Research Article

Cytotoxicity and Modes of Action of the Methanol Extracts of Six Cameroonian Medicinal Plants against Multidrug-Resistant Tumor Cells

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Introduction. The present study aims at evaluating the cytotoxicity of twelve parts from six Cameroonian medicinal plants on sensitive and drug-resistant cancer cell lines. We also studied the mode of action of the most active plants, *Gladiolus quartinianus*, *Vepris soyauxii*, and *Anonidium mannii*. *Methods*. The cytotoxicity of the extracts was determined using a resazurin assay. Flow cytometry was used for cell-cycle analysis and detection of apoptosis, analysis of mitochondrial membrane potential (MMP), and measurement of reactive oxygen species (ROS). *Results*. At 40 g/mL, three extracts showed a growth of CCRF-CEM leukemia cells by less than 50%. This includes the extracts from *G. quartinianus* (GQW; 25.69%), *Vepris soyauxii* leaves (VSL; 29.82%), and *Anonidium mannii* leaves (AML; 31.58%). The lowest IC_{50} values below 30 μg/mL were obtained with GQW, AML and VSL against 7/9, 8/9, and 9/9 tested cancer cell lines, respectively. The lowest IC_{50} values for each plant were 4.09 μg/mL, and 9.14 μg/mL (against U87MG.Δ*EGFR* cells), respectively, for VSL and AML and 10.57 μg/mL (against CCRF-CEM cells) for GQW. GQW induced cell cycle arrest between G0/G1 and S phases, whilst VSL and AML induced arrest in G0/G1. All three extracts induced apoptosis in CCRF-CEM cells by loss of MMP, whilst AML also enhanced production of ROS. *Conclusion*. The three active plants may be a source for the development of new anticancer drugs.

1. Introduction

Cancer is one of the major causes of death in humans representing the third leading cause of death worldwide (12.4%), the first being cardiovascular disease (30%) and the second being infectious diseases, including HIV/AIDS (18.8%) [1]. Chemotherapy remains the treatment of choice in many malignant diseases [2]. Nevertheless, the appearance of drug resistance, in particular multidrug resistance (MDR), can make many of the clinically established anticancer drugs ineffective [3]. Thus, MDR is one of the major concerns preventing cure of many cancer patients. Also, malignancies are increasingly recognized as a critical public health problem in Africa [4]. Worldwide, the number of new cancer cases will annually reach 15 million by 2020, 70% of which will occur in developing countries, where governments are less prepared to address the growing cancer burden and where survival rates

are often less than half of those in more developed countries [4]. It has been observed that throughout the continent, though infectious diseases continue to burden African population, noninfectious diseases require much more attention [4]. Currently, limited funding is available to tackle cancer in African countries. Awareness of this impeding epidemic in Africa deserves priority, and further resources should be mobilized to both prevent and treat cancer. Research on anticancer agents has become a worldwide effort in both developed and developing countries, since chemotherapy is a mainstay in the treatment of many malignancies [5]. The majority of standard anticancer drugs has been isolated or derived from natural sources, based on their use in traditional medicine [6]. Screenings of medicinal plants used as anticancer drugs have provided modern medicine with effective cytotoxic pharmaceuticals. More than 60% of the approved anticancer drugs in USA were from natural origin [7–9].

In Cameroon, the use of plants in traditional medicine systems has been extensively documented in the Cameroonian pharmacopoeia [10]. Evidence highlighting the importance of these plants for cancer therapy has been provided [11–16]. However, whether these plants are also effective in cells resistant to standard chemotherapy is largely unknown. It has been recommended that ethnopharmacological usages such as immune and skin disorders, inflammatory, infectious, parasitic, and viral diseases should be taken into account when selecting plants used to treat cancer, since these reflect disease states bearing relevance to cancer or cancer-like symptoms [17, 18]. Though the plants selected in the present studied are used in the Cameroonian traditional medicine to fight cancers, there is still a lack of published data regarding the use. Therefore, in our continuous search of the cytotoxic candidates from Cameroonian plants with unpublished ethnopharmacological information related to cancer use, we investigated the antiproliferative potential of six Cameroonian plants against cancer cell lines with different mechanisms of drug resistance, that is, ATP-binding cassette (ABC) transporters (P-glycoprotein, breast cancer resistance protein), tumor suppressors (p53), or oncogenes (epidermal growth factor receptor). The most cytotoxic extracts from *Gladiolus quartinianus* A. Rich. (Iridaceae), *Vepris soyauxii* Engl. (Rutaceae) and *Anonidium mannii* (oliv) Engl. et Diels. (Annonaceae) were further analyzed to study their mode of action regarding cell-cycle distribution, MMP, and ROS.

2. Materials and Methods

2.1. Plant Material. All medicinal plants used in the present work were collected in different areas of Cameroon between January and April 2012 (Table 1). The plants were identified at the National Herbarium (Yaounde, Cameroon), where voucher, specimens were deposited under the references numbers given in Table 1.

2.2. Extraction. The air-dried and powdered plant samples (1 kg) were soaked in methanol (3 L) for 48 h, at room temperature. The methanol extracts were concentrated *in vacuum* to obtain the crude extracts [12]. These extracts were then stored at 4°C until further use.

2.3. Chemicals. Doxorubicin, vinblastine, and daunorubicin were provided by the University Pharmacy of the Johannes Gutenberg University (Mainz, Germany) and dissolved in PBS (Invitrogen, Eggenstein, Germany) at a concentration of 10 mM. Geneticin (72.18 mM) was purchased from Sigma-Aldrich (Munich, Germany).

2.4. Preliminary Phytochemical Investigations. The major secondary metabolites classes such as alkaloids, anthocyanins, anthraquinones, flavonoids, phenols, saponins, sterols, and triterpenes (Table 2) were determined according to a common phytochemical methods previously described [34].

2.5. Cell Cultures. Drug-sensitive CCRF-CEM and multi-drug-resistant CEM/ADR5000 leukemia cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum in a humidified 5% CO_2 atmosphere at 37°C. Sensitive and resistant cells were kindly provided by Dr. Axel Sauerbrey (Department of Pediatrics, University of Jena, Jena, Germany). The generation of the resistant subline was described [35]. The specific overexpression of P-glycoprotein, but not other ABC transporters, has been reported [36, 37]. Breast cancer cells transduced with control vector (MDA-MB-231-pcDNA3) or with cDNA for the breast cancer resistance protein, *BCRP* (MDA-MB-231-*BCRP* clone 23) were maintained under standard conditions as described previously for CCRF-CEM cells. Human wild-type HCT116 ($p53^{+/+}$) colon cancer cells as well as knockout clones HCT116 ($p53^{-/-}$) derived by homologous recombination were a generous gift from Dr. B. Vogelstein and H. Hermeking (Howard Hughes Medical Institute, Baltimore, MD, USA). Human glioblastoma multiforme U87MG cells (nontransduced) and U87MG cell line transduced with an expression vector harboring an epidermal growth factor receptor (*EGFR*) gene with a genomic deletion of exons 2 through 7 (U87MG.Δ*EGFR*) were kindly provided by Dr. W. K. Cavenee (Ludwig Institute for Cancer Research, San Diego, CA, USA) [38]. MDA-MB-231-*BCRP,* U87MG.Δ*EGFR,* and HCT116 ($p53^{-/-}$) were maintained in DMEM medium containing 10% FBS (Invitrogen) and 1% penicillin (100 U/mL) streptomycin (100 μg/mL) (Invitrogen) and were continuously treated with 800 ng/mL and 400 μg/mL geneticin, respectively. Human HepG2 hepatocellular carcinoma cells and normal AML12 hepatocytes were obtained from American Type Culture Collection (ATCC, USA). The previous medium without geneticin was used to maintain MDA-MB-231, U87MG, HCT116 ($p53^{+/+}$), HepG2, and AML12 cell lines. The cells were passaged twice weekly. All experiments were performed with cells in the logarithmic growth phase.

2.6. Resazurin Reduction Assay. Resazurin reduction assay [16, 39] was performed to assess cytotoxicity of the studied samples toward cancer cells. The assay is based on reduction of the indicator dye, resazurin, to the highly fluorescent resorufin by viable cells. Nonviable cells rapidly lose the metabolic capacity to reduce resazurin and thus produce no fluorescent signal. Briefly, adherent cells were detached by treatment with 0.25% trypsin/EDTA (Invitrogen, Darmstadt, Germany) and an aliquot of 1×10^4 cells was placed in each well of a 96-well cell culture plate (Thermo Scientific, Langenselbold, Germany) in a total volume of 200 μL. Cells were allowed to attach overnight and then were treated with different concentrations of the studied sample. For suspension cells, aliquots of 2×10^4 cells per well were seeded in 96-wellplates in a total volume of 100 μL. The studied sample was immediately added in varying concentrations in additional 100 μL of culture medium to obtain a total volume of 200 μL/well. After 24 h or 48 h, 20 μL resazurin (Sigma-Aldrich, Schnelldorf, Germany) 0.01% w/v in ddH_2O was added to each well and the plates were incubated at 37°C for 4 h. Fluorescence was measured on an Infinite M2000

TABLE 1: Pharmacognosy of Cameroonian medicinal plants.

Samples, family, and herbarium number[a]	Traditional treatment	Part used in this study and extraction yield (%)[b]	Area of plant collection	Known bioactive (or potentially active) compounds	Screened activity for crude plant extract
Allanblackia gabonensis Pellegr. (Clusiaceae); 17275 SRF/Cam	Dysentery, cold, toothache [19, 20]; pain, rheumatism, inflammations [21], cancer (personal information)	Fruits (4.14%), leaves (10.16%), stem (8.42%) and roots bark (8.69%)	Lebialem, South West region of Cameroon	Allan Xanthones A and D; 1,3,6,7-tetrahydroxy-2-(3-methylbut-2-enyl)xanthone [22]	Antimicrobial against Gram-positive and Gram-negative bacteria, yeasts, and mycelial fungus [22]; analgesic and anti-inflammatory effect of aqueous extract of the stem bark [21].
Aroidium mannii (oliv) Engl. et Diels. (Annonaceae); 1918/SRFK	Sore feet, spider bite, bronchitis, dysentery, sterility caused by poison, gastroenteritis [23]; syphilis, infectious diseases [24]; diarrhea, snake bite, malaria [25], cancer (personal information)	Leaves (3.39%)	Bafoussam, West region of Cameroon	Not reported	Not reported
Gladiolus quartinianus A. Rich (Iridaceae); 17260/SRF/Cam	Gastrointestinal infection, cancer (personal information)	Whole plant (10.22%)	Lebialem, South West region of Cameroon	Not reported	Not reported
Peperomia fernandopoiana C.DC. (Piperaceae); 7171 SRF/Cam	Gastrointestinal infection, cancer (personal information)	Whole plant (7.28%)	Lebialem, South West region of Cameroon	Not reported	Not reported
Recinodindron heudelotii (Baill.) ex Pax. (Euphorbiaceae); 19695 SRF/Cam	Cough, antidote, intestinal diseases, dysentery [26–28]; malaria, anaemia, stomach pain, easy delivery, yellow fever, aphrodisiac [29], cancer (personal information)	Leaves (5.18%) and stems bark (5.72%)	Melon, littoral region of Cameroon	E-ferulic acid octacosylate, 3-methylmethylorsellinate, lupeol, heudoletinone, 1,2-dihydroheudoleunol [26], aleuritolic acid 1 and labda-8(17),13–dien 3β,15-diol 2 [30]	Antimicrobial activity of the stem bark against Streptococcus faecalis [30], Staphylococcus aureus, Bacillus cereus, Escherichia coli, Shigella dysenteriae, Shigella flexneri, Salmonella typhi, Pseudomonas aeruginosa, Klebsiella pneumoniae, Candida albicans [31], antioxidant [32].
Vepris soyauxii Engl. (Rutaceae); 18394 SFR/Cam	Antifibromyoma, stomachache, malaria [32], cancer (personal information)	Leaves (10.16%), stems (5.18%), and roots bark (9.26%)	Melon, littoral region of Cameroon	Not reported	Not reported

[a]Plants were identified at the Cameroon National Herbarium (HNC); [b]The percentage of the methanol extract.

TABLE 2: Chemical constituents and extraction yield of the studied plant extracts.

Studied samples	Phytochemical constituents								
	Alkaloids	Anthocyanins	Anthraquinones	Flavonoids	Phenols	Saponins	Tannins	Sterols	Triterpenes
Allanblackia gabonensis									
Leaves	+	+	−	+	+	−	+	−	−
Stem bark	+	+	+	+	+	+	+	−	+
Root bark	+	+	+	+	+	+	+	−	+
Fruits	+	+	−	+	+	+	+	−	+
Anonidium mannii									
Leaves	+	−	−	−	+	+	+	+	+
Gladiolus quartinianus									
Whole plant	+	+	−	+	+	−	+	+	+
Peperomia fernandopoiana									
Whole plant	+	+	−	+	+	−	+	+	−
Ricinodendron heudelotii									
Leaves	+	−	−	−	+	+	+	−	+
Stem bark	+	+	−	+	+	−	+	−	−
Vepris soyauxii									
Leaves	+	+	−	+	+	+	+	+	+
Stem bark	+	+	+	+	+	+	+	−	+
Roots bark	+	+	−	+	+	+	+	−	+

(+): present; (−): absent.

Pro plate reader (Tecan, Crailsheim, Germany) using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Each assay was done at least two times, with six replicate each. The viability was evaluated based on a comparison with untreated cells. IC_{50} values represent the sample's concentrations required to inhibit 50% of cell proliferation and were calculated from a calibration curve by linear regression using Microsoft Excel.

2.7. Flow Cytometry for Cell Cycle Analysis and Detection of Apoptotic Cells. Cell-cycle analysis was performed by flow cytometry using The Vybrant DyeCycle (Initrogen). The Vybrant DyeCycle Violet stain is a DNA-selective, cell membrane-permeant, and nonfluorescent dye for DNA content analysis in living cells. The Vybrant DyeCycle Violet stain is fluorescent upon binding to double-stranded DNA. Leukemia CCRF-CEM cells (1×10^6) were treated with the concentrations equivalent to the IC_{50} values of the crude extract for 24 h, 48, and 72 h. Following incubation, $1 \mu L$ of Vybrant DyeCycle Violet stain was added to 1 mL of cell suspension and incubated for 30 min at 37°C. Cells were measured on an LSR-Fortessa FACS analyzer (Becton-Dickinson, Germany) using the violet laser. Ten thousand cells were counted for each sample. Vybrant DyeCycle Violet stain was measured with 440 nm excitation. Cytographs were analyzed using FlowJo software (Celeza, Switzerland). All experiments were performed at least in triplicate.

2.8. Analysis of Mitochondrial Membrane Potential (MMP). The effects of extract on the MMP were analyzed by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) (JC-1; Biomol, Germany) staining. JC-1 is a dye that can selectively enter into mitochondria and exhibits an intense red fluorescence in healthy mitochondria with normal membrane potentials. In cells with reduced MMP, the red fluorescence disappears. Briefly, 1×10^6 CCRF-CEM cells treated with different concentrations of the test compounds or DMSO (solvent control) for 24 h were incubated with JC-1 staining solution according to the manufacturer's protocol for 30 min. Subsequently, cells were measured in an LSR-Fortessa FACS analyzer (Becton-Dickinson). For each sample, 1×10^4 cells were counted. The JC-1 signal was measured with 561 nm excitation (150 mW) and detected using a 586/15 nm bandpass filter. The compounds signal was analyzed with 640 nm excitation (40 mW) and detected using a 730/45 nm bandpass filter. All parameters were plotted on a logarithmic scale. Cytographs were analyzed using FlowJo software (Celeza, Switzerland). All experiments were performed at least in triplicate.

2.9. Measurement of Reactive Oxygen Species (ROS) by Flow Cytometry. 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFH-DA) (Sigma-Aldrich, Germany) is a probe used for the highly sensitive and quantifiable detection of ROS. The nonfluorescent H_2DCFH-DA diffuses into the cells and is cleaved by cytoplasmic esterases into 2′,7′-dichlorodihydrofluorescein (H_2DCF) which is unable to diffuse back out of the cells. In the presence of hydrogen peroxide, H_2DCF is oxidized to the fluorescent molecule dichlorofluorescein (DCF) by peroxidases. The fluorescent signal emanating from DCF can be measured and quantified by flow cytometry, thus providing an indication of intracellular ROS concentration [40, 41]. Briefly, 2×10^6 CCRF-CEM cells were resuspended in PBS and incubated with

$2\,\mu$M H$_2$DCFH-DA for 20 min in the dark. Subsequently, cells were washed with PBS and resuspended in RPMI 1640 culture medium containing different concentrations of extract or DMSO (solvent control). After 1 h of incubation, cells were washed and suspended in PBS. Subsequently, cells were measured in an FACS Calibur flow cytometer (Becton-Dickinson, Germany). For each sample 1×10^4 cells were counted. DCF was measured at 488 nm excitation (25 mW) and detected using a 530/30 nm bandpass filter. All parameters were plotted on a logarithmic scale. Cytographs were analyzed using FlowJo software (Celeza, Switzerland). All experiments were performed at least in triplicate.

3. Results

3.1. Chemical Composition of the Studied Extracts. The results of the qualitative analysis showed that each of the studied plant extract contained at least one class of secondary metabolites such as alkaloids, anthocyanins, anthraquinones, flavonoids, phenols, saponins, and triterpenes. All studied extracts contained alkaloids, phenols, and tannins (Table 2).

3.2. Cytotoxicity of the Studied Samples. The growth inhibition of CCRF-CEM cells induced by 12 extracts belonging to six medicinal plants is depicted in Figure 1. The extracts from *Gladiolus quartinianus* (whole plant; GQW; 25.69%), *Vepris soyauxii* (leaves; VSL; 29.82%), and *Anonidium mannii* (leaves; AML; 31.58%) inhibited cell growth by more than 50% at $40\,\mu$g/mL.

To investigate these extracts in more detail, their IC$_{50}$ values were determined in a panel of cancer cell lines. The VSL extract was active (IC$_{50}$ < $40\,\mu$g/mL) against all 9 sensitive or drug-resistant cell lines. IC$_{50}$ values below $30\,\mu$g/mL were obtained with GQW, AML, and VSLagainst 7/9, 8/9, and 9/9 tested cancer cell lines, respectively. The IC$_{50}$ values were in a range from $4.09\,\mu$g/mL (U87MG.ΔEGFR cells) to $13.60\,\mu$g/mL (HepG2 cells) for VSL from $10.57\,\mu$g/mL (CCRF-CEM) to $34.01\,\mu$g/mL (U87MG.ΔEGFR) for GQW, and from $9.14\,\mu$g/mL (U87MG.ΔEGFR) to $32.02\,\mu$g/mL (MDA-MB-231-*BCRP*) for AML. For the control drug doxorubicin, the IC$_{50}$ values were in a range from $0.11\,\mu$g/mL (CCRF-CEM cells) to $195.12\,\mu$g/mL (CEM/ADR5000 cells) (Table 3). High degrees of resistance to doxorubicin were observed for CEM/ADR 5000 cells (1772-fold), MDA-MB-231-*BCRP* cells (7.11-fold), and U87MG.ΔEGFR (5.76-fold) compared to their corresponding parental cell lines. HCT116 ($p53^{-/-}$) cells were weakly resistant to doxorubicin (2.84-fold) compared to HCT116 ($p53^{+/+}$) cells. Interestingly, the drug-resistant cell lines were not or only weakly resistant to the tested extracts ($\leq$2.53-fold). Remarkably, none of the tested extract inhibited the growth of more than 50% normal AML12 hepatocytes at a concentration of $40\,\mu$g/mL. Collateral sensitivity, which means that resistant cells are more sensitive than sensitive cells, was observed with the three extracts against U87MG.ΔEGFR with degree of resistances below 1. This was also noted for the VSL and AML extracts against HepG2 cells and AML extract against CEM/ADR5000 cells. All the plant
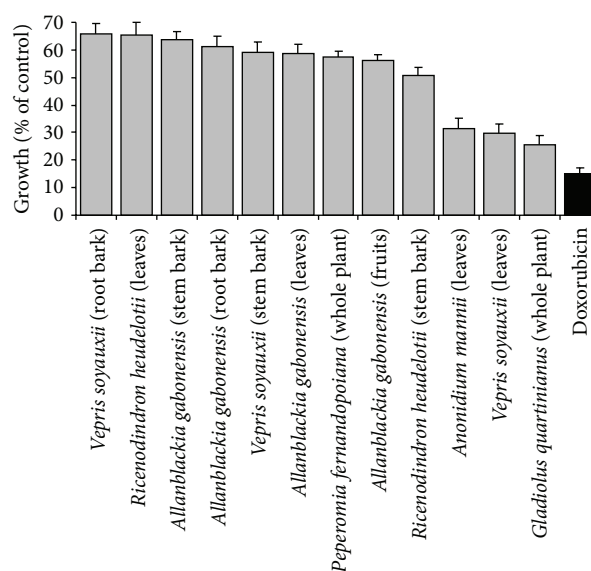

FIGURE 1: Growth (% of untreated control) of CCRF-CEM leukemia cells in the presence of plant extracts ($40\,\mu$g/mL) or doxorubicin ($10\,\mu$g/mL).

extracts showed higher IC$_{50}$ values in normal AML12 hepatocytes compared to HepG2 liver cancer cells. Furthermore, AML12 normal hepatocytes were more doxorubicin resistant than HepG2 cancer cells towards doxorubicin. None of the extracts inhibited normal AML12 hepatocytes by more than 50%.

3.3. Cell Cycle Distribution and Apoptosis. The cell-cycle distribution and induction of apoptosis of CCRF-CEM cells upon treatment with GQW, VSL AML, are depicted in Figure 2. Upon 72 h treatment, the GQWextract induced cell cycle arrest between G0/G1 and S phases whilst VSL and AMLextracts induced G0/G1 arrest. The three extracts led to a time-dependent increase of sub-G0/G1 cells, indicating induction of apoptosis. CCRF-CEM cells treated with concentrations equivalent to the IC$_{50}$ value of each studied extracts progressively underwent apoptosis, with percentages in sub-G0/G1 phase ranging from 11.2% (24 h) to 44.3% (72 h) for GQW, from 19.7% (24 h) to 53.2% (72 h) for VSL, and from 22.7% (24 h) to 76.2% (72 h) for AML. The values of the sub-G0/G1 phase recorded with AMLwere higher than those obtained with nontreated cells (range from 3.82% (24 h) to 9.37% (72 h)), but were comparable to those obtained for the control drug, doxorubicin (range from 59.4% (24 h) to 71.9% (72 h)) (see Supplementary Material available online at http://dx.doi.org/10.1155/2013/285903, Figure S1).

3.4. Effect on the Mitochondrial Membrane Potential (MMP). We assessed the effect of the GQW, VSL, and AML extracts on MMP in CCRF-CEM cells. As shown in Figure 3, percentage alterations of 13.5%, 28.9%, and 32.3% were induced by GQW, VSL, and AML extracts, respectively, after 24 h of treatment with twofold IC$_{50}$. The MMP value for untreated cells was 4.81%. Under similar experimental conditions, these values

TABLE 3: Cytotoxicity of the studied extracts towards sensitive and drug-resistant cancer cell lines and normal cells as determined by the resazurin assay.

Cell lines	Studied samples, IC$_{50}$ values (μg/mL)[a] and degree of resistance (in bracket)			
	Gladiolus quartinianus (Whole plant)	Vepris soyauxii (Leaves)	Anonidium mannii (Leaves)	Doxorubicin
CCRF-CEM	10.57 ± 2.08	9.28 ± 1.01	17.32 ± 2.27	0.11 ± 0.01
CEM/ADR5000	26.14 ± 1.97 (2.47)	11.72 ± 1.43 (1.26)	16.44 ± 1.76 (0.95)	195.12 ± 14.30 (1772)
MDA-MB-231	16.11 ± 1.62	7.52 ± 0.84	12.65 ± 1.49	1.10 ± 0.01
MDA-MB-231-BCRP	29.6 ± 3.19 (1.49)	12.93 ± 1.69 (1.71)	32.02 ± 3.16 (2.53)	7.83 ± 0.01 (7.11)
HCT116 $p53^{+/+}$	19.83 ± 1.66	8.59 ± 0.88	13.61 ± 1.79	1.43 ± 0.02
HCT116 $p53^{-/-}$	22.15 ± 1.97 (1.12)	9.70 ± 0.72 (1.12)	∗ (>2.94)	4.06 ± 0.04 (2.84)
U87MG	∗	8.75 ± 1.21	22.25 ± 2.76	1.06 ± 0.03
U87MGΔEGFR	34.01 ± 2.78 (<0.85)	4.09 ± 0.56 (0.47)	9.14 ± 1.77 (0.41)	6.11 ± 0.04 (5.76)
HepG2	∗ (n.a)	13.60 ± 1.22 (<0.34)	22.09 ± 2.42 (0.55)	1.41 ± 0.12 (<0.04)
AML12	∗	∗	∗	∗

[a]The degree of resistance was determined as the ratio of IC$_{50}$ value of the resistant/IC$_{50}$ sensitive cell line.
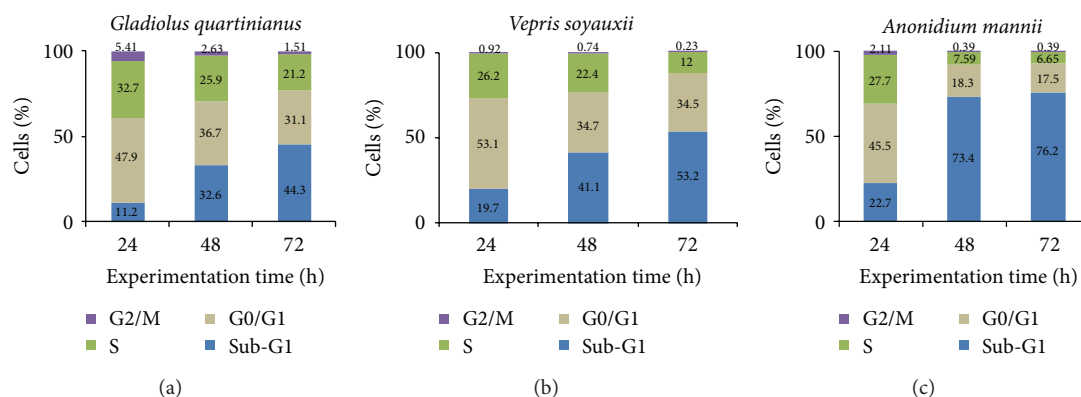(∗): >40 μg/mL; n.a: not applicable.

FIGURE 2: Cell-cycle distribution of CCRF-CEM cells treated with plant extractsordoxorubicin at their corresponding IC$_{50}$ values for 72 h. Data of control and doxorubicin obtained under similar experimental conditions were previously reported [33]. Flow cytometry histograms are available as supportive information (Figure S1).

were lower than that of the reference compound, vinblastine which yielded 48.6% as previously reported [33].

3.5. Effects on Reactive Oxygen Species (ROS). The effects of the GQW, VSL, and AMLextracts on ROS levels were investigated in CCRF-CEM cells after 24 h treatment (Figure 4). The control agent, H$_2$O$_2$, increased ROS level to 10.4%, while ROS production in nontreated cells was 0.94%. Only AML induced significant ROS production in CCRF-CEM cells treated with a concentration equivalent to 2 × IC$_{50}$ (8.42%).

4. Discussion

Drug resistance is a complex multifactorial phenomenon that can result from a number of biochemical mechanisms, including decreased drug uptake or increased drug efflux, perturbed expression of target enzymes or altered target enzymes, altered metabolism of drugs, increased repair of drug-induced DNA damage, or failure to undergo apoptosis [42, 43]. Resistance phenomena may lead to failure of therapy

with fatal outcome for cancer patients. Secondary metabolites play an important role in plant defense against herbivores, microbial infections, and other interspecies defenses and can be exploited to fight human diseases, including cancer [44]. Their antiproliferative properties have been broadly discussed [45]. In the present study, the classes of secondary metabolites detected in the tested plant extracts (Table 2) provide a preliminary explanation on their activities. The obtained results represent the first phytochemical data on the cytotoxic activity of *G. quartinianus*, *V. soyauxii*, and *A. manni*.

According to the criteria of the ATCC, 30 μg/mL represent the upper IC$_{50}$ limit considered promising for purification of a crude extract [46]. In the present work, the highest concentration tested (40 μg/mL) in our screening was slightly above this limit. Herein, we recorded IC$_{50}$ values below 30 μg/mL for GQW, AML, and VSL extracts towards the majority of the tested cancer cell lines (Table 3). This demonstrates that the crude extracts of GQW, AML, and VSL could serve as potential sources of cytotoxic compounds.
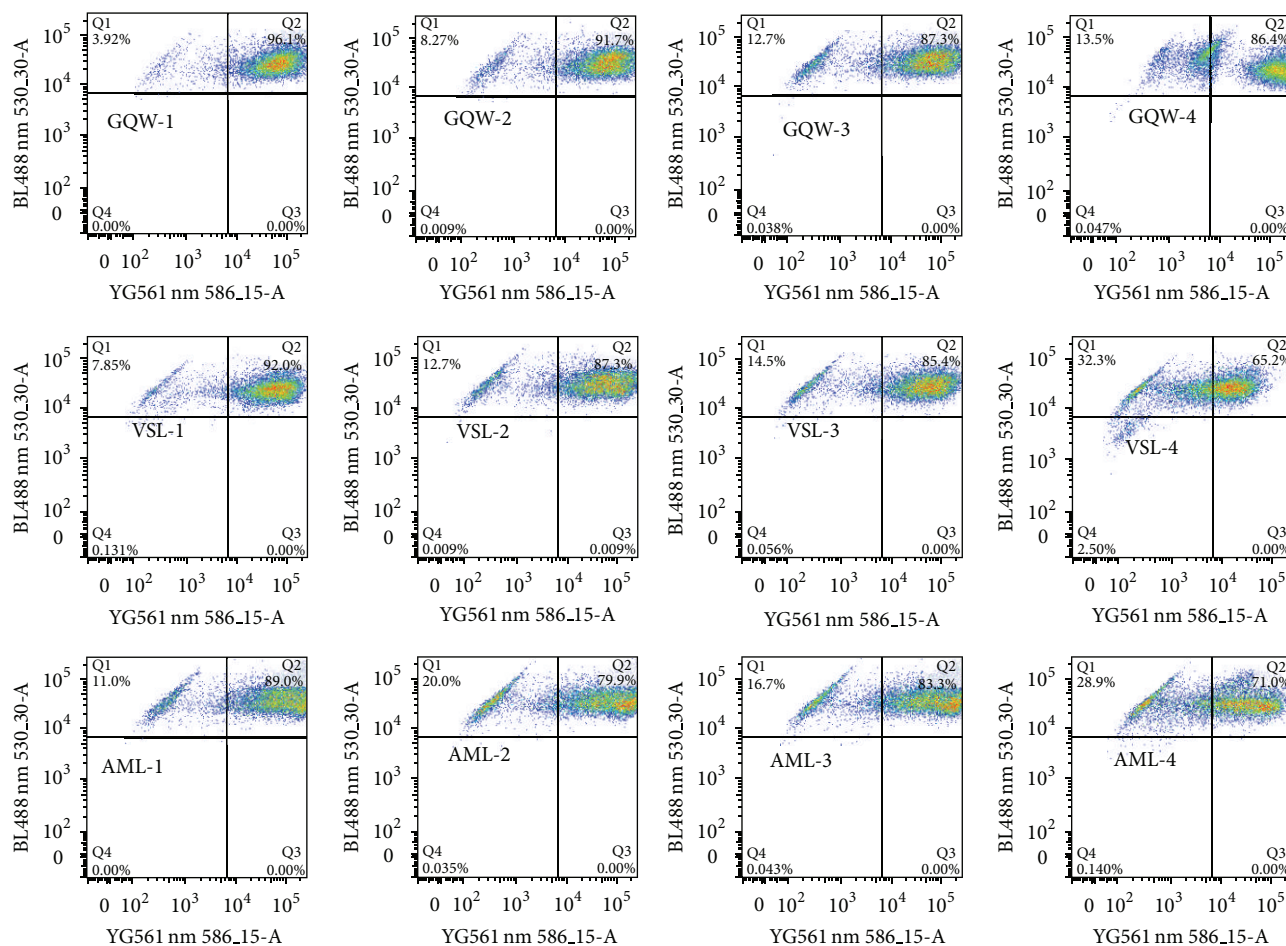
FIGURE 3: Effect of plant extracts and vinblastine (VIN) on the MMP of CCRF-CEM cells after 24 h of treatment. Data of control and vinblastine under similar experimental conditions were previously reported [33]. Samples were tested at their $1/4 \times IC_{50}$ (1), $1/2 \times IC_{50}$ (2), IC_{50} (3), and $2 \times IC_{50}$ (4) values. The IC_{50} values are 0.20 μM for VIN, 10.57 μg/mL (*Gladiolus quartinianus* whole plant, *GQW*), 9.28 μg/mL (*Vepris soyauxii* leaves, *VSL*), and 17.32 μg/mL (*Anonidium mannii* leaves, *AML*).

In addition to the identification of crude extracts with reasonable low IC_{50} values, we identified extracts capable of killing otherwise drug-resistant cancer cells. Having in mind that drug resistance is a major obstacle of chemotherapy in the clinic, the search for novel noncross-resistant cyto-toxic compound from natural sources is urgently warranted. Drug-resistant cell models overexpressing P-glycoprotein, BCRP, or ΔEGFR as well as p53 knockout cells were used to assess the suitability of the studied extracts to tackle multifactorial drug resistance. The degrees of resistance of the three extracts were generally lower than that of doxorubicin in corresponding drug-resistant cell lines (Table 3), clearly highlighting their possible role fighting multidrug resistance. It was pleasing that even collateral sensitivity was observed in several cases. This phenomenon is characterized by the fact that drug-resistant cells are more sensitive to a test compound than the parental sensitive cells [47, 48].

The objective of cancer chemotherapy is to kill cancer cells with as little damage as possible to normal cells [49].

The GQW, VSL, and AML extracts were more cytotoxic towards HepG2 liver carcinoma cells and the other cancer cell lines tested than towards normal AML12 hepatocytes. This highlights at least some specificity of the three plant extracts towards target malignant cells with little effects on normal cells.

We further found that the GQW, VSL, and AML extracts induced apoptosis by disruption of MMP, whilst in addition AML produced ROS. To the best of our knowledge, the cytotoxicity of GQW, VSL, and AML is being reported here for the first time. Therefore, the isolation of the active constituents from these plants is worthwhile for the better understanding of their activities towards cancer cells.

In conclusion, the present study provides evidence of the cytotoxic potential of GQW, VSL, and AML extracts on sensitive and drug-resistant cancer cell lines. The three extracts induced apoptosis in CCRF-CEM cells by loss of MMP and, in the case of AML, also enhanced ROS production. These plant extracts merit more detailed investigations to improve therapy of drug-resistant and refractory tumors in the future.
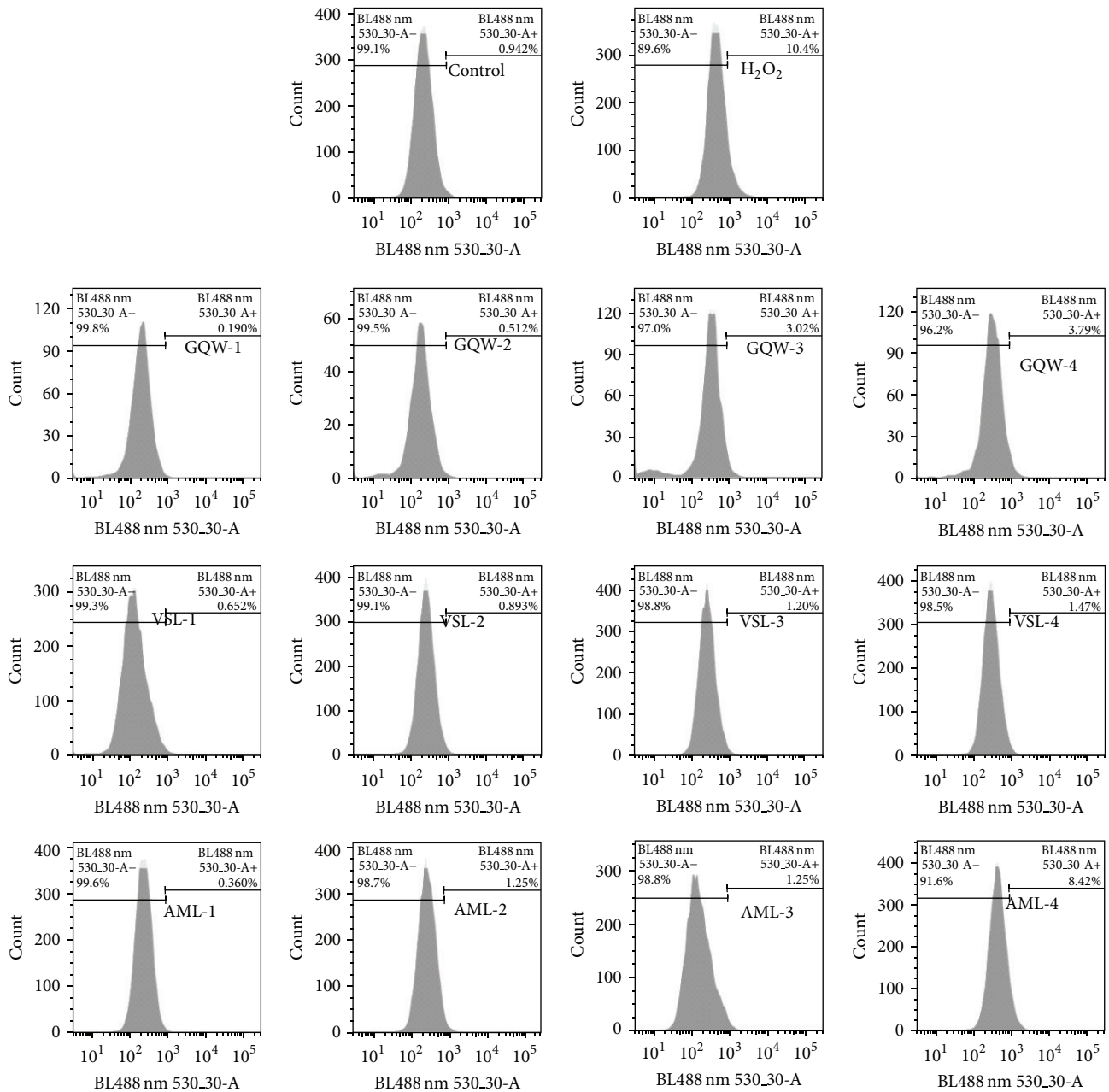
FIGURE 4: Effect of plant extracts and H_2O_2 (at 50 μM) on the ROS production of CCRF-CEM cells after 24 h treatment. Samples were tested at their $1/4 \times IC_{50}$ (1), $1/2 \times IC_{50}$ (2), IC_{50} (3), and $2 \times IC_{50}$ (4) values. The IC_{50} values are 10.57 μg/mL (*Gladiolus quartinianus* whole plant, *GQW*), 9.28 μg/mL (*Vepris soyauxii* leaves, *VSL*), and 17.32 μg/mL (*Anonidium mannii* leaves, *AML*).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Victor Kuete, Aimé G. Fankam, and Benjamin Wiench carried out the experiments. Victor Kuete and Thomas Efferth designed the study. Victor Kuete wrote the paper. Thomas Efferth supervised the work and provided the facilities for the study. All authors read and approved the final paper.

Acknowledgment

References

[1] C. Mathers, C. Boschi-Pinto, A. Lopez, and C. Murray, *Cancer Incidence, Mortality and Survival by Site for 14 Regions of the World*, World Health Organization, Lyon, France, 2001.

[2] H. Lage, N. Duarte, C. Coburger, A. Hilgeroth, and M. J. U. Ferreira, "Antitumor activity of terpenoids against classical and atypical multidrug resistant cancer cells," *Phytomedicine*, vol. 17, no. 6, pp. 441–448, 2010.

[3] E. Borowski, M. M. Bontemps-Gracz, and A. Piwkowska, "Strategies for overcoming ABC-transporters-mediated multidrug resistance (MDR) of tumor cells," *Acta Biochimica Polonica*, vol. 52, no. 3, pp. 609–627, 2005.

[4] V. Kuete, "Recent advances in medicinal plant research in sub-saharan Africa," in *Recent Progress in Medicinal Plants: Ethnomedicine and Therapeutic Validation*, J. Govil and G. Kaushik, Eds., vol. 32, pp. 73–131, Studium Press, Houston, Tex, USA, 2012.

[5] S. Carter and R. Livingston, "Principle of cancer chemotherapy," in *Principles of Cancer Treatment*, S. Carter, E. Glatstein, and R. Livingston, Eds., pp. 95–110, McGraw-Hill, New York, NY, USA, 1982.

[6] M. Cragg and D. Newman, *Natural Product Drugs Discovery in Next Millennium*, National Cancer Institute, Frederick, Md, USA, 2001.

[7] C. Stévigny, C. Bailly, and J. Quetin-Leclercq, "Cytotoxic and antitumor potentialities of aporphinoid alkaloids," *Current Medicinal Chemistry—Anti-Cancer Agents*, vol. 5, no. 2, pp. 173–182, 2005.

[8] D. J. Newman and G. M. Cragg, "Natural products as sources of new drugs over the last 25 years," *Journal of Natural Products*, vol. 70, no. 3, pp. 461–477, 2007.

[9] D. J. Newman and G. M. Cragg, "Natural products as sources of new drugs over the 30 years from 1981 to 2010," *Journal of Natural Products*, vol. 75, no. 3, pp. 311–335, 2012.

[10] J. Adjanohoun, N. Aboubakar, K. Dramane et al., *Traditional Medicine and Pharmacopoeia: Contribution to Ethnobotanical and Floristic Studies in Cameroon*, OUA/STRC, Lagos, Nigeria, 1996.

[11] V. Kuete, A. T. Mbaveng, M. Tsaffack et al., "Antitumor, antioxidant and antimicrobial activities of *Bersama engleriana* (Melianthaceae)," *Journal of Ethnopharmacology*, vol. 115, no. 3, pp. 494–501, 2007.

[12] V. Kuete, B. Krusche, M. Youns et al., "Cytotoxicity of some Cameroonian spices and selected medicinal plant extracts," *Journal of Ethnopharmacology*, vol. 134, no. 3, pp. 803–812, 2011.

[13] J. P. Dzoyem, A. H. L. N. Kuete, V. Kuete et al., "Cytotoxicity and antimicrobial activity of the methanol extract and compounds from *Polygonum limbatum*," *Planta Medica*, vol. 78, no. 8, pp. 787–792, 2012.

[14] A. T. Choumessi, M. Danel, S. Chassaing et al., "Characterization of the antiproliferative activity of *Xylopia aethiopica*," *Cell Division*, vol. 7, article 8, 2012.

[15] J. Dzoyem, S. Guru, C. Pieme et al., "Cytotoxic and antimicrobial activity of selected Cameroonian edible plants," *BMC Complementary and Alternative Medicine*, vol. 13, article 78, 2013.

[16] J. D. Tamokou, J. Chouna, E. Fischer-Fodor et al., "Anticancer and antimicrobial activities of some antioxidant-rich cameroonian medicinal plants," *PLoS ONE*, vol. 8, no. 2, Article ID e55880, 2013.

[17] G. A. Cordell, C. W. W. Beecher, and J. M. Pezzuto, "Can ethnopharmacology contribute to the development of new anticancer drugs?" *Journal of Ethnopharmacology*, vol. 32, no. 1–3, pp. 117–133, 1991.

[18] J. Popoca, A. Aguilar, D. Alonso, and M. L. Villarreal, "Cytotoxic activity of selected plants used as antitumorals in Mexican traditional medicine," *Journal of Ethnopharmacology*, vol. 59, no. 3, pp. 173–177, 1998.

[19] A. Raponda-Walker and R. Sillans, *Les plantes utiles du Gabon*, Paul Lechevalier, Paris, France, 1976.

[20] J. Vivien and J. Faure, *Fruitiers sauvages d'Afrique: especes du Cameroun*, Coopération Française, Paris, France, 1995.

[21] E. V. Ymele, A. B. Dongmo, and T. Dimo, "Analgesic and anti-inflammatory effect of aqueous extract of the stem bark of *Allanblackia gabonensis* (Guttiferae)," *Inflammopharmacology*, vol. 21, no. 1, pp. 21–30, 2013.

[22] A. G. B. Azebaze, B. M. W. Ouahouo, J. C. Vardamides et al., "Antimicrobial and antileishmanial xanthones from the stem bark of *Allanblackia gabonensis* (Guttiferae)," *Natural Product Research*, vol. 22, no. 4, pp. 333–341, 2008.

[23] J. Thomas, S. Bahuchets, A. Epelboin, and S. Furniss, *Encyclopédie des Pygmées Aka: techniques, langage et société des chasseurs-cueilleurs de la forêt centrafricaine (Sud-Centrafrique et Nord-Congo)*, Peeters-SELAF, Paris, France, 2003.

[24] E. Noumi and M. Eloumou, "Syphilis ailment: prevalence and herbal remedies in Ebolowa subdivision (South region, Cameroon)," *International Journal of Biomedical and Pharmaceutical Sciences*, vol. 2, no. 1, pp. 20–28, 2011.

[25] J. Betti, "An ethnobotanical study of medicinal plants among the Baka Pygmies in the Dja Biosphere Reserve, Cameroon," *African Study Monographs*, vol. 25, pp. 1–27, 2004.

[26] S. F. Kimbu, F. Keumedjio, L. B. Sondengam, and J. D. Connolly, "Two dinorditerpenoids from *Ricinodendron heudelotii*," *Phytochemistry*, vol. 30, no. 2, pp. 619–622, 1991.

[27] E. Noumi and A. Yomi, "Medicinal plants used for intestinal diseases in Mbalmayo Region, Central Province, Cameroon," *Fitoterapia*, vol. 72, no. 3, pp. 246–254, 2001.

[28] J. Momeni, M. Akam, S. Kimbu, and W. Kreiser, "Diterpenoids from *Ricinodendron heudelotii* (Euphorbiaceae)," *Journal of the Cameroon Academy of Science*, vol. 6, no. 1, pp. 119–124, 2006.

[29] J. Fondoun, T. Tiki, and J. Kengue, "*Recinodindron heudelotii* (Djansang): ethnobotany and importance for forest dwellers in Southern Cameroon," *Plant Genetic Resources Newsletter*, vol. 118, pp. 1–6, 1999.

[30] J. Momeni, R. D. Djoulde, M. T. Akam, and S. F. Kimbu, "Chemical constituents and antibacterial activities of the stem bark extracts of *Ricinodendron heudelotii* (Euphorbiaceae)," *Indian Journal of Pharmaceutical Sciences*, vol. 67, no. 3, pp. 386–389, 2005.

[31] E. Tekwu, A. Pieme, and V. Beng, "Investigations of antimicrobial activity of some Cameroonian medicinal plant extracts against bacteria and yeast with gastrointestinal relevance," *Journal of Ethnopharmacology*, vol. 142, no. 1, pp. 265–273, 2012.

[32] J. Momeni, W. P. D. D. Ntchatchoua, F. Fadimatou, M. T. Akam, and M. B. Ngassoum, "Antioxidant activities of some cameroonian plants extracts used in the treatment of intestinal and infectious diseases," *Indian Journal of Pharmaceutical Sciences*, vol. 72, no. 1, pp. 140–144, 2010.

[33] V. Kuete, P. D. Tchakam, B. Wiench et al., "Cytotoxicity and modes of action of four naturally occuring benzophenones: 2,2′,5,6′-tetrahydroxybenzophenone, guttiferone E, isogarcinol

and isoxanthochymol," *Phytomedicine*, vol. 20, no. 6, pp. 528–536, 2013.

[34] J. Harbone, *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis*, Chapman & Hall, London, UK, 1973.

[35] A. Kimmig, V. Gekeler, M. Neumann et al., "Susceptibility of multidrug-resistant human leukemia cell lines to human interleukin 2-activated killer-cells," *Cancer Research*, vol. 50, no. 21, pp. 6793–6799, 1990.

[36] T. Efferth, A. Sauerbrey, A. Olbrich et al., "Molecular modes of action of artesunate in tumor cell lines," *Molecular Pharmacology*, vol. 64, no. 2, pp. 382–394, 2003.

[37] J. Gillet, T. Efferth, D. Steinbach et al., "Microarray-based detection of multidrug resistance in human tumor cells by expression profiling of ATP-binding cassette transporter genes," *Cancer Research*, vol. 64, no. 24, pp. 8987–8993, 2004.

[38] H. S. Huang, M. Nagane, C. K. Klingbeil et al., "The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling," *The Journal of Biological Chemistry*, vol. 272, no. 5, pp. 2927–2935, 1997.

[39] J. O'Brien, I. Wilson, T. Orton, and F. Pognan, "Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity," *European Journal of Biochemistry*, vol. 267, no. 17, pp. 5421–5426, 2000.

[40] D. A. Bass, J. W. Parce, and L. R. Dechatelet, "Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation," *Journal of Immunology*, vol. 130, no. 4, pp. 1910–1917, 1983.

[41] A. Cossarizza, R. Ferraresi, L. Troiano et al., "Simultaneous analysis of reactive oxygen species and reduced glutathione content in living cells by polychromatic flow cytometry," *Nature Protocols*, vol. 4, no. 12, pp. 1790–1797, 2009.

[42] E. Teodori, S. Dei, C. Martelli, S. Scapecchi, and F. Gualtieri, "The functions and structure of ABC transporters: implications for the design of new inhibitors of Pgp and MRP1 to control multidrug resistance (MDR)," *Current Drug Targets*, vol. 7, no. 7, pp. 893–909, 2006.

[43] G. Szakács, J. K. Paterson, J. A. Ludwig, C. Booth-Genthe, and M. M. Gottesman, "Targeting multidrug resistance in cancer," *Nature Reviews Drug Discovery*, vol. 5, no. 3, pp. 219–234, 2006.

[44] N. Stamp, "Out of the quagmire of plant defense hypotheses," *Quarterly Review of Biology*, vol. 78, no. 1, pp. 23–55, 2003.

[45] V. Kuete and T. Efferth, "Pharmacogenomics of Cameroonian traditional herbal medicine for cancer therapy," *Journal of Ethnopharmacology*, vol. 137, no. 1, pp. 752–766, 2011.

[46] M. Suffness and J. Pezzuto, "Assays related to cancer drug discovery," in *Methods in Plant Biochemistry: Assays for Bioactivity*, K. Hostettmann, Ed., pp. 71–133, Academic Press, London, UK, 1990.

[47] K. M. Pluchino, M. D. Hall, A. S. Goldsborough, R. Callaghan, and M. M. Gottesman, "Collateral sensitivity as a strategy against cancer multidrug resistance," *Drug Resistance Updates*, vol. 15, no. 1-2, pp. 98–105, 2012.

[48] M. Saeed, H. Greten, and T. Efferth, "Molecular mechanisms of tumor cell resistance to chemotherapy," in *Collateral Sensitivity in Drug-Resistant Tumor Cells*, B. Bonavida, Ed., pp. 187–211, Springer, New York, NY, USA, 2013.

[49] B. Halliwell and J. Gutteridge, *Free Radicals in Biology and Medicine*, Charendon Press, Oxford, UK, 2nd edition, 1988.