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Gene Expression Modulation of Apoptotic and Oestrogen Receptor Alpha Genes by Active Fractions of Selected Nigerian Plants on Cervical Cancer Cell Line (HeLa)

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ABSTRACT

The roles of natural product in drug discovery and development cannot be over emphasised. It plays a vital role in human therapy and gives a better understanding on the cellular pathways.This study investigated the modulatory effects of partially purified fractions of *Piper guineense* Schumach. & Thonn. (Piperaceae), *Zanthoxylum zanthoxyloides* Lam. (Rutaceae), *Amaranthus viridis* L. (Amaranthaceae), *Costus afer* Ker-Gawl. (Zingiberaceae) and *Catharanthus roseus* (L.) G. Don. (Apocynaceae) on oestrogen receptor-α (ESR-α), tumour protein p53 (TP-53), retinoblastoma (RB) and NAD(P) H quinine oxidoreductase (NQO1) genes in cervical cancer cell line (HeLa cells). *n*-Hexane, ethylacetate, chloroform, and water fractions of 80% ethanol extracts of study plants were screened with brine shrimp lethality and water-soluble tetrazolium-1 (WST-1) cytotoxicity assay. HeLa cells were treated with 1:10 dilutions of IC_{50} concentrations of test fractions for 24 hours, total RNA was extracted, RNA quality was checked, and normalized to a baseline concentration. Gene expression were monitored by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Results showed that ESR-α was downregulated ($p < 0.05$) by *P. guineense*-hexane, *C. roseus*-chloroform and *A. viridis*-ethylacetate fractions. TP53 gene was up-regulated ($p < 0.05$) by *P. guineense*-hexane and *Z. zanthoxyloides*-ethylacetate fractions. None of the test fractions caused an up-regulation in the expression of retinoblastoma gene. NQO1 gene was down-regulated ($p < 0.05$) by *C. roseus*-chloroform, *P. guineense*-hexane, *A. viridis*, *C. afer*, and *Z. zanthoxyloides* ethylacetate fractions. Our study provides scientific evidence of the possible anti-proliferative potentials of *C. roseus*-chloroform, *P. guineense*-hexane, ethylacetate fractions of *A. viridis*, *C.afer*, and *Z. zanthoxyloides* in cervical cancer.

Keywords: Natural products; Gene expression; rt-pcr; Tp53; Retinoblastoma; Oestrogen receptor-α; NAD(P)H Quinine Oxidoreductase

Introduction

Despite the increase in the usage and coverage of vaccines, cervical cancer still remains a major public health concern in developing countries.[1, 2] The infectivity and integration of high-risk human papilloma virus (HPV) into the host genome is a critical step towards the progression of precancerous lesions and ultimately cancer.[3]

The genomes of all HPV types contain approximately eight open reading frames (ORFs) that are transcribed from only one DNA strand. The ORF´s are classified into three functional parts: the early (E) region that encodes proteins necessary for viral replication, the late (L) region that encodes the structural proteins (L1–L2) required for virion assembly as well as a largely non-coding part which is known as the long control region (LCR) that contains cis elements necessary for viral replication and transcription.[4] The E6 and E7 viral proteins play key roles in tumour progression in cervical cancers by abrogating the cell cycle control proteins such as- p53, Bak, Bax, retinoblastoma (Rb) protein inversely enhancing telomerase activity, steroid receptor co-activator (Src) family kinases, activator protein (AP-1) transcription complex, and activates histone deacetylases.[5]

Viral DNA of High-Risk HPV viruses integrate into the host genome and disrupt the E2 gene known to down-regulate the expression of E6 and E7 proteins. The disruption of E2 protein results in the increased expression of E6 and E7 proteins. The E6 protein in high-risk HPV binds to p53 protein and activates ubiquitination leading to degradation of p53 protein whereas the low-risk HPV do not bind to p53 protein at any detectable level. The consequential destruction of p53 destroys the normal activities that maintain genome stability, DNA repair, G1 arrest, and apoptosis. The HPV E7 protein binds to pRb (retinoblastoma protein) resulting into the disassociation of pRb from E2F-1 (a cellular transcription factor). E2F-1 activates the transcription of genes whose products are required for the cell to enter the S phase of cell cycle.[6] The inactivation of p53 and pRb proteins can lead to an increased proliferation rate and genomic instability. As

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a result of this, the host cell accumulates more and more damaged DNA that cannot be repaired, leading to transformed cancerous cells.[7]
One of the first indications that cervical cancer is oestrogen-dependent came from the observation that these tumours typically arose in the most oestrogen-responsive tissue.[8] Oestrogen receptors (ERα and ERβ) are coded by genes located on different chromosomes (6 and 11) and their expression is altered in different target tissues in which ERα is predominant in the breast, corpus, uterine cervix, and the vagina; whereas ERβ is dominant in the lungs, prostate, testis and ovaries.[9]
The modulation of oestrogen receptor-α (ER-α) gene is therefore associated with cervical cancer and its down-regulation reduces sensitivity to oestrogens.[10]
Natural phytochemicals have been utilized in the treatment of cancer over several decades and they still remain the strategy of choice in most developing countries due to their widely acclaimed cytotoxicity, safety, and low cost since cytotoxicity assay allow a real-time measurement of cell either the viability or via cytotoxicity.[11] More so, cancer cells can gradually become resistant to available chemotherapeutic treatments.[12] Hence, the need for improved approach from medicinal plants ensues with the potential of inhibiting or modulating the expressions of some genes involved in cervical cancer pathogenesis such as oestrogen receptor-α (ESR-1), tumour protein 53 (TP53), retinoblastoma (Rb) and NAD(P)H quinine oxidoreductase gene (NQO1).
This study investigated the modulatory effects of partially purified active fractions of *Piper guineense* Schumach. & Thonn. (Piperaceae), *Zanthoxylum zanthoxyloides* Lam. (Rutaceae), *Amaranthus viridis* L. (Amaranthaceae), *Costus afer* Ker-Gawl. (Zingiberaceae), and *Catharanthus roseus* (L.) G. Don. (Apocynaceae) on genes involved in cervical cancer pathogenesis.

Materials and Methods

Collection of Plants
Fresh mature specimens comprising of leaves and stem barks of *Piper guineense*, *Zanthoxylum zanthoxyloides*, *Amaranthus viridis*, *Costus afer* and *Catharanthus roseus* were collected from their predominant GPS locations in Southwest Nigeria in the month of October, 2011. The plants were identified and photographic records were taken. Specimens were authenticated by the herbarium's taxonomist and voucher specimens (herbarium numbers; LUH6310, LUH6317, LUH6322, LUH 6321 and LUH6323, respectively) were deposited at the herbarium in the Department of Botany, University of Lagos, Lagos-Nigeria.

Plant extraction and fractionation
About 100 g of collected plant materials were dried, pulverised and extracted for two-weeks by maceration in I litre of water/ethanol (80% ethanol and 20% water). Residual plant materials were separated from the solvent by filtration though a muslin bag. The filtrates were further filtered using low ash filter papers. Filtered aqueous-ethanol extracts were concentrated under reduced pressure at 40°C using a rotary evaporator (Laborota 4000®, Schwabach-Germany). The remaining water part of the 80% ethanol was gradually evaporated at low heat and vacuum suction to minimize the denaturing of phytochemicals. The aqueous-ethanol extracts of the plant samples were individually fractionated by organic solvent extraction in the order of increasing polarity index (*n*-hexane, chloroform, ethylacetate, and water). Brineshrimp assay was to pre-screen the fractions with lethality effect serving as hit fractions for mammalian cell line. The following fractions were selected for testing based on the condition of having an LC_{50} value less than 10 µg/mL in Brinesshrimp Lethality assays: *P. guineense* chloroform fraction (CF), *C. afer* ethylacetate fraction (EAF), *A. viridis* ethylacetate fraction (EAF), *P. guineense* hexane fraction (HF), *C. roseus* chloroform fraction (CF), and *Z. zanthoxyloides* ethylacetate fraction (EAF).

Brine shrimp lethality Test (Artemia salina)
Brine shrimp lethality assay Brine shrimp lethality assay for pre-cytotocity test was carried out using a standard method similar to *Artemia salina* method,([13]) with minor modifications. LC_{50} values for each test fractions were obtained. Extract and fractions of each plant were dissolved in 5 mL of filtered fresh seawater to give 5000 µg/mL solution from which further dilutions were made to obtain 500 µg/mL and 50 µg/mL solutions respectively. The lethality test was done using 1 mL of each stock solution (50, 500, 5000 µg/mL) in a test tube already calibrated to 5mL and made up to the mark with seawater to give an overall concentration of 10, 100 and 1000 µg/mL. Ten shrimp nauplii were added to each of the test tubes and this was done in triplicates. The control consisted of seawater to which only 10 nauplii were added. After 24 hours the number of shrimps that survived were counted using a magnifying lens and recorded (Ibrahim *et al*., 2013). LC_{50} values were calculated using graphpad prism5.

Cell lines
Two 75 cm^2 T-flasks containing HeLa (cervical cancer) cell lines were obtained from the Tissue Culture Unit of the Department of Biotechnology, University of the Western Cape, South Africa. Cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% Fetal Bovine serum and 1% Penicillin-streptomycin at 37°C in a humidified 5% CO_2 incubator. At confluence, cells were seeded at a density of 0.3×10^6 in a six well plate for testing. A 10-fold dilution of the IC_{50} values of the test fractions (*P. guineense* (CF): 23.02 µg/mL; *C. afer* (EAF): 92.54 µg/mL; *A. viridis* (EAF): 195.5 µg/mL; *P. guineense* (HF): 147.5 µg/mL; *C. roseus* (CF): 18.45 µg/mL; and *Z. zanthoxyloides*: 320.6 µg/mL) were prepared in a 5% maintenance culture medium. Camptothecin (5 µM) was used as the apoptosis inducing drug control. After 12 h of seeding, the culture media was replaced with the test media for another 12 h.

Water soluble tetrazolium salt (WST-1) cytotoxicity testing
Metabolic active cells were quantified by measuring the amount of soluble formazan formed spectrophotometrically using the WST-1 (Roche, Johannesburg-South Africa) (29). HeLa cells were seeded at a density of 1×10^4 cells/well in 100 µL growth medium (DMEM) into flat bottom 96 well plates. After 24 hours, cells were treated with different concentrations of test fractions (0.01-10,000 µg/mL) in triplicates and incubated at 37°C and 5% CO_2 for another 24 hours as described by NICEATEM.[14] 5 µM Camptothecin (Calbiochem, Germany) was used as an apoptosis-inducing reference compound. Subsequently, 10 µL of WST-1 reagent was added to each well and incubated for 4 h at 37°C and 5% CO_2. Plates were shaken thoroughly for 1 min prior to measurement of absorbance at 440 nm with a reference wavelength of 630 nm in a POLARstar Omega® microplate reader (BMG Labtech, USA).

Semi-quantitative RT-PCR
Total cellular RNA extracted from different samples were quantified and normalized to equal amounts. To compensate for variation of the amount of RNA input, both target gene(s) and a housekeeping gene (β-actin) that is not expected to vary in response to stimuli was quantified. The resultant difference in amplicon quantities were monitored by densitometric analysis.[15]

Total RNA Isolation
HeLa cells were scraped and lysed in 350 µL of RP1 Lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarkosyl and 200 mM β-mercaptoethanol (β-ME) and the Whole cell lysates were clarified by filtering through a 0.2 µM filter column. To create a suitable binding condition for the nucleic acids, 350 µL 70% ethanol was added to each filtered lysate. Samples were loaded unto the silica membrane columns and centrifuged at 11000 *g* for 45 sec. Filtrates were discarded and the silica membrane was washed twice with 500 µL DNA wash buffer (25% isopropanol, 25% ethanol, 100 mM, NaCl and 10 mM Tris-HCl, pH 8.0). Bound DNA on the columns was digested with 95 µL DNase at room temperature for 15 min. RNA bound columns were washed twice and centrifuged at 11000 *g* with RA buffer (wash buffer) for 2 min at 4°C. Bound RNA was eluted from the columns with 60 µL of DNase/RNase free water.

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RNA quality assessment and normalization

RNA concentrations and purity were checked using the Nanodrop spectrophotometer. The RNA quality and degradation were assessed by electrophoresis on 1.0% agarose gel using pre-cooled 1X Tris-acetate-EDTA buffer at 4°C. All the RNA sample concentrations were diluted to 90 ng/µL with RNase free TE buffer (10 mM Tris, 2 mM EDTA, pH 8.0).[16, 17]

Synthesis of cDNA by RT-PCR.

Complementary DNA (cDNA) was synthesized by RT-PCR. RNA (90 ng) of each sample was converted into cDNA in two separate reaction mixes. Mix I (20 µL) contained 10 µL (90 ng/µL) of isolated RNA with 10 µL (2.5 µM) of oligo-dT primers table 1) (JenaBioscience, Germany). Samples were incubated at 70°C for 5 min and immediately placed on ice. A 20 µL Mix II was prepared by mixing 5 µL water, 8 µL (1X) SCRIPT RT buffer, 2 µL (500 nM) dNTPs, 2 µL (5 mM) DTT, 2 µL (40 Units) RNase-OUT (Invitrogen®, USA) and 1.0 µL (100 Units) SCRIPT reverse transcriptase enzyme (JenaBioscience, Germany). Thermal conditions were set at 42°C for 10 min, 50°C for 30 min, 70°C for 10 min and 4°C hold.

Semi - quantitative PCR (qPCR)

Quantitative Polymerase Chain Reaction (qPCR) was performed in a 25 µL reaction volume containing 5 µg cDNA, 0.2 mM dNTP mix, 1X complete buffer (JenaBiosciences, Germany) and 0.3 µM of each target primer pair. Primer pairs used in this study are listed in Table 1. *β*-actin gene was used as the internal control (endogenous gene). PCR Reaction was done at 94°C for 5 min, 30 cycles of 94°C for 30 sec, 3°C<T_m for 30 sec, 72°C for 30 sec and a single hold at 72°C for 5 min. PCR amplicons were analyzed on 1.8% agarose gel electrophoresis. Gel images were captured with Biospectrum 610 Advanced Imaging system UVP (Analytik Jena, USA) and densitometric analysis was done using Gel Analyzer™ software (Gel analyzer, USA).

Data analysis

Densitometric data from the gel images for each study gene were used to calculate the relative gene expression using *β*-actin as the endogenous reference control and the formula below.[18]

$$\text{Relative fold change} = \frac{\left(\frac{\text{Study gene}}{\text{house keeping gene}}\right)\text{treatment}}{\left(\frac{\text{Study gene}}{\text{house keeping gene}}\right)\text{control group}}$$

All analyses were done in triplicates and statistics were performed with the Graphpad Prism 5 program. Parametric data were expressed as the mean ± S.E. To evaluate whether observed differences were significant, student's *t-test* for comparisons between two groups was used and $p < 0.05$ was considered to be significant for all analyses

Results and Discussion

Our study describes the semi-quantitative modulation of apoptotic and oestrogen Receptor alpha (ERα) genes using natural products as drug candidates in low-resource settings. However, it is a sensitive method if all the necessary conditions are carefully considered and controlled during the experiment.[19] The medicinal plants investigated in this study are commonly used as food condiments and anti-tumour natural medicines in Nigeria.[20]

Brineshrimp assay was used to pre-screen the fractions with lethality effect serving as hit fractions for mammalian cell line,[21] the LC_{50} values (Supplementary data) for all the fractions were less than 10 µg/mL. Water Soluble Tetrazolium salt (WST-1) Cytotoxicity assay was later done on the active fractions; *P. guineense*-chloroform; *C. afer*-ethylacetate; *A. viridis*-ethylacetate; *P. guineense*-hexane; *C. roseus*-chloroform; *Z. zanthoxyloides*-ethylacetate; and *Z. zanthoxyloides*-hexane fractions, the results as presented in Table 2. Shows that *P. guineense* (chloroform) fraction and *C. roseus* (chloroform) fractions has the most prominent cytotoxicities in cervical cancer cells (HeLa) as depicted by their low IC_{50} values.

For RNA quality and quantification, Figure 1 depicts confirmatory bands for 28S and 18S ribosomal RNA in the Total RNA isolated from all the treatment groups, thus indicating high quality samples with minimal degradation. The Nanodrop spectrophotometric concentrations of each sample corresponded to their respective band intensities. 5µM Camptothecin treated group on lane 2 had the lowest nanodrop concentration while *C. afer*-EAF (lane 5) had the highest concentration. RNA concentration values were used for the normalization of the different variations of RNA amounts in the treatment groups to an equal concentration prior to cDNA synthesis.

Figures 2-5, shows gel images and densitometry analyses of RT-PCR products of Oestrogen receptor-α (ESR-α), TP53, Retinoblastoma (Rb), and NAD(P)H: Oxidoreductase (NQO1) genes. Oestrogen receptor-α , which is necessary for the activation of cell proliferation in cervical cancer cells due to oestrogen responsiveness was significantly ($p<0.05$) down-regulated 1.0-fold by *P. guineense*-HF, 0.8-fold by *C. roseus*-CF and 0.6-fold by *A. viridis*-EAF treatments as shown in Figure 2. Messenger RNA (mRNA) of TP53 gene which codes for the tumour suppressor protein P53 "guardian of the genome" had a significant ($p<0.05$) 0.2-fold up-regulation by *P. guineense*-HF followed by a non-significant ($p>0.05$) up regulation by *Z. zanthoxyloides*-EAF; while other treatment groups showed down regulation of TP53 gene (Figure 3). There was no up-regulation of Retinoblastoma (Rb) gene in all the treatment groups as shown in Figure 4. The activities of HPV viral proteins E6 and E7 in cervical cancer are clearly linked to carcinogenesis due to their abilities to inactivate the p53 and retinoblastoma (pRb) tumour suppressors respectively.[22] The Retinoblastoma tumour suppressor gene (Rb) encodes a nuclear phosphoprotein (p105Rb or pRb) which is also referred to as "the pocket protein" derived from the conserved binding pocket region through which the protein bind viral oncoproteins such as HPV E7 protein, and the E2F transcription factors.[23] The up-regulation of Rb stops cell cycle progression at the G_1 phase by binding to E2F transcription factor preventing the activation of genes necessary for cell cycle to progress to the synthesis "S" phase. Our data shows no observable up-regulation of the Rb gene in all the treatment groups. Whereas, HeLa cells treated with *Z. zanthoxyloides* (EAF) and *P. guineense* (HF) caused an up-regulation of TP53 gene. Upregulation of TP53 gene abrogates the consequential effect of the HPV viral E6 protein which continuously targets p53 for degradation thus preventing the normal function of p53 which are of G_1 cell cycle arrest, apoptosis, and DNA repair.[24]

Oxidoreductase (NQO1) helps to mop up free radicals necessary for the activation of cell death in cancer cells; It is therefore usually up-regulated in cancer cells to prevent the activation of cell death via reactive oxygen species. In this study, significant ($p<0.05$) down-regulation of NQO1 gene was observed in groups treated with Camptothecin, *C. afer*-EAF, *C. roseus*-CF, *P. guineense*-HF, and *A. viridis*-EAF showing the most down-regulation effect of about 3.8-fold change (Figure 5). The gene for NAD(P)H: quinone oxidoreductase-1 (NQO1), also known as DT-diaphorase, is located on chromosome 16q22. It was found to be highly expressed in many solid tumours.[25,26] Its function has been proposed to include xenobiotic detoxification, superoxide scavenging, and maintenance of endogenous anti-oxidants.[27] This study has shown that all the test fractions caused a down-regulation of NQO1 gene expression with *A. viridis* (EAF) having the most significant ($p<0.05$) effect leading to the accumulation of reactive oxygen species. However, studies have shown that If NQO1 is highly expressed, ROS levels is low and vice versa. ROS increase has been known to induce intrinsic apoptosis.[28] Therefore, the generation of the reactive oxygen species (ROS) in excess is known to induce oxidative modification of cellular macromolecules, inhibit protein function and promote cell death.[29]

ROS triggers the intrinsic apoptotic cascade via the translocation of cytochrome c, AIF, or Smac/Diablo that activates caspase-dependent or caspase-independent cytosolic signaling events.[30] A study by Jeong and Joo(2016)[31] further affirms that the modulation of ROS by phytochemicals emerges as a crucial mechanism to regulate apoptosis in cancer pathogenesis.

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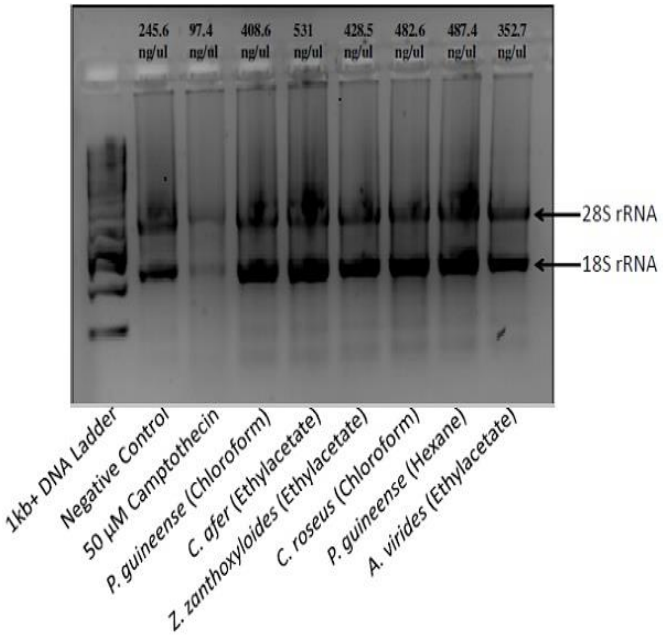

Figure 1: RNA quality Check using Agarose Gel electrophoresis and Nanodrop Spectrophotometer.
The sharp intense bands of 28S and 18S rRNA indicates minimal degradation of RNA species during isolation from treated HeLa Cells. RNA concentration (ng/µl) of each treatment group is indicated at the top of the 1 % agarose gel.

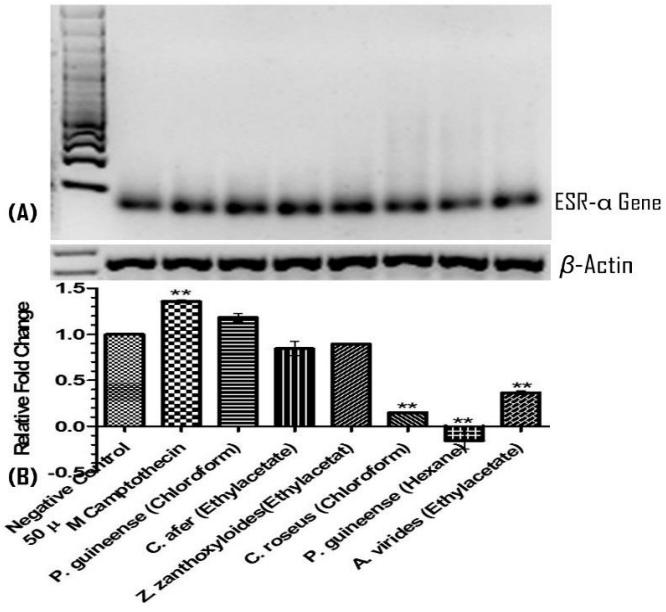

Figure 2: RT-PCR Gene Expression Analysis of Oestrogen Receptor-α (ESR-α).
(a) Gel image shows the amplification product (79 bp) of Oestrogen receptor-α gene in all the treatment groups. The lower panel shows the amplification product of the house keeping gene (endogenous control) *β*-actin in the treatment groups. **(b)** Densitometry analysis showing the relative fold change of ESR-α gene in comparison with the untreated control (negative) shows that the camptothecin treated group had a significant ($P < 0.05$) increase, while *C. roseus* (Chloroform), *A. viridis* (Ethylacetate), and *P. guineense* (Hexane) fractions had significant decreases ($P < 0.05$) in the relative fold change of ESR-α gene expression ($N = 3$).

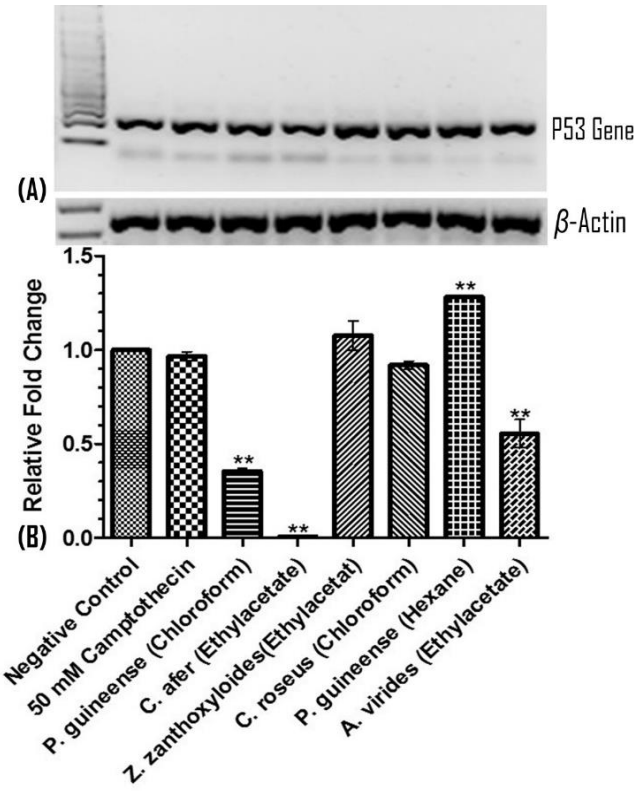

Figure 3: RT-PCR Gene Expression Analysis of TP53 Gene.
(a) Gel image shows the amplification product (199 bp) of TP53 gene in all the treatment groups. The lower panel shows the β-actin (endogenous control) amplification bands. **(b)** Densitometry analysis reveals that only *P. guineense* (Hexane) had a significant ($P < 0.05$) increase compared with untreated group. *Z. zanthoxyloides* (Ethylacetate) Fraction had a slight increase in the relative fold change but was not significant ($P > 0.05$) ($N = 3$).

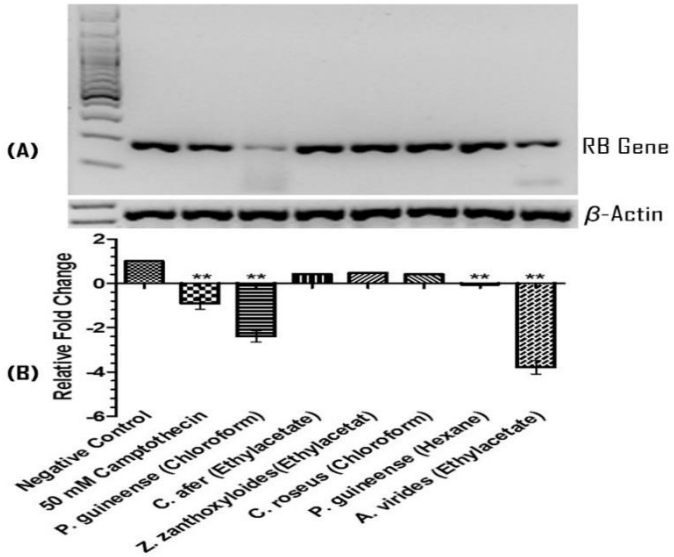

Figure 4: RT-PCR Gene Expression Analysis of Rb Gene.
(a) Gel image shows the amplification product (178 bp) of Retinoblastoma (Rb) gene in HeLa cells with its corresponding house-keeping gene (*β*-Actin) in the lower panel. **(b)** All the treatment groups had a very low expression of Rb gene necessary for the arrest of cell cycle.

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Table 1: List of PCR primers used in this study

S/N	Primers	Primer Sequence	NCBI Accession Number	Melting Temp. (Tm,^{o}C)	Band Size (bp)
1.	β-Actin fwd	5'-GGC ATG GGT CAG AAG GAT TC-3'	NM_001101.3	60	235
	β-Actin rvs	5'-ACA TGA TCT GGG TCA TCT TCT C-3'		60	
2.	ESR-1 fwd	5'-GAA GAG CTG CCA GGC CTG CC-3'	NM_001122742.1	67	94
	ESR-1 rvs	5'-CCA CCC TAT GCT TTT CTG GCT TTT-3'		65	
3.	TP53 fwd	5'-GCG CAC AGA GGA AGA GAA-3'	NM_001126118.1	60	199
	TP53 rvs	5'-CAA GGC CTC ATT CAG CTC TC-3'		60	
4.	RB fwd	5'-AGG ACC GAG AAG GAC CAA CT-3	NM_000321.2	60	178
	RB rvs	5'-AAG GCT GAG GTT GCT TGT GT-3'		58	
5.	NQO1 fwd	5'-GGT GGA GTC GGA CCT CTA TG-3'	NM_001025433	63	78
	NQO1rvs	5'-GGG TCC TTC AGT TTA CCT GT-3'		58	

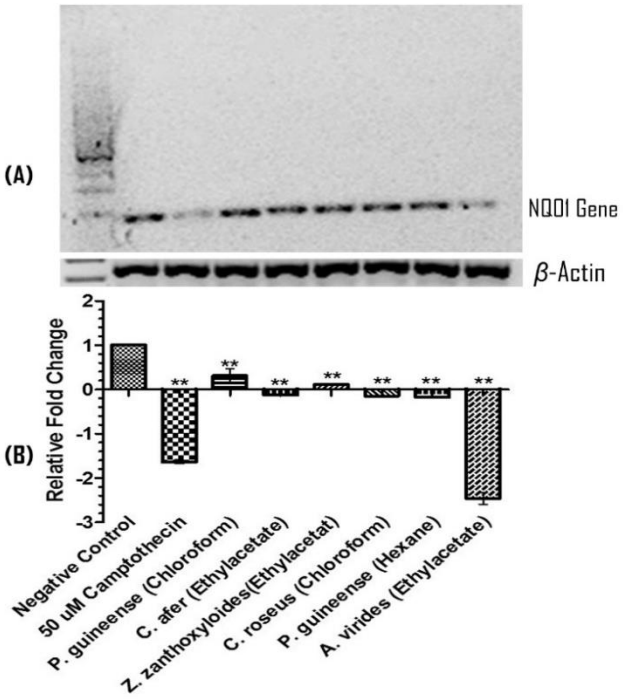

Figure 5: RT-PCR Gene Expression Analysis of NQO1 Gene. (a) Gel Image shows the amplification product (78 bp) of NAD(P)H quinine oxidoreductase (NQO1) gene in HeLa treated cells. The same (β-actin gene) was used as the endogenous control as shown in the lower panel. (b) Densitometry analysis of the gel image shows that all the test fractions induced significant ($P < 0.05$) decrease in the relative fold change of NQO1 gene.

Conclusion

Our findings provide evidence for the anti-proliferative gene expression modulation by *Piper guineense*, *Zanthoxylum zanthoxyloides*, *Amaranthus viridis*, and *Catharanthus roseus* as therapeutic candidate agents against cervical cancer.

Conflict of interest

The authors declare no conflict of interest.

Table 2: IC_{50} values of selected active fractions

S/N	Hit Fraction	IC_{50} (µg/mL)	1:10 Dilution of IC_{50} (µg/mL)
1.	*P. guineense* (CF)	230.2	23.02
2.	*C. afer* (EAF)	925.4	92.54
3.	*A. viridis* (EAF)	1955	195.5
4.	*P. guineense* (HF)	1475	147.5
5.	*C. roseus* (CF)	184.5	18.45
6.	*Z. zanthoxyloides* (EAF)	3206	320.6

IC_{50} values were determined based on data generated from WST-1 cytotoxicity assay. A 1:10 dilution was done for each fraction for easy monitoring of early apoptosis.

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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