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Original Research Article

Antiproliferative Potential of Ethanol Leaf Extract of *Motandra guineensis* (Thonn.) A.DC. (Apocynaceae) against Human Melanoma and Ovarian Cancer Cells

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ABSTRACT

In ethnomedicine, *Motandra guineensis* (Thonn.) A.DC. (Apocynaceae) is used to massage abscessed gums and manage pain. This study aimed to evaluate the antiproliferative effect of leaves of *M. guineensis* against human ovarian (OVCAR3) and melanoma (MDA-MB-435) skin cancer cells. Crude ethanol extract and fractions of *M. guineensis* leaves were evaluated for antiproliferative effect on OVCAR3 and MDA-MB-435 cells using cell viability assay at 2 and 20 µg/ml. In addition, the inhibitory effect of extract and fractions on nitric oxide (NO) production with lipopolysaccharide (LPS) stimulation (Greiss assay) on murine macrophage cells (RAW 264.7) at 0.3125 – 10 µg/ml was evaluated. Cell viability was evaluated using murine macrophage cells while toxicity assessment was done on Vero cells. Antiproliferative activity profile (% cell death) of extract and fractions followed the order: butanol (30% and 23%) > aqueous (28% and 21%) > ethyl acetate (22% and 19%) > crude (22% and 0%) at 20 µg/ml in OVCAR3 and MDA-MB-435 cell lines respectively. Percentage NO inhibitory activity showed hexane fraction with the highest activity and aqueous fraction with the least activity at 10 µg/ml. Extract and fractions were less toxic in Vero cells when compared to the standard drug, Tamoxifen. Toxicity assessment using murine macrophage cells showed no significant difference in cell viability when compared to standard. Results indicate antiproliferative potential, NO inhibitory potential and safety of crude ethanol leaf extract and fractions of *M. guineensis*.

***Keywords*:** Antiproliferative, nitric oxide inhibition, *Motandra guineensis*, Ovarian cancer, Human melanoma skin cancer, Vero cells, murine macrophage cells

Introduction

From ancient times, man has used plants to treat human and animal diseases mostly through trial and error. Man has made preparations from these plants either processed as extracts or in whole form as dried plant parts. The high cost of orthodox and modern drugs has necessitated the search for affordable, safe yet effective treatment options from nature. Globally, 80% of the world population uses traditional medicine while up to 88% in Sub-Saharan Africa depend on traditional, complementary, and alternative medicine especially plants for their healthcare needs.[1]

Motandra guineensis (Thonn) A.DC. (Apocynaceae) is a climbing shrub or liana that grows on trees as its support and is found in parts of West, Central and East Africa in countries such as Ivory Coast, Nigeria, Senegal, Congo and Angola. The plant is called Agba doje or Bodekadun in the Yoruba language. It is used as mouthwash massage into abscessed gums and in the management of pain as well as a sedative in the management of insanity in ethnomedicine.[2] Ethanol extracts of aerial parts were shown to cause central nervous system (CNS) depressant activity in mice.[3] The seed extract was also shown to elevate total cholesterol, oestrogen and progesterone in pregnant rabbits.[4]

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The plant was reported to contain flavonoids, terpenoids, tannins and steroids while phytol, hexadecenoic acid and ethyl ester are the volatile components of the plant after GC-MS analysis.[3] Existing scientific literature showed a scarcity of information on pharmacological effects and a dearth of chemical data on the plant. Cancer is the leading cause of death worldwide as of 2020. Ten million deaths annually are due to cancers majorly lung, colorectal, liver, stomach and breast cancers.[5,6] The cost of managing cancer even in Africa is huge as there were 800,000 new cases and 520,000 deaths due to cancers in Sub-Saharan Africa majorly breast and cervical cancers.[7,8] Cancer causes over 72,000 deaths annually in Nigeria with the most common ones being breast and cervical cancer.[9] Risk factors implicated in cancer development include benzene and other chemicals, excess alcohol intake and environmental toxins amongst others. The mechanisms with which these risk factors lead to cancers and malignancies are not yet fully established.[10] There is hope that the reduction of these risks as well as increased screening habits especially for breast, colorectal and prostate cancers may in coming years cause a corresponding decline in cases and mortality arising from them however slow diagnosis and therapy may cause an increase in advanced-stage disease and death.[11]

Inflammation has been implicated as a hallmark in the pathogenesis of cancer and tumorigenesis. There are many inflammatory mediators of which nitric oxide is one of the most notable. Inducible nitric oxide synthase (iNOS) produces the highest amounts of nitric oxide independent of calcium. Nitric oxide has a crucial involvement in inflammatory response. The role of nitric oxide in the biological process of cancer development includes cytoprotective effects on tumour cells and triggering carcinogenesis through the activation of oncogenic pathways.[12,13] Treatment of cancer involves many interventions of which chemotherapy with the use of chemical agents is a mainstay. However, cancer chemotherapy is non-specific and may lead to adverse effects that cause reduced quality of life in patients.[10] Hence, this has led to the search for alternative therapy from nature such as medicinal

plants that may be safer yet effective. Medicinal plants have been used traditionally to treat illnesses and chronic conditions such as cancers as well as proliferative conditions. An example of such plants is the Rose periwinkle, *Catharanthus roseus* L. (Apocynaceae) which is the source of the *Vinca* alkaloids, vincristine and vinblastine.[10]
To understand the antiproliferative potential and nitric oxide inhibitory effect of crude extract and fractions of *Motandra guineensis* against human ovarian and melanoma skin cancer cells and murine macrophage cells with LPS stimulation respectively, the study evaluated and assessed these activities using standard *in vitro* models. Furthermore, the study was designed to predict safety using toxicity assay on green monkey kidney Vero cells and murine macrophage cells.

Materials and Methods

Plant material collection and authentication
Plant leaves (Figure 1) were collected from the Obafemi Awolowo University campus, Ile-Ife, Osun state (Latitude 7.5196263º, Longitude 4.5216767º), Nigeria, in July 2022, identified and authenticated by Mr. I.I. Ogunlowo of the Obafemi Awolowo University Pharmacognosy Department, Ile-Ife, Osun state. A voucher specimen was prepared and deposited at the Medicinal Plant herbarium, Department of Pharmacognosy, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Osun state, Nigeria. The voucher number assigned to the specimen was FPI 2351.

Extraction and liquid-liquid partitioning of extract
The leaves of the plant were dried using a drying oven at 40°C for 48 hours and pulverised using a grinding machine (Viking) to obtain 3.15kg of the dried plant material. It was then macerated using 54L of 95% ethanol and filtered. The hydroethanolic extract was dried under reduced pressure at 30°C using a rotary evaporator to give 210g (6.7% yield). The extract was suspended in 1.5L of deionised water and partitioned in increasing order of polarity with 6L each of hexane, ethyl acetate and butanol to obtain the hexane, ethyl acetate, butanol and aqueous fractions respectively which were dried under reduced pressure.[14]

Cell viability assay on human ovarian and human melanoma cancer cells
Human melanoma (MDA-MB-435) and human ovarian cancer (OVCAR3) cells were purchased from the American Type Culture Collection (Manassas, VA). The cell lines were cultivated at 37°C in 5% Carbon dioxide in RPMI 1640 medium and supplemented with fetal bovine serum (10%), penicillin (100 units/ml), and streptomycin (100 µg/ml). Cells at the log growth stage were collected by trypsinisation followed by two washes to ensure the absence of enzyme. A complete count of 5,000 cells was seeded per well of a 96-well plate (Microtest 96®, Falcon) and incubated all night at 37°C in 5% Carbon dioxide. Test samples were dissolved in DMSO, diluted and added to the appropriate wells. The cells were grown in the presence of the test substances for 72 h at 37°C and assessed for viability with CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega Corp, Madison, WI), a commercial absorbance assay that measures viable cells. Cell viability was expressed in percentage comparatively to the solvent (DMSO) control.[15] Percentage cell death was the difference between 100 and survival percentage. The crude extract and fractions were analysed in three biological replicates, with 10% cell death at 20 µg/ml indicative of activity.

Nitric Oxide Inhibitory assay
The murine macrophage RAW 264.7 cell line was obtained from the American Type Culture Collection (VA, USA) and preserved in Dulbecco's modified Eagle's medium (DMEM, Gibco, NY, USA) containing 10% fetal bovine serum (FBS, Gibco) and 1% antibiotics (Gibco). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (LPS from *Escherichia coli* O111:B4). RAW 264.7 cells (5 × 104) were seeded to a 6-well plate. After 24 h incubation, the cultured media was replaced with 200 µl FBS-free DMEM that contained 100 ng/ml LPS in the presence of test substances. Epigallocatechin gallate, EGCG (Sigma) was used as positive control. The concentration of Nitric oxide in the supernatant was measured according to the Griess assay. The harvested cultured media (100 µl) was then transferred into a 96-well plate and mixed with the same volume of Griess reagent (0.04 g/mL in distilled water, Sigma). Developed diazonium was measured using a Synergy HT microplate reader (BioTek, VT, USA) at 548 nm. Varying concentrations of nitrite (0, 20, 40, 80, and 100 µg/ml) were used to obtain a standard curve. All the experiments were performed in triplicates and analysed using SigmaPlot 14.0 (Systat Software, CA, USA) unless otherwise stated.[16] Percentage inhibition of Nitric oxide was calculated relative to DMSO.

Toxicity assay on Vero cells
Green monkey kidney cells (Vero cell CCL- 81) were used to assess the cytotoxicity of the crude extract and fractions. Cultured Vero cells in Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) were augmented with the addition of a penicillin and streptomycin antibiotic mix. The cells were microscopically quantified into a density of 2×10^5 cells/ml. A 100 µl portion of cell suspension was added to the test substances and incubated for 3 days at 5% Carbon dioxide and 37 °C. At the end of the incubation, 20 µl of 0.6 mM resazurin was added, and the fluorescence was measured following incubation for 4 h to calculate IC_{50}.[14]

Toxicity Assay on Murine Macrophage cells
Toxicity assay was performed in RAW 264.7 cells without LPS stimulation. After 24 h treatment of extract and fractions (from nitric oxide inhibitory assay), Water Soluble Tetrazolium 1 (WST-1, Roche, Mannheim, Germany) (20 µl) was added to each well. Coloured formazan was measured at 490 nm using a microplate reader.[16] Percentage cell viability was evaluated relative to DMSO.

Statistical analysis
Data in triplicates were processed using Microsoft Excel to obtain mean and standard errors of the mean. Data for nitric oxide inhibitory activity and murine macrophage toxicity were analysed using two-way repeated measures analysis of variance (ANOVA) and Dunnett's multiple comparison tests The results were considered significant at $p < 0.05$. GraphPad Prism 9 (San Diego, CA, USA) was used for the analysis.

Figure 1: Leaves and fruit of *Motandra guineensis* (Thonn) A.DC.

Results and Discussion

Yield of extract and fractions
The percentage yield of the dried extract of *M. guineensis* was 6.7% which was quite similar to the 6.4% that was reported by a recent study.[3] The hexane fraction (122g) gave the highest yield which indicates a high content of non-polar constituents in the extract compared to ethyl acetate (8g), butanol (11g) and aqueous (48g) fractions which may contain less non-polar, moderately polar and polar constituents.

Antiproliferative assay of Motandra guineensis extract and fractions
Table 1 shows the results of cell viability assay on human ovarian and human melanoma skin cancer cells as a measure of antiproliferative activity. The extract and fractions apart from the hexane fraction were active against human ovarian and melanoma skin cancer cells. The hexane fraction which is the non-polar fraction of the plant had the least activity while the other fractions (butanol, aqueous, ethyl acetate) which had polar components had better activity. The butanol fraction was the most active against human ovarian cancer cells with cell death (29.56%) followed by the aqueous fraction, crude extract, ethyl acetate fraction and the hexane fraction.

Table 1: Antiproliferative assay of *Motandra guineensis* (Thonn) A.DC. extract and fractions

Results (% Cell Viability)					Cell death (% Cell death)			
	OVCAR3		MDA-MB-435		OVCAR3		MDA-MB-435	
	2µg/ml	20µg/ml	2µg/ml	20µg/ml	2µg/ml	20µg/ml	2µg/ml	20µg/ml
CEE	91.10	78.24	87.44	>100	8.90	21.76	12.56	<0
HF	91.82	>100	95.65	>100	8.80	<0	4.35	<0
EF	86.53	78.46	90.49	80.85	13.47	21.54	9.51	19.15
BF	84.24	70.44	82.82	77.49	15.76	29.56	17.18	22.51
AF	77.93	71.57	85.58	79.11	22.07	28.43	14.42	20.89

CEE = Crude ethanol extract, HF = Hexane fraction, EF = Ethyl acetate fraction, BF = Butanol fraction, AF = Aqueous fraction

The butanol fraction was the most active with cell death (22.51%) against human melanoma skin cancer cells followed by aqueous fraction, ethyl acetate fraction, crude extract and hexane fraction. All the fractions had some measure of activity on both cancer cell lines apart from the hexane fraction which had no activity on the cell lines even at higher concentrations (20 µg/ml). The poor activity of the hexane fraction may be attributed to the non-polar nature of its constituents while the presence of activity in other fractions may be due to their polar nature. Hence, it may be suggested that the polar nature of the constituents may be responsible for antiproliferative activity in this plant. The results obtained from the assays suggest that the extract contains compounds that may possess antiproliferative activity.

Although, there is a paucity of information on the genus, the results from other members of the Apocynaceae family such as *Catharanthus roseus* L., *Mondia whitei* (Hook.f.) Skeels and *Tabernaemontana divaricata* (L.) R.Br. which have been reported to be cytotoxic to brine shrimp, human colorectal adenocarcinoma (HT-29) and cervical cancer (HeLa) cell lines and human epidermoid larynx carcinoma cancer (Hep 2) respectively support the antiproliferative activity being observed in *M. guineensis* .[17,18,19]

Toxicity assay of Motandra guineensis extract and fractions

Table 2 shows the results of toxicity testing on green monkey Vero cells as a measure of safety. Crude ethanol extract and fractions were less toxic in Vero cells than the standard drug, Tamoxifen. The butanol and aqueous fractions (IC_{50} > 100 µg/ml) were the least toxic when compared to the standard drug, Tamoxifen (5.31 µg/ml). This was followed by the hexane fraction (83.47 µg/ml), crude extract (56.23 µg/ml) and ethyl acetate fraction (55.65 µg/ml). These results suggest that the more polar fractions (butanol and aqueous) may contain compounds that may be less toxic to Vero cells when compared with the other fractions. Therefore, the extent of toxicity to Vero cells may be attributed to the polarity of the fractions.

Table 3 shows the results of toxicity testing in murine macrophage cells as a measure of safety. The nature of the solvent used to partition the extract did not influence the toxicity to murine macrophages as the crude ethanol extract and fractions were non-toxic in murine macrophage cells. There was no significant difference in toxicity of the extract and fractions in murine macrophage cells compared to the standard drug, EGCG ($p > 0.05$). The results from this study are consistent with a recent study that showed that there were no signs of toxicity in mice when they were treated with ethanol extract from the aerial parts of the plant.[3] Metabolites from *Tabernaemontana catharinensis* A.DC., another member of the Apocynaceae family, have also been reported to be non-toxic to normal cells but toxic to tumour cells.[20]

Nitric oxide inhibitory assay of Motandra guineensis extract and fractions

Table 4 shows the results of percentage nitric oxide inhibition in murine macrophage cells as a measure of reduction in inflammation. The results showed that nitric oxide inhibitory activity increased with concentration. The hexane fraction (13.17%) at the highest concentration of 10 µg/ml was the most active followed by crude extract (11.32%), the butanol fraction (8.22%), the ethyl acetate fraction (5.45%) and the aqueous fraction (1.47%) respectively. The activity observed can be said to be influenced by the polarity of the constituents in the fraction as the least polar, hexane fraction had the highest activity and the most polar, aqueous fraction had the least activity. Hexane is known to extract non-polar phytoconstituents such as steroids. An earlier study has reported the presence of steroids in the crude extract of *M. guineensis*.[3] Steroids have been reported to have anti-inflammatory activity[21]. Hence, this may be responsible for the better activity observed in the hexane fraction in this model. Other members of the Apocynaceae family such as *Rauvolfia tetraphylla* L. and *Plumeria rubra* L. have been reported to inhibit nitric oxide production in murine macrophage cells[22] and exhibit good anti-inlammatory activity[23] respectively.

Table 2: Toxicity assay (IC_{50}) of *Motandra guineensis* (Thonn) A.DC. extracts and fractions in Vero cells

Extract and fractions	IC_{50} (µg/ml)
Crude Ethanol extract	56.23
Hexane	83.47
Ethyl acetate	55.75
Butanol	>100
Aqueous	>100
Tamoxifen	5.31

Table 3: Toxicity assay of *Motandra guineensis* (Thonn) A.DC. extract and fractions in Murine macrophage cells

Concentration (ug/ml)	Crude extract	Hexane fraction	Ethyl acetate fraction	Butanol fraction	Aqueous fraction	EGCG (3.125 - 100uM)
0.3125	105.08 ± 1.63	106.33 ± 0.73	109.01 ± 4.59	105.24 ± 1.45	111.77 ± 2.30	108.27 ± 2.73
0.625	100.35 ± 2.18	96.40 ± 1.20	100.88 ± 4.83	96.48 ± 1.88	102.81 ± 3.40	99.69 ± 5.69
1.25	93.17 ± 0.54[a]	93.30 ± 2.30	87.90 ± 6.48	87.86 ± 0.40[a]	100.16 ± 0.33	100.96 ± 1.43
2.5	94.09 ± 5.38	100.10 ± 0.71	99.09 ± 2.28	98.19 ± 0.96	101.94 ± 3.32	99.62 ± 0.60
5	91.19 ± 1.72	92.30 ± 1.01	110.27 ± 3.42[a]	96.02 ± 2.49[a]	101.86 ± 1.48[a]	91.63 ± 2.41
10	92.70 ± 2.33	89.52 ± 3.25	97.74 ± 3.28	97.80 ± 5.14	99.93 ± 3.18	96.17 ± 6.51

Data expressed as Mean ± Standard error of mean at 6 concentrations
[a] = Significantly different from standard EGCG at $p \leq 0.05$ (95% confidence interval)

Table 4: Nitric oxide inhibitory assay of *Motandra guineensis* (Thonn) A.DC. extract and fractions

Concentration (ug/ml)	Crude extract	Hexane fraction	Ethyl acetate Fraction	Butanol fraction	Aqueous fraction	EGCG (3.125 - 100uM)
0.3125	5.31 ± 5.30	5.65 ± 5.99	1.46 ± 8.91	4.40 ± 7.52	2.90 ± 6.73	7.21 ± 5.13
0.625	-4.40 ± 8.51	4.08 ± 0.86	0.85 ± 1.88	8.70 ± 2.85	3.75 ± 2.92	5.72 ± 1.64
1.25	-2.57 ± 8.69	-3.23 ± 11.39	2.73 ± 4.17	8.31 ± 2.13	1.93 ± 2.59	5.85 ± 2.74
2.5	8.37 ± 3.96	1.39 ± 5.76	-4.21 ± 2.29	6.48 ± 3.90	4.75 ± 2.44[a]	11.86 ± 2.55
5	11.68 ± 5.44	8.97 ± 4.82	8.32 ± 2.73	10.39 ± 4.70[a]	6.81 ± 2.36	16.89 ± 4.95
10	11.32 ± 5.57	13.17 ± 0.49	5.45 ± 2.63[b]	8.22 ± 4.45	1.47 ± 2.85 [a]	34.44 ± 1.05

Data expressed as Mean ± Standard error of mean at 6 concentrations
[a] = Significantly different from standard EGCG at $p \leq 0.05$ (95% confidence interval)
[b] = Significantly different from standard EGCG at $p \leq 0.01$ (95% confidence interval

Conclusion

Based on the results, it can be concluded that *Motandra guineensis* possesses antiproliferative activity against human ovarian and human melanoma skin cancer cells as well as Nitric oxide inhibitory activity. The study also provided data on the toxicity of the plant on normal cells and provided a basis for the isolation of active constituents from the plant.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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