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Cytotoxicity of 15 Cameroonian medicinal plants against drug sensitive and multi-drug resistant cancer cells

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ABSTRACT

Ethnopharmacological relevance: Cameroonian medicinal plants are traditionally used to treat many ailments, including cancer and related diseases. Cancer is characterized as a condition with complex signs and symptoms. 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 for anticancer screenings, since these reflect disease states bearing relevance to cancer or cancer-like symptoms.
Aim of the study: The present study aims at investigating 20 methanol extracts from 15 Cameroonian medicinal plants on a panel of human cancer cell lines, including various drug-resistant phenotypes. Possible modes of action of the of the most active plant were analyzed.
Materials and methods: Methanol extracts from different plant parts (leaves, bark, roots, fruits or whole plant) were evaluated for their cytotoxicity using resazurin reduction assay on a panel of nine sensitive and multi-drug resistant (MDR) cancer cell lines. Cell cycle, apoptosis, mitochondrial membrane potential (MMP), and reactive oxygen species (ROS) were measured by flow cytometry.
Results: Prescreening of extracts at 80 μg/mL showed that 6 extracts out of 20 inhibited more than 50% proliferation of leukemia CCRF-CEM cells; these include extracts from *Anthocleista schweinfurthii* fruits (ASF; 48.28%), *Morus mesozygia* bark (MMB; 42.76%), *Nauclea latifolia* bark (NLB; 38.75%), *Tridesmostemon omphalocarpoides* bark (TOB; 38.53%), *Nauclea latifolia* leaves (NLL; 35.17%) and *Erythrina sigmoidea* bark (ESB; 33.77%). Subsequent investigations revealed IC_{50} values below or around 20 μg/mL for extracts from MMB, NLB, NLL and ESB towards sensitive CCRF-CEM cells and its resistant P-glycoprotein over-expressing subline CEM/ADR5000. The best extract, ESB also displayed IC_{50} values below 20 μg/mL colon carcinoma HCT116 ($p53^{+/+}$) cells with an IC_{50} value of 19.63 μg/mL and it resistant p53 knockout subline HCT116 ($p53^{-/-}$) with an IC_{50} value of 16.22 μg/mL.
Conclusion: *Erythrina sigmoidea*, *Anthocleista schweinfurthii*, *Morus mesozygia*, *Nauclea latifolia*, *Tridesmostemon omphalocarpoides* used in African traditional medicine are good cytotoxic plants that can be exploited to develop phytomedicine to fight cancers including MDR phenotypes.

1. Introduction

Cancer is a worldwide disease and is now recognized as a serious public health concern in Africa. The number of new cancer cases will reach 15 million every year by 2020 worldwide, 70% of which will be 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 (Vorobiof and Abratt, 2007). Clear attention should be given to cancer diseases though communicable diseases continue to burden African population (Vorobiof and Abratt, 2007). Fighting malignancies should take into consideration the fact that cancer cells rapidly acquire multidrug resistance (MDR), mainly due to

Abbreviations: ASF, *Anthocleista Schweinfurthii* Fruits; DCF, Dichlorofluorescein; DMSO, Dimethylsufoxide; ESB, *Erythrina Sigmoidea* Bark; H_2DCFH-DA, 2′,7′-Dichlorodihydrofluorescein Diacetate; IC_{50}, Inhibitory Concentrations 50%; JC-1, 5,5′,6,6′-Tetrachloro-1,1′,3,3′-Tetraethylbenzimidazolylcarbocyanine Iodide; MDR, Multi-Drug Resistant; MMB, *Morus mesozygia* Bark; MMP, Mitochondrial Membrane Potential; NLB, *Nauclea Latifolia* Bark; NLL, *Nauclea Latifolia* Leaves; PBS, Phosphate Buffer Saline; ROS, Reactive Oxygen Species; TOB, *Tridesmostemon Omphalocarpoides* Bark

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the presence of two adenosine triphosphate-binding cassette (ABC) transporters (Biedler and Spengler, 1994; Efferth, 2001; Efferth et al., 2003b; Gillet et al., 2007; Shen et al., 2011), such as the breast cancer resistance protein (BCRP/ABCG2) and P-glycoprotein (P-gp/MDR1/ABCB1) (Shen et al., 2011) as well as the oncogene epidermal growth factor receptor (EGFR) (Biedler and Spengler, 1994; Efferth et al., 2003a, 2003b) and the deletions or inactivation of tumor suppressor gene p53 (el-Deiry, 1997). Medicinal plants have been used since ancient time to manage various human ailments including cancers. However, in the particular case of cancer, there is a lack of validation of potential antineoplastic plants throughout the world. To fill this gap, it has been recommended that plants used traditionally in case of immune and skin disorders, inflammatory, infectious, parasitic and viral diseases should be taken into account, if selecting plants for anticancer screenings, since these reflect disease states bearing relevance to cancer or cancer-like symptoms (Cordell et al., 1991; Popoca et al., 1998). This recommendation was lately corroborated in the pharmacogenomic studies of Cameroonian medicinal plant for cancer therapy where it was shown that most plant having promising cytotoxic compounds were not used traditionally to treat cancers (Kuete and Efferth, 2011). Besides, plant extracts consist of a mosaic of compounds displaying more than one mode of action on several target and therefore might be better treatment option than synthetic drugs, as multi-factorial activities may decrease the probability of emergence of resistant tumor clones (Efferth and Koch, 2011). Previously, several African medicinal plants, independently to the direct traditional use in the management of malignancies displayed good cytotoxicity towards drug-sensitive and drug-resistant cancer cell lines. These plants include *Aframomum arundinaceum, Aframomum. alboviolaceum*, *Aframomum kayserianum*, *Aframomum polyanthum* (Kuete et al., 2014a), *Echinops giganteus, Xylopia aethiopica, Piper capense, Imperata cylindrical* (Kuete et al., 2011a, 2013c). *Gladiolus quartinianus, Vepris soyauxii* (Kuete et al., 2013a), *Polygonum limbatum* (Dzoyem et al., 2012), *Polycias fulva*, *Beilschmiedia acuta*, *Crinum zeylanicum, Dioscorea bulbifera*, *Elaoephorbia drupifera* (Kuete et al., 2013e), *Solanum aculeastrum, Albizia schimperiana, Zanthoxylum giletii* and *Strychnos usambarensis* (Omosa et al., 2015). In our ongoing search of antiproliferative plants from African flora, the present study was designed to evaluate the cytotoxicity of 15 Cameroonian medicinal plants used traditionally to manage cancer or disease states bearing relevance to cancer or cance-liker symptoms.

2. Material and methods

2.1. Plant material and extraction

All medicinal plants used in the present work were collected in different regions of Cameroon between January and March 2012. The plants were identified at the National Herbarium (Yaoundé, Cameroon), where voucher specimens were deposited under the reference numbers shown in Table 1. The air-dried and powdered plant material was soaked in methanol for 48 h, at room temperature. The methanol extract was concentrated under reduced pressure to give the crude extract. This extract was then conserved at 4 °C until further use.

2.2. Chemicals

Doxorubicin 98.0% and vinblastine $\geq$96% from Sigma-Aldrich (Munich, Germany) were provided by the University Pharmacy of the Johannes Gutenberg University (Mainz, Germany) and dissolved in phosphate buffer saline (PBS; Invitrogen, Eggenstein, Germany) at a concentration of 10 mM. Geneticin $>$98% (Sigma-Aldrich) was stored at a stock concentration of 72.18 mM.

2.3. Cell cultures

The cell lines used in the present study, their origins and their treatments were previously reported. They include drug-sensitive leukemia CCRF-CEM and multidrug-resistant P-glycoprotein-overexpressing subline CEM/ADR5000 cells (Efferth et al., 2003b; Gillet et al., 2004; Kimmig et al., 1990), breast cancer MDA-MB-231-pcDNA3 cells and its resistant subline MDA-MB-231-*BCRP* clone 23 (Doyle et al., 1998), colon cancer HCT116 ($p53^{+/+}$) cells and its knockout clone HCT116 ($p53^{-/-}$), glioblastoma U87MG cells and its resistant subline U87MG.Δ*EGFR* (Kuete et al., 2013b, 2013c, 2013d).

2.4. Resazurin reduction assay

The cytotoxicity of the tested samples was performed by resazurin reduction assay as previously described (O'Brien et al., 2000; Kuete et al., 2013d). All extracts were previously tested at a single concentration of 80 μg/mL against the sensitive leukemia CCRF-CEM cells and those inhibiting more than 50% growth were further investigated for IC_{50} determinations in all studied cell lines. Doxorubicin was used as positive control while dimethylsulfoxide (DMSO), used to dissolve the samples was used as negative control. The highest concentration of DMSO was less than 0.4%. Fluorescence was measured on an Infinite M2000 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 (Kuete et al., 2011b; Dzoyem et al., 2012).

2.5. Flow cytometry for cell cycle analysis and detection of apoptotic cells

Extract from *Erythrina sigmoidea* bark (ESB) that displayed the best cytotoxicity and doxorubicin were used to treat CCRF-CEM cells (1×10^6) at their IC_{50} values. The cell cycle was then analyzed after incubation for 24 h, 48 h and 72 h. All reagents, experimental conditions and apparatus were identical to those previously reported (Kuete et al., 2013b; Kuete et al., 2013c). Briefly, cell cycle analysis was performed by flow cytometry using Vybrant® DyeCycle™ (Invitrogen, Darmstadt, Germany). Cells were measured after Vybrant® DyeCycle™ Violet staining (30 min at 37 °C) on a LSR-Fortessa FACS analyzer (Becton-Dickinson, Heidelberg, Germany) using the violet laser. 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.

2.6. Caspase-Glo 3/7, caspase-Glo 8 and caspase-Glo 9 assay

Caspase activity in CCRF-CEM leukemia cells treated ESB was detected using Caspase-Glo 3/7, Caspase-Glo 8 and Caspase-Glo 9 Assay kits (Promega, Mannheim, Germany) as previously described (Kuete et al., 2014b).

2.7. Analysis of mitochondrial membrane potential (MMP)

Leukemia CCRF-CEM cells were treated with ESB and vinblastine and the MMP was analyzed using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1;