
ALR-03	50	31.7338	33.12486
ALR-04	50	31.9457	35.94833
BCR-01	50	49.63911	73.38319
BCR-02	50	49.78038	72.81215
BCR-03	50	15.87683	12.53569
BCR-04	50	16.05342	6.5307
OSR-01	50	78.42181	79.98187
OSR-02	50	80.08167	82.01224
OSR-03	50	29.19104	35.63109
OSR-04	50	8.460435	40.86562
OSS-01	50	48.75621	68.52935
OSS-02	50	75.49056	60.75685
OSS-03	50	10.61472	20.97439
OSS-04	50	15.38241	4.160435

razolium (MTT) by the mitochondrial NAD(P)H-dependent cellular oxidoreductase enzyme. The cleavage leads to the conversion of yellow MTT to purple formazan which can be solubilized by dimethyl sulpoxide (DMSO) and absorbance taken at 490 nm with a multi-well scanning spectrophotometer (Multiskan, Thermo Fisher Scientific, Waltham, MA). The intensity of formazan formed is directly proportional to the number of viable cells (14). MTT assay measures both cytotoxicity and proliferation as it can quantify cell viability and proliferation.

According to the United States National Cancer Institute (US. NCI), an upper cutoff of <30 µg/mL was set for crude extracts from medicinal plants (15). Seven extracts met this criterion for RD and two for MCF-7 cell lines with IC50 values less than 30 µg/mL. Bioactivity-guided fractionation of active extracts revealed their cytotoxic principles are concentrated in the non-polar regions (hexane and dichloromethane fractions) for both cancer cell lines. Generally, RD cell was observed to be more sensitive to the extracts than MCF-7 cell.

The non-cancerous African green monkey kidney (VERO) cell was used to determine the Selective Index (SI) of the extracts. Since SI measures differential activity of an extract, the higher the value of SI, the more selective an extract is. SI less than 2 is considered as general toxicity (16). Selective Index study carried out on the active extracts revealed they are more active on the cancer cell lines than normal cells. The primary setback of currently available chemotherapy is indiscriminate destruction of both cancerous and non-cancerous cells. Selective extracts will be able to kill cancer cells without destroying normal cells. From the studies, ALR is about 3 times more toxic on MCF-7 and about 5 times more toxic on RD than non-cancerous cells. BCR, CR and OSS had greater than 10 selectivity indices on RD which means they are regarded as safe or non-toxic. Although most of the selected extracts had good selectivity indices, the susceptibility of normal cell line- Vero to OSR extract indicated that it has no selectivity between non-cancerous and cancerous cells, especially on MCF-7 and RD. However, there exists a possibility of isolating more selective compounds from a crude extract that is weakly selective (16, 17). Therefore, toxicity studies of *Olax subscorpioidea* root on cancer cell need to be well-defined.

Hassan et al. (2018) (19) reported cytotoxicity of leaves and bark extracts of *Anogeissus leiocarpus* but no serious work has been reported

on the root extracts. *Calliandra portoricensis* and *Olax subscorpiodea* have been reported to be used in the treatment of various types of cancer in Western Nigeria (20). The root of *Calliandra portoricensis* with the root of *Olax suscorpioidea*, seeds of *Lagenaria breviflora* and leaves of *Andrographis paniculata* are cooked together as a recipe for the treatment of prostate cancer (6). A study has suggested that the methanolic root fraction of *Calliandra portoricensis* induced apoptosis in prostatic LNCap and DU-145 by alteration of mitochondrial integrity and cell cycle arrest (21). Cytotoxicity of leaf extracts of *Byrsocarpus coccineus* has been established (22, 23). However, the cytotoxic potential of the root extracts has been ill-defined.

5. Conclusion

This research work has demonstrated the cytotoxicity of nineteen selected medicinal plant extracts with two showing significant activity on breast adenocarcinoma (MCF-7) and seven showing significant activity on rhabdomyosarcoma (RD) cell lines. Six of the extracts which showed strong cytotoxicity (some on MCF-7 and some on RD) have good cancer cells selectivity. Bioactivity-guided solvent fractionation of four selected extracts revealed that the cytotoxic properties lied in the non-polar regions (n-hexane and dichloromethane fractions). Efforts are underway to isolate and characterize the bioactive cytotoxic principles of the active fractions. This work provided credence to the folkloric medicinal use of these plants and hope for the discovery of anticancer drugs from locally available plants.

Authors' Contributions

Adekunle YA and Samuel BB conceived and designed the experiments. Adekunle YA carried out the extraction and fractionation of the plant materials. Adekunle YA drafted the manuscript and it was reviewed by Samuel BB and Ogbole OO. Adekunle YA, Akinleye TE and Ogbole OO carried out the MTT assay. Adeniji JA provided laboratory and Cell culture materials. All the authors read the manuscripts.

Conflict of Interest

The authors have no conflict of interest.

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