https://dx.doi.org/10.4314/njnpm.v28i2.1

SELECTED MEDICINAL PLANTS EXHIBIT SELECTIVE CYTOTOXICITY AGAINST BREAST (MCF-7) AND RHABDOMYOSARCOMA (RD) CANCER CELL LINES

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Keywords: Breast cancer, Phytochemical, Rhabdomyosarcoma, Selective index

ABSTRACT

Cancer is one of the most frightening diseases threatening the health of mankind, with poor prognosis and treatment. Current cancer treatment options, though costly to develop, have not met expectations largely due to issues like recurrence, drug resistance, poor accessibility, and serious side effects. This necessitates the search for new potent chemotherapeutic agents with high selectivity. Therefore, Phytochemical constituents and cytotoxic potentials of four selected medicinal plants were investigated. *In vitro* cytotoxic activity of the selected plant extracts using MTT (3(4,5-dimethylthiazol-2-yl)-2-diphenyltetrazolium bromide) colorimetric assay was carried out against human breast cancer (MCF-7) and Rhabdomyosarcoma (RD) cell lines. The selective index (SI) of the extracts was also determined using the Vero cell line. *Indigofera nummularriifolia* (INW) exhibited the highest inhibition on the growth of MCF-7 (IC_{50} = 1.22 µg/mL) and RD (IC_{50} = 6.51 µg/mL) cell lines as compared with the standard reference drug, vincristine with IC_{50} = 0.25 and 2.89 µg/mL on MCF-7 and RD cancer cell lines, respectively. Determination of SI using the Vero cell line indicated that INW displayed a relatively high degree of selectivity against the cancer cells under investigation. These plants especially *I. nummularriifolia* exhibited cytotoxic effects and thus could contribute to anticancer drug discovery and many other disease conditions due to their phytoconstituents.

INTRODUCTION

Cancer is one of the most frightening diseases that has a bad prognosis for treatment and poses a threat to human health. 80–90% of cases of cancer that are in an advanced stage result in mortality because of inadequate access to cancer therapy [1]. In Nigeria, there are 72,000 cancer-related fatalities and 102,000 new cancer cases per year. Complex diseases like cancer are currently emerging as significant healthcare concerns in Nigeria, a country with a population of around 200 million [2]. Lung cancer has been the main cause of cancer-related fatalities in men worldwide. However, with an estimated 1.7 million new cases and 521,900 deaths in 2012 compared to 1.38 million new cases and 458,000 deaths in 2008, and 2.3 million new cases and 670,000 deaths in 2022, breast cancer remained the most common disease diagnosed in women and the leading cause of cancer death globally [3,4]. In the US, it was anticipated that in 2015 there would be 231,840 (29%) new cases of invasive breast cancer diagnosed in women, compared to 105,590 (13%) cases of lung cancer in the same population [5]. According to estimates from the World Health Organization, 5,884,000 years of life were lost worldwide in 2004 as a result of female breast cancer [3]. According to Joko-Fru *et al*. [6], this accounted for little over 1% of all female premature deaths overall, with rates varying from about 8% in some regions of Europe to less than 0.5% in Africa. The most frequent soft tissue sarcoma in children and adolescents under the age of 20 is rhabdomyosarcoma (RD). Five cases per million children and adolescents are diagnosed with RD annually in the US, and more than half of all cases occur in the first ten years of life [7]. Rhabdomyosarcoma is a prevalent cause of mortality in this age group in Nigeria and frequently affects children. The head and neck areas are the most often affected anatomical sites, and the most common variant is embryonal rhabdomyosarcoma [8]. Considering the disease's high-profile nature, treating it has been an ongoing battle with only moderately successful results [9]. Current cancer treatment options include surgical excision of the malignancy and radiation therapy for the extensive accumulated cancer biomass, usually followed by systemic chemotherapy for maintenance. The most often used chemotherapy drugs are antimetabolites, such as methotrexate, DNA-interactive drugs, such as cisplatin and doxorubicin, and anti-tubulin drugs, such as taxanes, hormones, and molecular targeting drugs [10]. Recurrence of cancer, drug resistance, and severe side effects on tissues that aren't being targeted are chemotherapy's main drawbacks. These concerns can limit the use of anticancer medications and lower patients' quality of life [9]. The hunt for novel, promising anticancer agents with greater efficacy and lesser side effects continues in order to address the issues with current therapy.

Plant-based phytochemicals and their derivatives hold promise for increasing cancer patients' response rates and reducing side

effects. Many of these phytochemicals, including alkaloids, flavonoids, phenolics, tannins, glycosides, gums, resins, and oils, are biologically active substances that are present in nature and have strong anticancer potential [11]. Despite the fact that numerous anticancer agents have been made from medicinal plants, many undiscovered plants still have anticancer potential [12]. In various cancer institutes, around 114,000 plant extracts are being examined for their anticancer properties [13]. In order to add to the pool of anticancer compounds that could be turned into chemotherapeutic agents, it is imperative to do additional research on various medicinal plant extracts to see whether they demonstrate anticancer potential [14]. Four (4) plants from two (2) families (table 1) were chosen from the literature review for the evaluation of their cytotoxic effects and their phytochemical constituents.

Table 1. Selected Medicinal Plants analyzed for cytotoxicity

Botanical name	Common name	Local name	FHI number	Part used	Habit	Pharmacological actions
Alternanthera sessilis (L.) R.Br. ex DC. (Amaranthaceae)	Sessile joyweed	Ewé Rèkùrekù Dagunro (Yoruba)	112838	whole	Herb	Wound healing, antioxidant, antiallergic, anti-inflammatory, antibacterial [15]
Chamaecrista rotundifolia var. *grandiflora* (Benth) H.S.Irwin & Barneby (Leguminosae)	Round Leaf Cassia	Epa-Ile (Yoruba)	112837	whole	Herb	Anticoccidial [16]
Indigofera nummularriifolia (Linn). Livera ex Alston (Leguminosae)	Coinwort indigo	Cuùcún kàryaá (Hausa)	112560	whole	Herb	Antimicrobial [17]
Zornia glochidiata DC. (Leguminosae)	Zornia plant	Maraek-emori (Hausa)	112559	whole	Herb	Spasmolytic and antimicrobial, anticancer [18,19]

RESULTS

MTT assay and selectivity studies

The results obtained showed that from the selected plant extracts, the extract of INW had the highest cytotoxic effect on MCF-7, with an IC_{50} = 1.22 µg/mL, followed by ASW extract with an IC_{50} = 3.74 µg/mL, as compared to the standard reference drug, vincristine (IC_{50} = 0.25 µg/mL). Similarly, on RD, INW had the highest cytotoxic effect with an IC_{50} = 6.51 µg/mL, followed by the extract of ZGW with an IC_{50} = 25.52 µg/mL, as compared to vincristine (IC_{50} = 2.89 µg/mL).

The SI result revealed that INW had a SI= 8.2 on MCF-7 when compared to vincristine with SI= 0.3. Similarly, INW had a SI= 1.5 compared to vincristine with SI = 0.026.

DISCUSSION

Since time immemorial, many cultures in the world have utilised medicinal plants to treat several ailments, especially cancer. With regard to this, the cytotoxic effects of four selected medicinal plants were assessed *in vitro* using MTT colorimetric assay against breast cancer (MCF) and rhabdomyosarcoma (RD).

Phytochemicals from plants are thought to be more versatile, less resistant, and more effective than drugs with synthetic origins. They also have more positive biological properties such as antibacterial, antioxidant, antidiarrheal, anticancer, and analgesic action [23, 24]. Natural products derived from medicinal plants can be a suitable starting point for the creation of prospective anti-cancer therapies that are effective both *in vitro* and *in vivo*. The phytochemical screening of ASW, CRW, INW, and ZGW revealed that they contain constituents such as saponins, steroids/sterols, tannins, alkaloids, coumarins, flavonoids, and cardenolides. Numerous phytochemicals have been shown to have a variety of functions that may aid in the prevention of chronic diseases. For instance, saponins prevent hypercholesterolemia and exhibit antibacterial properties whereas steroids and terpenoids exhibit analgesic and antipyretic properties. Alkaloids protect against chronic diseases like malaria and also exhibit anticancer properties. Tannins have astringent properties that speed up the healing of wounds and inflamed mucous membranes [25, 26].

The MTT assay is a colorimetric assay that assesses cell viability and is sensitive, quantitative, and reproducible. The ability of the cellular mitochondrial dehydrogenase enzyme to transform the yellow water-soluble substrate (MTT) into the deep blue/purple water-insoluble formazan product provides the basis for this assay. In a variety of cell lines, the amount of formazan generated is directly related to the cell number [27]. The American National Cancer Institute (NCI) defines cytotoxicity for crude extracts as a CC_{50}<30 µg/mL in a preliminary assessment after a 72-hour exposure interval [28]. The result obtained showed that the four extracts had cytotoxic effect with IC_{50} less than 30 µg/mL on MCF-7, while INW and ZGW extracts had cytotoxicity with IC_{50} less than 30 µg/mL on RD. However, INW had the best activity against the cancer cells. Either or a combination of the chemical constituents

Table 2. Preliminary phytochemical evaluation of the selected plant extracts

Tests	Test plants			
	A. sessilis	*C. rotundifolia*	*I. nummulariifolia*	*Z. glochidiata*
Carbohydrate				
Molisch test	+	+	+	+
Saponins				
Frothing test	+	+	+	+
Tannins				
Ferric chloride test	+	+	+	-
Flavonoids				
General test	+	+	+	+
Lead acetate test	+	+	+	+
Cardenolides				
Kedde test	+	+	+	+
2-Deoxy Sugars				
Keller-Killiani test	+	+	+	+
Anthraquinone				
Borntrager's test	-	+	-	+
Alkaloids				
Wagner	+	+	+	+
Mayer	+	+	+	+
Dragendorff	+	+	+	+
Coumarins	+	+	+	+
Sterols/Steroids	+	+	+	+
Terpenoids	+	+	+	+

+ = present; - = absent

Table 3. Cytotoxic activity of crude extracts of the selected plant extracts on human cancer and normal cells

Sample	VERO	MCF-7		RD	
	CC_{50}±SD (µg/mL)	CC_{50}±SD (µg/mL)	SI	CC_{50}±SD (µg/mL)	SI
A. sessilis	5.93±0.02	3.74±0.84	1.6	61.0±1.20	0.1
C. rotundifolia	11.59±0.95	5.49±1.01	2.1	101.3±1.89	0.1
I. nummulariifolia	10.06±1.31	1.22±0.03	8.2	6.51±0.81	1.5
Z. glochidiata	14.52±1.45	4.22±0.12	3.4	25.52±0.17	0.5
Vincristine	0.07±0.00	0.25±0.01	0.3	2.89±0.23	0.026

SI= selective index; CC_{50}= 50% cytotoxic concentration; SD= Standard Deviation
MTT method, with the cells incubated with the test agents for 72 h (IC_{50} ± SD, n = 3) by nonlinear regression

present in the INW extract [17] could be implicated in the cytotoxic activity of the plant extract. Alkaloids, flavonoids, lignans, saponins, terpenes, taxanes, vitamins, minerals, glycosides, gums, etc., all play important roles in either inhibiting cancer cells activating proteins, enzymes, and signaling pathways or by activating DNA repair mechanism, stimulating the formation of protective enzymes, inducing antioxidant action (antioxidant enzymes e.g. GSH, GST and GPxn), thus showing strong anticancer effects in terms of their efficacy on the proteins, enzymes and signaling pathways [29]. No cytotoxic constituents have been isolated from INW; however, previous studies have shown constituents such as β-sitosterol and β-sitosterol-glucoside from *I. zollingeriana* to exhibit cytotoxic effects against HepG2 and Huh7 (Hepatocellular cancer), but not against normal human primary fibroblasts cells [30].

Cancer chemotherapy's main objective is to specifically target cancer cells without causing harm to healthy cells. This is the limitation of the use of numerous conventional chemotherapy agents; as a result, selective toxicity must be considered in the search for leads for the treatment of cancer [31]. The selectivity index (SI) of the extracts was calculated to see if the extracts would be cytotoxic to cancer cell lines with very little or no damage to normal cells. *In vitro* studies classify SI values between 1 and 10 as weakly selective, below 1 as non-selective (toxic), and above 10 as safe (non-toxic) [7]. From the selectivity studies, INW was approximately 8 times more toxic to the MCF-7 and approximately 2 times more toxic to the RD compared to normal cells (Vero), an indication that its cytotoxic activity is weakly selective for the cancer cells under investigation as compared with vincristine, which is non-selective (toxic).

Indigofera nummulariifolia is a medicinal plant found in tropical and subtropical regions of the world, such as India, Nigeria, Madagascar, Australia and parts of Asia. According to Santosh *et al*. [32], it was allegedly utilized by ethnic communities to treat liver ailments. This supports Okafor and Noah's [17] examination into the phytochemical components and antibacterial potentials of the entire plant part. However, a wide range of pharmacological properties are present in numerous different *Indigofera* species, according to research. For instance, *I. tinctoria* has demonstrated antioxidant, free radical scavenging, and anti-dyslipidemic action, *I. pulchra* is reported to have anti-venom capabilities [33], *I. heterantha* is used as herbal medicine as well as folk medicine to treat digestive disorders and stomach pain, and *I. oblongifolia* proven to possess antimicrobial, hepatoprotective and strong lipoxygenase inhibitory activity [34, 35]. Additionally, compounds such as kaempferol 3-O-α-l-rhamnopyranoside, kaempferol 7-O-α-l-rhamnopyranoside, kaempferol 3-O-α-l-rhamnopyranoside-7-O-α-l-rhamnopyranoside, kaempferol 3-O-α-l-arabinopyranoside-7-O-α-l-rhamnopyranoside, indigo, and indirubin have been isolated from *I. truxillensis*, while quercetin 7-O-β-d-glucopyranoside, quercetin 3-O-[β-d-xylopyranosyl-(1→2)-β-d-galactopyranoside], quercetin 3-O-[α-l-rhamnopyranosyl-(1→6)-β-d-glucopyranoside] and quercetin 3-O-[β-d-glucopyranosyl-(1→2)-β-d-glucopyranoside], indigo, and indirubin have also been isolated from *I. suffruticosa* [36]. These compounds were tested for mutagenic activity by *Salmonella/*microsome assays and it was revealed that indigo and indirubin were mainly responsible for the activity.

CONCLUSION

The observed cytotoxic activity of extracts of the medicinal plants against the two selected human cancer cells has lent credence to ethno-medicinal uses of the study plants especially *Z. glochidiata* used to treat gastric cancer. This also strongly suggests a broad-spectrum potency of the active plant extracts. Therefore, further analysis to evaluate the cytotoxic activity using different fractions, along with the isolation and elucidation of their cytotoxic compounds. Also, evaluating their potential for treating other conditions is essential, especially given the limited scientific data available on the studied plants.

MATERIALS AND METHODS

Plant collection and extraction

Alternanthera sessilis whole (ASW) was collected from Obafemi Awolowo Hall field, the University of Ibadan, *C. rotundifolia* whole (CRW), *I. nummulariifolia* whole *(*INW*)*, and *Z. glochidiata* whole (ZGW) were collected in the month of May, 2019, from the University of Ilorin environs Mr. Adeyemo of the Forest Herbarium Institute (FHI), Ibadan identified and validated the plants by comparison with appropriate voucher specimens. The plants collected were dried in a shade and ground into a coarse powder at room temperature. Plant material (200 g each) was macerated in redistilled methanol for 72 hours with occasional stirring. Filtration was carried out and the extracts were concentrated to dryness using a rotary evaporator. After drying, the extracts were stored at 4 °C until further analysis.

Phytochemical Analysis

The methanol extracts of the selected plants were subjected to different chemical tests for the detection of different phytoconstituents using standard procedures [20, 21].

Carbohydrate Molisch Test

To 1 mL of the extracts, 5 mL of water was added and 2-3 drops of 20% ethanolic α-naphtol. Then 1 mL of concentrated H_2SO_4 was added drop-wisely to the side of the test-tube. A brown, red, or violet ring at the inter-phase is positive for all carbohydrates.

Alkaloids

The crude extract (0.5 g) was added to 10 mL of 10% Hydrochloric acid. This was placed in a water bath for 10 minutes; the extract was filtered and allowed to cool. The pH was adjusted to about 6-7 by adding 10% ammonium hydroxide. Then 5 mL of the filtrate was taken into separate test tubes and a small quantity of Wagner's, Mayer's, and Dragendorff' reagents were added and observed. The presence of colored (Wagner- yellow; Mayer- cream; and Dragendorff reddish- brown) precipitates indicates the presence of alkaloids.

Phenols/Tannins

Ferric Chloride test: The crude extract (0.5 g) was added to 20 mL of distilled water, boiled for 10 minutes, and filtered whilst hot. The filtrate obtained is allowed to cool and 1 mL of $FeCl_3$ reagent (5%) is added to the filtrate. An intense coloration ranging from blue-black, green or blue-green, indicates the presence of phenols and tannins.

Anthraquinone Glucosides

Bontrager's test: To 0.5 g of the extract, 2 mL of 10%

Hydrochloric acid was added and placed in a water bath for 10 minutes. This is filtered while hot and allowed to cool. The filtrate is then partitioned with 2.5 mL of dichloromethane (DCM) and shaken gently. The DCM layer was transferred into a clean test tube and an equal volume of ammonium hydroxide was added and shaken gently. The presence of a delicate rose-pink layer on the test solution indicates the presence of anthraquinones.

Flavonoids

General test: To 0.2 g of the extract, 5 mL of ethanol and 3 drops of Ferric chloride was added. The presence observation of a dark-green color confirms the presence of flavonoids and flavonoids.

Lead acetate test: Extract 5 mg is added to 2 mL of distilled water and warmed in a water- bath for 5 minutes. After cooling, a few drops of 10% lead acetate solution were added. The appearance of yellow-colored precipitates indicates the presence of flavonoids.

Saponins

Extract (1 mg) is diluted with 20 mL of distilled water. This was shaken well in a graduated cylinder for 10 minutes. The formation of foam to a length of 1 cm is indicative of the presence of saponins and steroids.

Cardenolides

To 0.5 g of dried alcohol extract was added 2 drops of 15% lead acetate solution and 3mL of Dichloromethane and evaporated to dryness. Then 1mL of 2% 3,5-dinitrobenzoic acid in methanol (Kedde's Solution A) and 2-3 drops of 5% NaOH (Kedde's Solution B). The formation of dirty pink color indicates the presence of cardenolides).

2-Deoxy Sugars

To the extract (0.5 g) is added 3 mL of ferric chloride reagent (0.3 mL of 10% $FeCl_3$ in 50 mL glacial acetic acid) in a clean test tube, then 2 mL of concentrated H_2SO_4 was added carefully down the sides of the test tube.

Sterols

Salkowski's test: The extract (10 mg) was dissolved in 2 mL of DCM; 2 mL of concentrated H_2SO_4 was added along the side of the test tube. The presence of sterols and steroids is indicated if the upper layer turned red while the lower layer turns yellow. A confirmation is when a green fluorescence is obtained under UV light.

Coumarins

To 0.2 g of the extract is added 2 mL distilled water and warmed for 5 minutes and allowed to cool. Then 2 mL of 10 % Sodium hydroxide is added to the aqueous extract. The formation of yellow color indicates the presence of coumarins.

Terpenoids

0.5 mL of the extract was treated with 2 mL of DCM and concentrated H_2SO_4. The formation of red-brown coloration indicates the presence of terpenoids.

Effect of plant extracts on cell proliferation by MTT assay

Cell culture

Human rhabdomyosarcoma (RD), breast cancer (MCF-7), and normal Vero cells were obtained from the World Health Organization (WHO) reference polio laboratory, University College Hospital, Ibadan, Nigeria. Eagle's minimum essential medium supplemented with 10% Foetal Bovine Serum, 100 units/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine, 0.07% $NaHCO_3$, and 1% non-essential amino acids and vitamin solution were used to culture the cells. Cultures were kept at 37 °C in a humidified environment with 5% CO_2 and passaged every two weeks.

Cytotoxicity assay

Using the methods described by Mosmann [22], the ability of the cells to cleave the tetrazolium salt MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] [Sigma, Chem, St. Louis, MO]) by the mitochondrial enzyme succinate dehydrogenase was evaluated. Each extract was pre-solubilized in dimethyl-sulphoxide (DMSO) at 37 °C and serially diluted in ten-folds to give different concentrations (1000–0.01 µg/mL), with the last concentration of DMSO in the tested dilutions not exceeding 1%. For 24 hours, confluent monolayers of the cells were grown in 96 well-microtitre plates. At 37 °C in a CO_2 environment, cells were incubated with varying concentrations of the extracts, Vincristine (positive control), and a negative control (growth medium alone instead of plant extract) in triplicate for 72 hours. Furthermore, the viability of the cell was evaluated microscopically for the cytopathic effect (CPE) whether it is present or absent. At the end of the 72-hour treatment period, the supernatants were removed from the wells, and 25 µL of MTT solution (2 mg dissolved in 1 mL of PBS) was applied to each well. To dissolve the formazan crystals, the plates were incubated at 37 °C for 2 hours before 75 µL of DMSO was applied to each well. The microtitre plates were agitated for 15 min, and the optical density was measured using a multi-well spectrophotometer (Multiskan, Thermo Fisher Scientific, Waltham, MA) at 492 nm. The extract concentration required to reduce cell viability by half was identified as the 50% cytotoxic concentration (CC_{50}). A non-linear regression curve included in the GraphPad Prism statistical software was used to compute the CC_{50} value and the standard error mean (SEM).

Selectivity index (SI)

The normal African green monkey kidney epithelial cell line (Vero) was used to measure the SI. The SI of the extracts was calculated as the ratio of the cytotoxic effect on normal cell lines to the cytotoxic effect on cancer (MCF-7 and RD) cells [7].

Statistical analysis

The data collected was analyzed using Microsoft Excel 2010, followed by GraphPad Prism 6.0. To determine IC_{50} values, the data were normalized so that the highest value in the dataset was aligned with 100%, and the lowest value was set to 0%. The standard and extracts' concentrations were log-transformed and plotted against the dose-response to determine the IC_{50} values through nonlinear regression. For this analysis, the log (inhibitor) vs normalized response with the Variable slope option was selected.

Author contributions

All authors were involved in the concept, design, and collection of data, interpretation, writing and critically revising the article. All authors approved the final version of the article.

Acknowledgements

The authors sincerely appreciate the WHO Polio Laboratory,

Department of Virology, College of Medicine, University of Ibadan, Nigeria for providing the equipment and materials for this study. We also acknowledge the staff of the Forest Herbarium Ibadan (FHI) for the identification of plants.

Funding
No funding

Conflict of Interest
The authors declare no conflict of interest.

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