Botanicals from the leaves of *Acacia sieberiana* had better cytotoxic effects than isolated phytochemicals towards MDR cancer cells lines

Carine M.N. Ngaffo a, Rodrigue S.V. Tchangna b, Armelle T. Mbaveng a, Justin Kamga b,c, Freya M. Harvey c, Bonaventure T. Ngadjuib, Christian G. Bochet c, Victor Kuete a

a Department of Biochemistry, Faculty of Science, University of Dschang, Dschang, Cameroon
b Department of Chemistry, Faculty of Science, University of Yaounde I, Yaounde, Cameroon
c University of Fribourg, Faculty of Science and Medicine, Department of Chemistry, Chemin du Musée 9, CH-1700 Fribourg, Switzerland

ARTICLE INFO

Keywords:
Acacia sieberiana
Apoptosis
Cytotoxicity
Fabaceae
Multi-drug resistance
Phytochemicals
Metabolite
Pharmacological scientific
Cancer research
Toxicology
Pharmacology

ABSTRACT

The efficiency of cancer chemotherapy is seriously hampered by the development of resistance of neoplastic cells to cytotoxic agents. In the present investigation, the cytotoxicity of the dichloromethane-methanol (1:1) extract of *Acacia sieberiana* (ASL), fractions (ASLa-c) from the leaves and isolated compounds: chrysoeriol-7-O-rutinoside (1), luteolin-7-O-rutinoside (2), chrysoeriol-7-O-D-glucopyranoside (3), Apigenin-7-O-D-glucopyranoside (4), luteolin-7,4′-dimethoxy-7′-O-D-glucoside (5) and luteolin (6) was investigated. The study was extended to the assessment of the mode of induction of apoptosis by ASL. The resazurin reduction assay (RRA) was used for cytotoxicity studies. Assessments of cell cycle distribution, apoptosis, and reactive oxygen species (ROS) were performed by flow cytometry. A caspase-Glo assay was used to evaluate caspase activities. Botanicals ASL, ASLb and ASLc as well as doxorubicin displayed observable IC50 values towards the nine tested cancer cell lines while ASLa and compounds 1–7 had selective activities. The IC50 values ranged from 13.45 μg/mL (in CCRF-CEM leukemia cells) to 33.20 μg/mL (in CCRF-CEM leukemia cells) to 29.64 μg/mL (against MDA-MB-231-breast adenocarcinoma cells) for ASL, from 16.42 μg/mL (in CCRF-CEM cells) to 29.64 μg/mL (against MDA-MB-231-pcDNA cells) for ASLa, and from 22.94 μg/mL (in MDA-MB-231-BCRP cells) to 40.19 μg/mL (against HCT116 (p53−/−) colon adenocarcinoma cells) for ASLb (Table 1), and from 0.92 μM (against CCRF-CEM cells) to 122.96 μM (against CEM/ADR5000 cells) for doxorubicin. ASL induced apoptosis in CCRF-CEM cells, mediated by ROS production. *Acacia sieberiana* is a good cytotoxic plant and should be further explored to develop an anticancer phytomedicine to combat both sensitive and drug resistant phenotypes.

1. Introduction

The effectiveness of herbal medicines in the treatment of cancer is well established. Plants have been the source of many established anticancer drugs. Clinically used secondary metabolites in cancer chemotherapy include combretastatins isolated from the Combretaceae plant, *Combretum caffrum* (Eckl. & Zeyh.) Kuntze, [1], vinblastine and vincristine isolated from Madagascar periwinkle, *Catharanthus roseus* (L.) G. Don., (Apocynaceae) [2], camptothecin from *Camptotheca acuminata* Decne (Nyssaceae) [3], paclitaxel isolated from a tree of the Cephalotaxaceae, *Cephalotaxus harringtonia* & (Cephalotaxaceae) [3]. Regarding the high diversity of phytochemicals in the plant kingdom, medicinal plants still constitute an undeniable source of novel anticancer agents. In the last decade, intensive studies have been performed on the anticancer potential of African medicinal plants. Considering the rapid development of resistance in cancer cell lines to cytotoxic drugs, several botanicals from African flora were successfully screened against multi-drug resistant phenotypes. A number of these botanicals showed a good potential to fight recalcitrant cancers. Some of such cytotoxic plants include *Pachypodanthium staudtii* Engl. & Diels (Annonaceae) [5], *Albizia adianthifolia* (Schumach.) W. Wight (Fabaceae) [6], *Dichrostachys cinerea* (L.) Wight & Arn. (Fabaceae) [7], *Fagara tessmannii* Engl. (Rutaceae) [8], *Withania obtusifolia* L. Dunal (Solanaceae), *Jasonia candidans* (Delile) Botsch. (Asteraceae), *Centaura lippii* L. (Asteraceae) and *Pulicaria undulata* (Forsk.) Hook.f. ex Benth., Oliv. & Hiern (Asteraceae) [9]. In the continuation of our research program aimed at discovering new...
botanicals to combat cancer multidrug resistance (MDR), the present work was designed to assess the cytotoxicity of the dichloromethane-methanol (1:1) extract and fractions from the leaves of *Acacia sieberiana* var Woodii (Fabaceae). This study was extended to the evaluation of possible cellular modes of action of the crude extract (ASL). *Acacia sieberiana* is traditionally used to treat bilharzia, tape-worm, haemorrhage, rheumatism, gonorrhea, syphilis, ophtalmia, stomach-ache, urethral diseases, oedema, and diarrhea [10]. The rationale of the present study comes to from fact that, there are recommendations that, ethnopharmacological usages such as immune and skin disorders, inflammatory diseases as well as infectious, parasitic and viral diseases should be taken into account when selecting plants that treat cancer; this is because these reflect disease states bearing relevance to cancer or cancer-like symptoms [11, 12, 13]. Previous phytochemical studies from the leaves of *Acacia sieberiana* harvested from Saudi Arabia afforded seven phenolics, namely 6,7,8-trihydroxy-3,4'-dimethoxy dihydroflavone, ellagic acid, gallic acid, isoferulic acid, quercetin, kaempferol, quercetin 3-0-β-D-glucoside and kaempferol 3-α-L-arabinoside [14]. The cytotoxicity of *Acacia sieberiana* is being reported for the first time.

2. Materials and methods

2.1. General procedure

Optical rotations were taken with a Polarimeter (Perkin-Elmer) using a sodium lamp operating at 589 nm. Infrared (IR) spectra (KBr disc) were recorded on a Perkin-Elmer 881 spectrometer. UV spectra were obtained on a Kontron model spectrophotometer. The nuclear magnetic resonance (NMR) spectra were performed on a Bruker DRX-400 MHz (400 MHz for ^1^H-NMR and 100 MHz for ^13^C-NMR) with tetramethylsilane (TMS) as internal reference. High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) was carried out in the positive-ion mode. Thin Layer Chromatography (TLC) employed precoated silica gel plates 60F254 (Merck). For flavonoids, the TLC solvent system CHCl3-MeOH-H2O (80-20-02 to 70-30-03) was used. Detection by UV at λ = 254 and 365 nm was followed by spraying with H2SO4–H2O (80-20). Column chromatography (CC) and Flash cartridge (Isolera) were carried out with SiO2 gel (63–200, 60 Å) in normal phase, and DIAION HP-20.

2.2. Chemicals

The tested phytochemicals were: chrysoeriol-7-O-rutinoside (1), luteolin-7-O-rutinoside (2), chrysoeriol-7-O-β-D-glucopyranoside (3), Apigenin-7-O-β-D-glucopyranoside (4), luteolin-3',4'-dimethoxyether-7-O-β-D-glucoside (5) and luteolin (6) (Figure 1). They were isolated from the dichloromethane (CH2Cl2); methanol (MeOH) (1:1) extract of the leaves of *Acacia sieberiana* var Woodii (Fabaceae) as described below. Doxorubicin (purity: 98.0%) purchased from Sigma-Aldrich (Munich, Germany) was obtained from the Johannes Gutenberg University Medical Center (Mainz, Germany). Geneticin >98% (used at 800 ng/mL and 400 μg/mL in culture media for MDA-MB-231 overexpressing breast cancer resistance protein (BCRP), U87MG with deleted epidermal growth factor receptor (EGFR), U87MG with deleted epidermal growth factor receptor (EGFR) and HCT116 p53/C0/C0 respectively) was obtained from Sigma-Aldrich and stored at 72.18 mM. Hydrogen peroxide (H2O2) was purchased from Sigma-Aldrich (Taufkirchen, Germany). DMSO (Sigma-Aldrich) was used to dissolved the test samples and the final concentration in all experiments was not greater than 0.1%.

Figure 1. Chemical structure of phytochemicals isolated from the leaves of *Acacia sieberiana*. Chrysoeriol-7-O-rutinoside (1), luteolin-7-O-rutinoside (2), chrysoeriol-7-O-β-D-glucopyranoside (3), apigenin-7-O-β-D-glucopyranoside (4), luteolin-3',4'-dimethoxyether-7-O-β-D-glucoside (5), luteolin (6), sitosterol-3-O-β-D-glucoside (7).
2.3. Plant material

The leaves of Acacia sieberiana var Woodii (Fabaceae) were collected in February 2017 at Kâlé (Far North Region, Cameroon; 10°6′00″N 14°27′00″E) and were identified by the botanist, Nana Victor of the National Herbarium of Cameroon, where a voucher specimen has been deposited under the registration number 49882/HNC.

2.4. Extraction and isolation of phytochemicals from the leaves of Acacia sieberiana

The leaves of Acacia sieberiana were reduced to a fine powder (930 g) and extracted by maceration in a mixture of dichloromethane (CH2Cl2): methanol (MeOH) (1:1) (8 L for 48 h) at room temperature. The solution was filtered and the solvents were evaporated in vacuo to afford a brown residue that constituted the crude extract (ASL; 66 g). Part of ASL (60 g) was dissolved in water (H2O; 300 mL) and successively partitioned with a mixture of CHCl3:MeOH:H2O (70:30:03); 115 new sub-fractions was dissolved in water (H2O; 300 mL) and successively partitioned with CHCl3:MeOH:H2O (90:10:01 to 70:30:03). 115 new sub-fractions was dissolved in water (H2O; 300 mL) and successively partitioned with CHCl3:MeOH:H2O (80:20:02). The mixture of sub-fractions eluted with CHCl3:MeOH:H2O (80:20:02) was further pooled into two main sub-fractions (8 mg; sub-frs 59 to 67), CHCl3:MeOH:H2O (70:30:03); 120 fractions (frs) of 20 mL each were collected as follows: CHCl3:MeOH:H2O (90:10:01): sub-frs 1 to 32; CHCl3:MeOH:H2O (90:10:01): sub-frs 1 to 71; CHCl3:MeOH:H2O (80:20:02): sub-frs 72 to 87; CHCl3:MeOH:H2O (70:30:03): sub-frs 88 to 120; Sub-frs eluted with CHCl3:MeOH:H2O (80:20:02) yielded compound 1 (11 mg; sub-frs 88 to 120); CHCl3:MeOH:H2O (70:30:03): sub-frs 91 to 115. They were further pooled into six sub-fractions on the basis of their thin layer chromatography (TLC) profile. The distribution of cycle phases of CCRF-CEM cells after application of the crude extract (ASL) or doxorubicin was analyzed by the flow cytometry [25, 26]. We followed the methods of Mbaveng et al. [23]. Additionally, flow cytometry was also applied with annexin V/propidium iodide (PI) staining to detect apoptotic cells as previously described [25, 26]. Briefly, CCRF-CEM cells (1 × 10^6 cells) were treated with various concentrations of ASL, doxorubicin or DMSO (solvent control). After 24 h incubation in the standard culture condition described above, the cell cycle was analyzed using a BD Accuri C6 Flow Cytometer (BD Biosciences, Heidelberg, Germany) by measuring the propidium iodide fluorescence of individual nuclei. For the annexin V/PI staining, apoptosis was also assessed after 24 h incubation using fluorescein isothiocyanate (FITC)-conjugated annexin V/PI assay kit (eBioscience™ Annexin V; Invitrogen, San Diego, USA) and BD Accuri C6 Flow Cytometer (BD Biosciences). Cells were stained with 5 μL FITC-conjugated annexin V (10 mg/mL) and 10 μL PI (50 mg/mL) and incubated for 15 min in the dark at room temperature (RT) and then analyzed using BD Accuri C6 Flow Cytometer (BD Biosciences) [25, 26]. At least three independent experiments with three repetitions each were done.

2.5. Investigated cell lines

The total of 10 cell lines (9 cancer cells a normal AML12 hepatocyte) used in this study. Previous reports provided their origins and their characteristics; both drug-sensitive CCRF-CEM leukemia cells and its multidrug-resistant P-glycoprotein-overexpressing subline CEM/ADR5000 cells [15, 16, 17], HCT116 p53+/− colon cancer cells and its knockout clone HCT116 p53−/−, U87.MG glioblastoma cells and its EGFR-transfected U87.MGΔEGFR subline, MDA-MB-231-pcDNA3 breast cancer cells and BCRP-transfected, multidrug-resistant MDA-MB-231-BCRP clone 23 cells [18] were investigated. Besides HepG2 liver cancer cells [19], and normal AML12 hepatocytes were also used; AML12 hepatocytes were used with HepG2 cells [19].

2.6. Cytotoxicity assay

The cytotoxicity of botanicals (ASL, ASLa-ASLc), phytochemicals (1–6) and doxorubicin was determined using the well-described resazurin reduction assay (RRA) [20, 21] at identical experimental conditions as documented earlier [22, 23]. After 72 h incubation in the standard culture condition (humidified 5% CO2 atmosphere at 37 °C), the fluorescence was measured with Infinite M2000 Pro™ plate reader (Tecan, Crailsheim, Germany) at 544 nm as excitation wavelength and 590 nm as detection wavelength. The IC50 was defined as the concentrations of botanicals, phytochemicals or doxorubicin required to inhibit 50% of the cell proliferation, and were determined by linear regression using Microsoft Excel 2007 [24]. If both sensitive cells and their corresponding resistant counterparts were tested, the degree of resistance (D.R.) against the tested samples was determined as the ratio of the IC50 value of the resistant cell line divided by that of the corresponding parental sensitive cell line. The selectivity index (S.I.) was determined as the ratio of the IC50 value of the normal AML12 hepatocyte divided by that of HepG2 hepatocarcinoma cells.

2.7. Analysis of cell cycle distribution and detection of apoptotic cells by flow cytometry

The distribution of cycle phases of CCRF-CEM cells after application of the crude extract (ASL) or doxorubicin was analyzed by the flow cytometry [25, 26]. We followed the methods of Mbaveng et al. [23]. Additionally, flow cytometry was also applied with annexin V/propidium iodide (PI) staining to detect apoptotic cells as previously described [25, 26]. Briefly, CCRF-CEM cells (1 × 10^6 cells) were treated with various concentrations of ASL, doxorubicin or DMSO (solvent control). After 24 h incubation in the standard culture condition described above, the cell cycle was analyzed using a BD Accuri C6 Flow Cytometer (BD Biosciences, Heidelberg, Germany) by measuring the propidium iodide fluorescence of individual nuclei. For the annexin V/PI staining, apoptosis was also assessed after 24 h incubation using fluorescein isothiocyanate (FITC)-conjugated annexin V/PI assay kit (eBioscience™ Annexin V; Invitrogen, San Diego, USA) and BD Accuri C6 Flow Cytometer (BD Biosciences). Cells were stained with 5 μL FITC-conjugated annexin V (10 mg/mL) and 10 μL PI (50 mg/mL) and incubated for 15 min in the dark at room temperature (RT) and then analyzed using BD Accuri C6 Flow Cytometer (BD Biosciences) [25, 26]. At least three independent experiments with three repetitions each were done.

2.8. Detection of caspases activation

Caspases activity in CCRF-CEM cells treated with ASL was determined by measuring the luminescence using spectrophotometric method. After application of ASL to the cells, followed by 6 h incubation under standard culture conditions, the activities of caspases were evaluated using Caspase-Glo 3/7, 8 and 9 Assay kits (Promega, Mannheim, Germany) with an Infinite M2000 Pro™ plate reader (Tecan) as previously reported [19]. The protocol was followed as described by Mbaveng et al. [23].

2.9. Flow cytometric analysis of ROS production

The production of reactive oxygen species (ROS) in CCRF-CEM cells after application of ASL was determined by the flow cytometry. For instance, CCRF-CEM cells were treated with ASL, DMSO (solvent
control, or hydrogen peroxide (H$_2$O$_2$; positive control). After 24 h incubation in standard culture conditions, cells were resuspended in Phosphate Buffer Saline (PBS; Sigma-Aldrich) and the production of ROS was evaluated using 2’,7’-dichlorofluorescein diacetate (H$_2$DCFH-DA) (Sigma-Aldrich) staining as earlier described [27, 28, 29]. The protocol was followed as described by Mbaveng et al. [23].

### 2.10. Statistics

Graph pad prism 5 software was used for statistical analyses. Representative data from three independent experiments are shown as mean value ± S.E.M. One-way Analysis Variance (ANOVA) followed by post hoc Tukey’s test was used to determine the significance of the difference between mean values relative to the control. The p-value was calculated to determine significant differences (p-value < 0.05).

### 3. Results

#### 3.1. Phytochemistry

The phytochemical investigations of the leaves of *Acacia sieberiana* led to the isolation of six phenolic including one flavone: luteolin (6) and five flavone glycosides: chrysoeriol-7-O-rutinoside (1), luteolin-7-O-rutinoside (2), chrysoeriol-7-O-β-D-glucopyranoside (3), apigenin-7-O-β-D-glucopyranoside (4), luteolin-3’,4’-dimethoxyether-7-O-β-D-glucoside (5) and a terpenoid, the sterol glycoside identified as sitosterol-3-O-β-D-glucoside (7) (Figure 1).

#### 3.2. Cytotoxicity of botanicals and phytochemicals

The cytotoxicity of the crude extract, ASL and its fractions ASLa to ASLc was first performed against a panel of 9 cancer cells lines and the normal AML12 hepatocytes. The results are summarized in Table 1. Botanicals ASL, ASLa and ASLb displayed observable IC$_{50}$ values towards all tested cancer cell lines while ASLa had selective activity. The recorded IC$_{50}$ values ranged from 13.45 μg/mL (in CCRF-CEM leukemia cells) to 33.20 μg/mL (against the resistant MDA-MB-231-BCRP breast adenocarcinoma cells) for ASL, from 16.42 μg/mL (in CCRF-CEM cells) to 29.64 μg/mL (against MDA-MB-231-pDNA breast adenocarcinoma cells) for ASLa, and from 22.94 μg/mL (in MDA-MB-231-BCRP cells) to 40.19 μg/mL (against the resistant HCT116 (p53$^{-/-}$) colon adenocarcinoma cells) for ASLa. Table 1, and from 0.02 μM (against CCRF-CEM cells) to 122.96 μM (against CEM/ADR5000 leukemia cells) for doxorubicin (Table 2).

Hypersensitivity of HCT116 p53$^{-/-}$ cells (D.R. of 0.91) and U87MG,ΔEGFR glioblastoma cells (D.R. of 0.86) to ASL compared to their sensitive counterparts HCT116 p53$^{+/+}$ cells and U87MG cells was observed meanwhile that of the MDR MDA-MB-231-BCRP cells and U87MG,ΔEGFR cells to ASLb (D.R. of 0.72 and 0.74, respectively) and ASLc (D.R. of 0.68 and 0.75, respectively) compared to their respective sensitive counterparts was also noted (Table 1). Observable IC$_{50}$ values were obtained with ASLa on 6/9 cancer cell lines tested. This fraction (ASLa) was less active than ASLb and ASLc and was not further processed for the isolation of its active constituents. The purification of ASLc led to phytochemicals 1–5 and 7 while that of ASLb afforded compounds 6 and 7. The cytotoxicity of phytochemicals 1–6 was further evaluated on the tested panel of cancer cell lines and AML12 hepatocytes. The results summarized in Table 2 show that all the tested compounds had selective cytotoxic effects, with observable IC$_{50}$ values obtained in 7/9, 7/9, 6/9, 3/9, 3/9 and 1/9 for 3, 6, 5, 1, 2 and 5, respectively. It is worth noting that the hypersensitivity CEM/ADR5000 leukemia cells (D.R. of 0.90), HCT116 p53$^{-/-}$ cells (D.R. of 0.93) and U87MG,ΔEGFR cells (D.R. of 0.85) to compound 3 compared with their respective sensitive counterparts was also achieved (Table 2). From the cytotoxicity data, it appears that the best activity was obtained with the crude extract, ASL. Consequently, the cellular mode of action of ASL was investigated towards the most sensitive CCRF-CEM cells.

#### 3.3. Cell cycle distribution and apoptosis

The effects of botanical ASL and doxorubicin on the distribution of the cell cycle of CCRF-CEM cells after 24 h treatment are depicted in Figure 2. ASL and doxorubicin modified the cell cycles’ distribution in concentration-dependent manner. ASL induced cycle arrest in the GO/G1 phase in CCRF-CEM cells, whilst doxorubicin induced S and G2/M phase arrest (Figure 2). ASL also increased significantly the amount of cells in the sub-G0/G1 phase in a range from 3.35% (3.36%) towards of drug sensitive and MDR cancer cells lines after 72 h incubation.

### Table 1. IC$_{50}$ values botanicals from the leaves of *Acacia sieberiana* towards of drug sensitive and MDR cancer cells lines after 72 h incubation.

| Cell lines | Samples, IC$_{50}$ values in μg/mL and degrees of resistance* or selectivity index** | Crude extract | Fractions | ASL | ASLa | ASLb | ASLc |
|------------|------------------------------------------------------------------------------------|---------------|-----------|-----|------|------|------|
| CCRF-CEM   |                                                                                   | 13.45 ± 2.12  | 44.17 ± 3.58 | 23.08 ± 1.72 | 16.42 ± 1.33 |
| CEM/ADR5000|                                                                                   | 14.26 ± 0.88  | >80        | 26.55 ± 3.22 | 20.17 ± 1.17 |
| Degree of resistance |                                                                                   | 1.06          | >1.81     | 1.15 | 1.23 |
| MDA-MB-231-pDNA|                                                                  | 27.76 ± 4.09  | 33.61 ± 1.83 | 31.82 ± 1.72 | 29.64 ± 2.55 |
| MDA-MB-231-BCRP|                                                                                   | 33.20 ± 3.13  | 38.12 ± 2.40 | 22.94 ± 3.01 | 20.27 ± 1.16 |
| Degree of resistance |                                                                                   | 1.20          | 1.13      | 0.72 | 0.68 |
| HCT116 (p53$^{-/-}$) |                                                                                   | 18.85 ± 1.10  | 28.14 ± 0.96 | 34.52 ± 2.37 | 16.71 ± 1.84 |
| HCT116 (p53$^{+/+}$) |                                                                                   | 17.23 ± 2.01  | >80        | 40.19 ± 3.51 | 23.87 ± 1.76 |
| Degree of resistance |                                                                                   | 0.91          | >2.84     | 1.16 | 1.41 |
| U87MG      |                                                                                   | 19.44 ± 1.89  | 44.16 ± 4.78 | 32.12 ± 2.46 | 28.09 ± 1.81 |
| U87MG,ΔEGFR|                                                                                   | 16.78 ± 1.72  | 27.30 ± 3.28 | 23.66 ± 1.19 | 21.11 ± 1.97 |
| Degree of resistance |                                                                                   | 0.86          | 0.62      | 0.74 | 0.75 |
| HepG2      |                                                                                   | 21.65 ± 2.09  | >80        | 38.95 ± 2.87 | 23.19 ± 2.08 |
| AML12      |                                                                                   | 67.53 ± 4.76  | >80 nd    | >80   | 67.86 ± 4.91 |
| Selectivity index** |                                                                                   | 3.12          | 2.05      | 2.93 |     |

(*): The degree of resistance was determined as the ratio of IC$_{50}$ value in the resistant divided by the IC$_{50}$ in the sensitive cell line; CEM/ADR5000, MDA-MB-231-BCRP, HCT116 (p53$^{-/-}$) and U87MG,ΔEGFR were used as the corresponding resistant counterpart for CCRF-CEM, MDA-MB-231-pDNA, HCT116 (p53$^{+/+}$), U87MG respectively; (**): The selectivity index was determined as the ratio of IC$_{50}$ value in the normal AML12 hepatocytes divided by the IC$_{50}$ in HepG2 hepatocarcinoma cells; In bold: Significant cytotoxic effect [30, 34, 35]; nd: not determined; ASL: crude CH2Cl2:MeOH (1:1) extract from the leaves of *Acacia sieberiana*, ASLa: Hexane fractions from ASL; ASLb: ethyl acetate fraction from ASL; ASLc: n-butanol fraction from ASL; nd: not determined; The data for doxorubicin used as positive control in similar experimental conditions are summarized in Table 2.

---

C.M.N. Ngaffo et al. Heliyon 6 (2020) e05412
respectively; (**): The selectivity index was determined as the ratio of IC50 value in the normal AML12 hepacytes divided by the IC50 in HepG2 hepatocarcinoma cells; In bold: Significantly cytotoxic compound in HepG2 hepatocarcinoma cells.

3.4. Activation of caspases

The effects of ASL on the activation of caspases in CCRF-CEM cells was also investigated. The results depicted in Figure 4 show that, though some significant differences were observed, treatment of cells with ASL does not importantly regulate the activity of caspases, with only 1.02-fold, 1.11-fold and 1.03-fold increases, respectively, for caspases 3/7, 8 and 9 at 26.9 μg/mL. Hence, ASL is not an intrinsic (caspase 3/7 and 9) nor extrinsic (caspase 8) caspases modulator.

3.5. Production of ROS

After treatment of CCRF-CEM cells with ASL or H2O2, increase of ROS level was observed as compared to cells treated with solvent control, DMSO (Figure 5). Botanical ASL significantly enhanced the production of ROS in the range of 3.36% (3.36 μg/mL) to 26.9% (26.9 μg/mL). Treatment of the cells with the reference compound, H2O2, increased the ROS levels to 94.30% at 50 μM, while ROS production in non-treated cells was 0.6%.

4. Discussion

Carcinogenic cancers are the major cause of treatment failures in cancer patients suffering from malignant diseases. Therefore, the use of resistant cell lines when searching for novel cytotoxic drugs is an interesting strategy. In the present study, several models of MDR cancer cell lines were used. They included a transfected cell line harboring a
mutation-activated EGFR gene (ΔEGFR), p53 knockout cell line, breast cancer resistance protein (ABCG2/BCRP) and ATP-binding cassette (ABC)-transporter-overexpressing MDR-mediating-P-glycoprotein (P-gp; ABCB1/MDR1). The cross-resistance profile of CEM/ADR5000 cells to Vinca alkaloids, anthracyclines, epipodophyllotoxins or taxanes, has been demonstrated [30]; that of the MDA-MB-231-BCRP has been shown towards doxorubicin, daunorubicin, mitoxantrone and topotecan [18, 22]; cross-resistance of HCT-116 p53⁻/⁻ was reported towards 5-fluorouracil, 6-thioguanine, gemcitabine, oxaliplatin, doxorubicin, etoposide, irinotecan paclitaxel, bleomycin, bortezomib [31, 32]; The resistance of U87MG.ΔEGFR cells was reported towards doxorubicin, cisplatin, erlotinib, and homoharringtonine [32]. Interestingly, collateral sensitivity or hypersensitivity of HCT-116 p53⁻/⁻ colon adenocarcinoma cells and U87MG.ΔEGFR cells to ASL, and as well as that of MDA-MB-231-BCRP breast adenocarcinoma cells and U87MG.ΔEGFR glioblastoma cells to ASLb and were observed (Table 1). These data clearly indicate that these botanicals can be exploited in the fight against refractory tumors. More importantly, IC₅₀ values below 20 μg/mL were obtained with ASL against 6/9 tested cancer cell lines and with ASLc against 2/9 cancer cell lines (Table 1). In effect, the cutoff point for good botanicals has been set below 20 μg/mL upon 48 h or 72 h incubation by the National Cancer Institute USA (NCI) [33]. It can therefore be confirmed that the crude extract ASL, and in lesser extent fraction ASLc are interesting cytotoxic agents. These data also show that fractions ASLb and had better cytotoxic activity than ASLa, explaining why they were selected for further isolation of their bioactive constituents. The purification of ASLc afforded phytochemicals 1–5 while that of ASLb led to 6 and 7. Unfortunately, none of these compounds had a good cytotoxic activity, as they generally displayed IC₅₀ values above 10 μM (Table 2). In effect, the cytotoxicity of phytochemicals is considered significant if the recorded IC₅₀ value is below 10 μM [7, 33, 34]. This might be indication that the good activity of the crude extract and in lesser extent that of the

Figure 3. Apoptotic effect of the crude extract (ASL) and doxorubicin as determined by the annexin V/PI assay in CCRF-CEM cells after 24 h. Flow cytometric measurements were performed after annexin V-PI double staining. IC₅₀ values were 13.45 μg/mL for ASL and 0.02 μM for doxorubicin. Shown are mean values ±SD of three independent experiments. Control cells were treated with DMSO to a final concentration of 0.1%.

Figure 4. Effects of the crude extract (ASL) on caspase activities in CCRF-CEM cells treated for 6 h. Caspase activity is expressed as percentage (%) compared to untreated cells. Shown are mean values ±SD of three independent experiments. Untreated cells were treated only with DMSO to a final concentration of 0.1%. (**): values are significantly different to that of untreated control (P < 0.05).
fractions ASLc and ASLb could be due the synergistic effects of their various constituents, rather than to the prominent inhibitory activity of individual compounds. It is important to note that several flavonoids such chrysoeriol-7-O-rutinoside (1), luteolin-7-O-rutinoside (2), chrysoteriol-7-O-β-D-glucopyranoside (3), apigenin-7-O-β-D-glucopyranoside (4), luteolin-3',4'-dimethoxyether-7-O-β-D-glucoside (5) and luteolin (6) were isolated from ASL. Their modes of action were not evaluated in the present study, in regards to their low or moderate activity towards the tested cancer cell lines. However, various flavone aglycones such artocarpesin gancaroonin Q, 6-prenylapigenin or 6,8-diprenylferiodictyol have earlier been shown to induce apoptosis in CCRF-CEM, through caspase activation, MMP alteration or ROS production [35, 36]. Luteolin has also been reported to induce apoptosis in SMMC-7721 human liver cancer cells [37], and A375 human melanoma cells via suppression of MMP-2 and MMP-9 through the PI3K/AKT pathway [38]. Chrysoeriol also had cytotoxic effects towards A549 human lung cancer cells via activation of autophagy, sub-G1/G0 cell cycle arrest, cell migration and invasion inhibition, and modulation of MAPK/ERK signalling pathway [39]. Apigenin induced apoptosis and autophagy in HepG2 cells through inhibition of PI3K/Akt/mTOR pathway [40]. It is worth noting that sitosterol-3-O-β-D-glucoside (7) was not tested in the present study, because a previous work had documented its poor cytotoxicity towards the cancer cell lines tested herein, with all IC_{50} values above 100 μM [8]. Consequently, only the crude extract, which had the best cytotoxic activity, was further selected for mechanistic studies. The cellular modes of action of ASL, including cell cycle distribution, apoptosis, caspases activation and ROS level evaluations, were determined towards the most sensitive CCRF-CEM leukemia cell line.

Apoptosis induction in cancer cells has been documented as a major mode of cellular death provoked by cytotoxic drugs including botanicals and phytochemicals [23, 25, 41, 42]. In the present study, it was found that ASL induced apoptosis in CCRF-CEM cells and provoked cycle arrest in the G0/G1 phase (Figure 2) and this was later confirmed by the annexin V/PI results (Figure 3). ASL did not activate neither intrinsic caspase 3/7 and 9 nor the extrinsic caspase 8 (Figure 4), suggesting a poor involvement of intrinsic and extrinsic pathways in the apoptotic process induced by this botanical. The increase production of ROS has also been reported in the apoptotic process induced by various botanicals and phytochemicals in cancer cells [8, 41, 43, 44]. Treatment of CCRF-CEM cells with the crude extract, ASL, led to increased in ROS level (Figure 5). This suggests that ROS production contributes to ASL-induced cell death. Finally, the present study shows that ASL extract induces apoptosis and increases the production of ROS, which is closely related to apoptosis, but is not caspase-dependent. Such phenomenon have previously been identified for several botanicals [29, 44, 45]. Caspase-independent pathways have also been reported in cadmium-induced apoptosis renal in proximal tubule (RPT) cells [46]; In effect, caspase-independent apoptotic pathway, known as the apoptosis-inducing factor (AIF)/Endo G pathway has earlier been discussed. Caspase-independent apoptosis is activated by Bcl-2/adenovirus E1B 19-kDa interacting protein 3 (BNIP-3), which induces mitochondrial AIF release; Endo G acts as a modulator. Forced BNIP-3 expression by plasmid transfection results in mitochondrial Endo G release and nuclear translocation, leading to cell death [47].

5. Conclusion

The results of the present investigation demonstrate that Acacia sieberiana is a good cytotoxic plant that can help to fight cancers, including MDR phenotypes. Its active constituents: chrysoeriol-7-O-rutinoside, luteolin-7-O-rutinoside, chrysoteriol-7-O-β-D-glucopyranoside, apigenin-7-O-β-D-glucopyranoside, luteolin-3',4'-dimethoxyether-7-O-β-D-glucoside, luteolin and sitosterol-3-O-β-D-glucoside may act synergically to contribute to the cytotoxicity of the plant. The crude extract induced apoptosis in CCRF-CEM cells through increased ROS production. This extract should be further explored to develop a new drug to fight cancers.

Declarations

Author contribution statement

V. Kuete: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.
C.M.N. Ngaffo: Performed the experiments; Contributed reagents, materials, analysis tools or data.
R.S.V. Tchangna: Performed the experiments.
A.T. Mbaveng: Conceived and designed the experiments; Performed the experiments; Conceived and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.
J. Kama: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.
F.M. Harvey and C.G. Bochet: Contributed reagents, materials, analysis tools or data.
B.T. Ngadjui: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.
[43] A.T. Mbaveng, V. Kuete, T. Efferth, Potential of Central, Eastern and Western Africa medicinal plants for cancer therapy: spotlight on resistant cells and molecular targets, Front. Pharmacol. 8 (2017) 345.

[44] V. Kuete, T. Efferth, African flora has the potential to fight multidrug resistance of cancer, BioMed Res. Int. 2015 (2015) 914813.

[45] V. Kuete, L.P. Sandjo, B. Wiench, T. Efferth, Cytotoxicity and modes of action of four Cameroonian dietary spices ethno-medically used to treat Cancers: Echinops giganteus, Xylopia aethiopica, Imperata cylindrica and Piper capense, J. Ethnopharmacol. 149 (2013) 245–253.

[46] G. Liu, H. Zou, T. Luo, M. Long, J. Bian, X. Liu, J. Gu, Y. Yuan, R. Song, Y. Wang, J. Zhu, Z. Liu, Caspase-dependent and caspase-independent pathways are involved in iodine-induced apoptosis in primary rat proximal tubular cell culture, PloS One 11 (2016), e0166823.

[47] Z. Zhang, X. Yang, S. Zhang, X. Ma, J. Kong, BNIP3 upregulation and EndoG translocation in delayed neuronal death in stroke and in hypoxia, Stroke 38 (2007) 1606–1613.