Cytotoxicity of Plumbagin, Rapanone and 12 other naturally occurring Quinones from Kenyan Flora towards human carcinoma cells

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Abstract

Background: Cancer is a major public health concern globally and chemotherapy remains the principal mode of the treatment of various malignant diseases.

Methods: This study was designed to investigate the cytotoxicity of 14 naturally occurring quinones including; 3 anthraquinones, 1 naphthoquinone and 10 benzoquinones against 6 human carcinoma cell lines and normal CRL2120 fibroblasts. The neutral red uptake (NR) assay was used to evaluate the cytotoxicity of the compounds, whilst caspase-Glo assay was used to detect caspases activation. Cell cycle and mitochondrial membrane potential (MMP) were all analyzed via flow cytometry meanwhile levels of reactive oxygen species (ROS) were measured by spectrophotometry.

Results: Anthraquinone: emodin (2), naphthoquinone: plumbagin (4), and benzoquinones: rapanone (9), 2,5-dihydroxy-3-pentadecyl-2,5-cyclohexadiene-1,4-dione (10), S-O-methylembelin (11), 1,2,4,5-tetraacetate-3-methyl-6-(14-nonadecenyl)-cyclohexadi-2,5-diene (13), as well as doxorubicin displayed interesting activities with IC₅₀ values below 100 μM in the six tested cancer cell lines. The IC₅₀ values ranged from 37.57 μM (towards breast adenocarcinoma MCF-7 cells) to 99.31 μM (towards small cell lung cancer A549 cells) for 2, from 0.06 μM (MCF-7 cells) to 1.14 μM (A549 cells) for 4, from 2.27 μM (mesothelioma SPC212 cells) to 46.62 μM (colorectal adenocarcinoma DLD-1 cells) for 9, from 8.39 μM (SPC212 cells) to 48.35 μM (hepatocarcinoma HepG2 cells) for 10, from 22.57 μM (MCF-7 cells) to 61.28 μM (HepG2 cells) for 11, from 9.25 μM (MCF-7 cells) to 47.53 μM (A549 cells) for 13, and from 0.07 μM (SPC212 cells) to 1.01 μM (A549 cells) for doxorubicin. Compounds 4 and 9 induced apoptosis in MCF-7 cells mediated by increased ROS production and MMP loss, respectively.

Conclusion: The tested natural products and mostly 2, 4, 9, 10, 11 and 13 are potential cytotoxic compounds that deserve more investigations towards developing novel antiproliferative drugs against human carcinoma.

Keywords: Carcinoma, cytotoxicity, Mode of action, Plumbagin, Quinones, Rapanone

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Background

Cancer is a major public health problem globally killing about 3500 million people and representing 2–3% of the annual deaths [1]. Due to limited resources and other pressing public health problems, including communicable diseases such as acquired immune deficiency syndrome (AIDS), malaria, and tuberculosis, cancer continues to receive low public health priority in Africa, despite the growing burden of the disease [2]. Chemotherapy remains the principal mode of the treatment of various malignant diseases. In recent years, the search of antineoplastic compounds of natural origin has become more and more important. Many investigations are being carried out to identify new drugs or to find new lead structures from the flora of Africa to develop novel therapeutic agents for the treatment of human diseases such as cancer [3]. In our continuous quest of lead molecules to fight cancer, we designed the present study to investigate the cytotoxicity of 14 quinones including 3 anthraquinones, one naphthoquinone and 10 benzoquinones, previously isolated from African medicinal plants. The study was extended to the study of the mode of action of the most active compounds including: plumbagin (4; a naphthoquinone) and rapanone (9; a benzoquinone). Quinones are secondary metabolites isolated principally from plants and having an aromatic di-one or di-ketone systems. Naturally occurring quinones are widely distributed and include benzoquinones, naphthoquinones, anthraquinones and polyquinones [4]. They exhibit numerous biological activities such as: neurological, antibacterial, antiplasmodial, antioxidant, trypanocidal, antitumor, antiviral activities [4, 5]. Amongst plant secondary metabolites, quinones comprise the second largest class of anticancer agents [6]. Some quinones isolated from African medicinal plants previously displayed anticancer activities. They include sargarhoquinic acid isolated from Sargassum heterophyllum [7], 2-acetylfuro-1,4-naphthoquinone isolated from Newbouldia laevis [8], and anthraquinones such as damacanthal, damacanthol, 3-hydroxy-2-hydroxymethyl anthraquinone and schimperiquinone B obtained from Pentas schimperi [9].

Methods

Chemicals

The quinones (Fig. 1) used in this study were obtained from the chemical bank of the natural products research laboratory of the Chemistry Department, University of Nairobi, Kenya. Their isolation and identification were previously reported from the following plants: Rumex dentatus, R. abyssinicus, R. usambarensis, R. bequaertii, R. ruwenzoriensis, R. crispus; Plumbago zeylanica, Myrsine Africana, Maesa lanceolata, Rapanee melanphloes, Aloe saponaria [10]. They include anthraquinones: chrysophanol (1), emodin (2), 3,6,8-trihydroxy-1-methyl-lanthraquinone-2-carboxylic acid methyl ester (3), a naphthoquinone: 5-hydroxy-2-methyl-1,4-naphthalenedione or plumbagin (4), benzoquinones; 2,5-dihydroxy-3-ethyl-2,5-cyclohexadiene-1,4-dione (5), 2,5-dihydroxy-3-propyl-2,5-cyclohexadiene-1,4-dione (6), 2,5-dihydroxy-3-butyl-2,5-cyclohexadiene-1,4-dione (7), 2,5-dihydroxy-3-heptyl-2,5-cyclohexadiene-1,4-dione (8), 2,5-dihydroxy-3-tridecyl-2,5-cyclohexadiene-1,4-dione or rapanone (9), 2,5-dihydroxy-3-pentadecyl-2,5-cyclohexadiene-1,4-dione (10), 2-hydroxy-5-methoxy-3-undecyl-1,4-benzoquinone or 5-O-methylumbellin (11), 2,5 dimethoxy-6-(14-nonadecenyl)-1,4-benzoquinone (12), 1,2,4,5-tetraacetate-3-methyl-6-(14-nonadecenyl)-cyclohexadi-2,5-diene (13) and ardisiaquinone B (14) [10]. Doxorubicin 98.0% was purchased from Sigma-Aldrich (Munich, Germany) and used as reference drug.

Cell lines and culture

Six human cancer cell lines and one normal cell line were used in this study. They included A549 human non-small cell lung cancer (NSCLC) cell line, obtained from the Institute for Fermentation, Osaka (IFO, Japan) and provided by Prof. Dr. Tansu Koparal (Anadolu University, Eskisehir, Turkey), SPC212 human mesothelioma cell line obtained from American Type Culture Collection (ATCC) and provided by Dr. Asuman Demiroğlu Zergeroğlu (Gebze Technical University, Kocaeli, Turkey), DLD-1 colorectal adenocarcinoma cell lines obtained from ATCC (CCL-221), Caco2 colorectal adenocarcinoma cells (ATCC, HTB-37) obtained from the SAP Institute of Turkey (Ankara), HepG2 hepatocarcinoma cells (ATCC, HB-8065) and MCF-7 breast adenocarcinoma cells (ATCC, HTB-22) were provided by Prof. Dr. Tansu Koparal (Anadolu University, Eskisehir, Turkey). The normal CRL2120 human skin fibroblasts were obtained from ATCC [CCL1094S (ATCC, CRL-2120)]. The cells were maintained as a monolayer in DMEM medium (Sigma-aldrich, Munich, Germany) medium supplemented with 10% fetal calf serum and 1% penicillin (100 U/mL) streptomycin (100 μg/mL) in a humidified 5% CO₂ atmosphere at 37 °C.

Neutral red (NR) uptake assay

The cytotoxicity of samples was performed by NR uptake assay as previously described [11, 12]. This method is based on the ability of viable cells to incorporate and bind the supravital dye NR in the lysosomes. The procedure is cheaper and more sensitive than other cytotoxicity tests [13]. Samples were added in the culture medium so that dimethylsulfoxide (DMSO) used prior for dilution did not exceed 0.1% final concentration. Briefly, cells were detached by treatment with 0.25% trypsin/EDTA (Invitrogen) and an aliquot of 1 x 10⁴ cells
was placed in each well of a 96-well cell culture plate (Thermo Scientific, Germany) in a total volume of 200 μL. The cells were allowed to attach overnight and subsequently treated with different concentrations of the 14 compounds. Each of the studied samples was immediately added in varying concentrations in additional 100 μL of culture medium to obtain a total volume of 200 μL/well. After 72 h incubation in humidified 5% CO2 atmosphere at 37 °C, the medium was removed and 200 μL fresh medium containing 50 μg/mL NR was added to each well and incubation continued for an additional 3 h at 37 °C in 5% CO2 atmosphere. The dye medium was then removed and each well was then washed rapidly with 200 μL phosphate buffer saline (PBS) followed by addition of 200 μL of acetic acid-water-ethanol in water (1:49:50). The plates were kept for 15 min at room temperature to extract the dye and then shaken for a few minutes on a GFL 3012 shaker (Gesellschaft für Labortechnik mbH, Burgwedel, Germany). Absorbance was measured on ELx 808 Ultra Microplate Reader (Biotek) equipped with a 540 nm filter. Each assay was done at least three times, with three replicates each. The viability was evaluated based on a comparison with untreated cells. The IC50 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 [14].

Flow cytometry for cell cycle analysis and detection of apoptotic cells

The cell-cycle analysis was performed by flow cytometry using BD cyctest™ Plus DNA Kit Assay (BD Biosciences, San Jose, USA). The BD Cyctest™ Plus DNA kit provides a set of reagents for isolating and staining cell nuclei. Flow cytometric analysis of differentially stained cells is used to estimate the DNA index (DI) and cell-cycle phase distributions. Briefly, MCF-7 cells (3 mL, 1 × 105 cells/mL) were seeded into each well of 6-well plates and allowed to attach for 24 h. The cells which were treated with ¼ × IC50, ½ × IC50 and IC50 concentrations of compounds 4, 9 and the standard drug, doxorubicin were then grown in 6-well plates for 72 h. The untreated cells (control) were also included in the assay. They were further trypsinized and suspended in 1 mL
PBS, then centrifuged at 400 g for 5 min at room temperature (RT). The cells were further processed according to the manufacturer protocol: addition of 250 μL of solution A (trypsin buffer), 10 min incubation at RT followed by the addition of 200 μL of solution B (trypsin inhibitor and RNAse buffer), 10 min incubation at RT followed by the addition of 200 μL of solution C (2–8 °C) (propidium iodide stain solution), 10 min on ice. The cells were further measured on a BD FACS Aria I Cell Sorter Flow Cytometer (Becton-Dickinson, Germany). For each sample, 104 cells were counted. For PI excitation, an argon-ion laser emitting at 488 nm was used. Cytographs were analyzed using BD FACSDiva™ Flow Cytometry Software Version 6.1.2 (Becton-Dickinson).

**Caspase-Glo 3/7 and caspase-Glo 9 assay**

Caspases activity in MCF-7 cells was detected using Caspase-Glo 3/7 and caspase-Glo 9 assay kits (Promega, Mannheim, Germany) as previously reported [15–17]. Cells were treated with compounds 4 and 9 at their 2 × IC50 and IC50 values with DMSO as solvent control for 6 h. Luminescence was measured using an BioTek Synergy HT multi-detection microplate reader. Caspase activity was expressed as percentage of the untreated control.

**Analysis of mitochondrial membrane potential (MMP)**

The MMP was analyzed in MCF-7 cells by 5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Biomol, Hamburg, Germany) staining as previously reported [15–17]. Cells (3 mL, 1 × 105 cells/mL) treated for 72 h with different concentrations (¼ × IC50, ½ × IC50 and IC50) of compounds 4, 9 and doxorubicin (drug control) or DMSO (solvent control) were incubated with JC-1 staining solution for 30 min according to the manufacturer’s protocol as reported in earlier. Subsequently, cells were measured in a BD FACS Aria I Cell Sorter Flow Cytometer (Becton-Dickinson, Germany). The JC-1 signal was measured at an excitation of 561 nm (150 mW) and detected using a 586/15 nm band-pass filter. The signal was analyzed at 640 nm excitation (40 mW) and detected using a 730/45 nm bandpass filter. Cytographs were analyzed using BD FACSDiva™ Flow Cytometry Software Version 6.1.2 (Becton-Dickinson). All experiments were performed at least in triplicate.

**Measurement of reactive oxygen species (ROS)**

The 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFH-DA) (Sigma-Aldrich) was used for the detection of ROS in MCF-7 cells treated with compounds 4, 9 and doxorubicin (drug control) or DMSO (solvent control) using OxiSelect™ Intracellular ROS Assay Kit (Green Fluorescence) as recommended by the manufacturer, Cell Biolabs Inc. (San Diego, USA). This is a cell-based assay for measuring hydroxyl, peroxyl, or other reactive oxygen species activity within a cell. The assay employs the cell-permeable fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA is diffused into cells and is deacetylated by cellular esterases to non-fluorescent 2′,7′-dichlorodihydrofluorescein (DCFH), which is rapidly oxidized to highly fluorescent 2′,7′-dichlorofluorescein (DCF) by ROS. Cells (1 × 104 cells) were treated with samples at ¼ × IC50, ½ × IC50 and IC50 for 24 h. After addition of 100 μL 1 × DCFH-DA/DMEM solution to cells and incubation at 37 °C for 30–60 min, the fluorescence was measured using SpectraMax® M5 Microplate Reader (Molecular Devices, Biberach, Germany) at 480/530 nm. All experiments were performed at least in triplicate.

**Results**

The fourteen investigated compounds included three anthraquinones; chrysophanol C13H10O4 (1; m/z: 254.0579), emodin C13H10O5 (2; m/z: 270.0528), 3,6,8-trihydroxy-1-methylandraquinone-2-carboxylic acid methyl ester C17H17O6 (3; m/z: 328.0583), one naphthaquinone; 5-hydroxy-2-methyl-1,4-naphthaledione or plumbagin C11H9O4 (4; m/z: 188.0473), and benzoquinones; 2,5-dihydroxy-3-ethyl-2,5-cyclohexadiene-1,4-dione C6H8O2 (5; m/z: 168.0423), 2,5-dihydroxy-3-propyl-2,5-cyclohexadiene-1,4-dione C8H10O2 (6; m/z: 182.0579), 2,5-dihydroxy-3-butyl-2,5-cyclohexadiene-1,4-dione C10H12O2 (7; m/z: 196.0736), 2,5-dihydroxy-3-heptyl-2,5-cyclohexadiene-1,4-dione C13H14O4 (8; m/z: 238.1205), 2,5-dihydroxy-3-tridecyl-2,5-cyclohexadiene-1,4-dione or rapanone C19H32O4 (9; m/z: 322.4450), 2,5-dihydroxy-3-pentadecyl-2,5-cyclohexadiene-1,4-dione C21H34O4 (10; m/z: 350.2457), 2-hydroxy-5-methoxy-3-undecyl-1,4-benzoquinone, 5-O-methylmellebin C19H18O4 (11; m/z: 308.1988), 2,5-dimethoxy-6-(14-nonenadecenyl)-1,4-benzoquinone C27H44O4 (12; m/z: 432.3240), 1,2,4,5-tetraacetate-3-methyl-6-(14-nonenadecylyl)-cyclohexadi-2,5-diene C34H56O8 (13; m/z: 590.3819), ardisiaquinone B C40H40O8 (14; m/z: 528.2723) [10]. These compounds are available in the Chemical bank of the Department of Chemistry, University of Nairobi, Kenya.

**Cytotoxicity**

The cytotoxicity of the 14 quinones and doxorubicin was determined by the NR uptake assay and the recorded IC50 values are summarized in Table 1. The selectivity index was determined as the ratio of IC50 value in the CRL2120 normal fibroblast divided by the IC50 in the cancer cell line. Compounds 2, 4, 9, 10, 11 and 13 as well as doxorubicin displayed IC50 values below 100 μM in the six tested cancer cell lines. Compounds 3, 5 and 12 were not active with IC50 values above 120 μM in all cancer cell lines meanwhile 1, 6, 7,
Table 1  Cytotoxicity of tested compounds and doxorubicin towards cancer cell lines and normal cells as determined by the neutral red assay

| Compounds | Cell lines, IC50 values in μM and selectivity index a (in bracket) |
|-----------|---------------------------------------------------------------|
|           | AS40  | SPC212 | DLD-1 | Caco-2 | MCF-7 | HepG2 | CRL2120 |
| 1         | 52.24 ± 5.51 (>3.01) | 145.63 ± 10.63 (>1.08) | >157.48 | >157.48 | >157.48 | >157.48 | >157.48 |
| 2         | 66.30 ± 6.19 (2.23) | 99.31 ± 8.46 (1.49) | 77.28 ± 8.77 (>1.92) | 73.63 ± 3.52 (>2.01) | 37.57 ± 2.59 (>3.94) | 71.7 ± 4.52 (>2.07) | >148.15 |
| 3         | >121.95 | >121.95 | >121.95 | >121.95 | >121.95 | >121.95 | >121.95 |
| 4         | 1.14 ± 0.02 (59.35) | 0.27 ± 0.01 (250.59) | 0.98 ± 0.11 (70.47) | 0.07 ± 0.01 (966.57) | 0.06 ± 0.01 (1127.67) | 1.01 ± 0.08 (66.99) | 67.66 ± 6.49 |
| 5         | >238.10 | >238.10 | >238.10 | >238.10 | >238.10 | 95.51 ± 5.64 | >238.10 |
| 6         | >222.22 | >222.22 | >222.22 | >222.22 | >222.22 | 161.92 ± 11.37 | >222.22 |
| 7         | 68.62 ± 7.55 (>2.97) | 87.91 ± 5.66 (2.32) | >204.08 | 89.72 ± 8.80 (>2.27) | 64.59 ± 13.42 (3.16) | 176.17 ± 28.5 (>1.16) | >204.08 |
| 8         | 107.52 ± 17.95 (1.09) | 8.05 ± 1.46 (1.09) | >168.07 | 63.93 ± 5.97 (2.27) | 38.8 ± 3.33 (3.16) | 94.22 ± 2.7 (1.16) | 117.27 ± 1.22 |
| 9         | 27.35 ± 1.46 (3.48) | 2.27 ± 1.52 (41.96) | 46.62 ± 5.38 (2.04) | 22.95 ± 0.13 (4.15) | 16.94 ± 4.65 (5.62) | 32.69 ± 0.61 (2.91) | 95.24 ± 6.65 |
| 10        | 43.32 ± 2.72 (>2.87) | 8.39 ± 0.48 (>14.81) | 51.21 ± 5.54 (>2.43) | 27.81 ± 2.03 (>4.47) | 30.37 ± 8.64 (>4.09) | 48.35 ± 3.73 (>2.57) | >124.24 |
| 11        | 50.26 ± 2.00 (0.88) | 38.28 ± 3.23 (1.15) | 45.37 ± 4.89 (0.97) | 38.89 ± 2.33 (1.14) | 22.56 ± 1.57 (1.96) | 61.28 ± 5.53 (0.72) | 44.20 ± 0.2 |
| 12        | 129.45 | >129.45 | >129.45 | >129.45 | >129.45 | >129.45 | >129.45 |
| 13        | 47.53 ± 3.56 (1.96) | 36.21 ± 3.08 (2.57) | 25.09 ± 1.50 (3.72) | 24.63 ± 2.71 (3.78) | 9.25 ± 0.16 (10.08) | 18.17 ± 1.46 (5.13) | 93.21 ± 1.17 |
| 14        | 21.68 ± 1.50 (3.29) | 3.14 ± 0.78 (22.72) | 114.17 ± 3.68 (0.62) | >123.08 | >123.08 | 114.60 ± 4.29 (0.62) | 71.34 ± 5.23 |
| Doxorubicin | 1.01 ± 0.19 (0.58) | 0.07 ± 0.01 (8.43) | 0.37 ± 0.05 (1.59) | 0.72 ± 0.13 (0.82) | 0.35 ± 0.05 (1.69) | 0.18 ± 0.03 (3.28) | 0.59 ± 0.01 |

*: The selectivity index was determined as the ratio of IC50 value in the CRL2120 normal fibroblasts divided by the IC50 in the cancer cell lines. Chrysophanol (1), emodin (2), 3,6,8-trihydroxy-1-methylnaphthaquinone-2-carboxylic acid methyl ester (3), plumbagin (4), 2,5-dihydroxy-3-ethyl-2,5-cyclohexadiene-1,4-dione (5), 2,5-dihydroxy-3-propyl-2,5-cyclohexadiene-1,4-dione (6), 2,5-dihydroxy-3-butyl-2,5-cyclohexadiene-1,4-dione (7), 2,5-dihydroxy-3-heptyl-2,5-cyclohexadiene-1,4-dione (8), 2,5-dihydroxy-3-tridecy1-2,5-cyclohexadiene-1,4-dione or rapanone (9), 2,5-dihydroxy-3-pentadecyl-2,5-cyclohexadiene-1,4-dione (10), 2-hydroxy-5-methoxy-3-undecyl-1,4-benzoquinone, 5-O-methylembelin (11), 2,5 dimethoxy-6-(14-nonadecenyl)-1,4-benzoquinone (12), 1,2,4,5-tetraacetate-3-methyl-6-(14-nonadecenyl)-cyclohexadi-2,5-diene (13), ardisiaquinone B (14). In bold: significant activity [3, 21, 22, 25]
8, and 14 displayed selective activities. The recordable IC50 values were obtained in 1/6 tested cancer cell lines for 6, 2/6 for 1, 4/6 for 14 and 5/6 for 7 and 8. Concerning the most active compounds, IC50 values ranged from 37.57 μM (towards breast adenocarcinoma MCF-7 cells) to 99.31 μM (towards small cell lung cancer A549 cells) for 2, from 0.06 μM (towards MCF-7 cells) to 1.14 μM (against A549 cells) for 4, from 2.27 μM (towards mesothelioma SPC212 cells) to 46.62 μM (against colorectal adenocarcinoma DLD-1 cells) for 9, from 8.39 μM (towards SPC212 cells) to 48.35 μM (towards hepatocarcinoma HepG2 cells) for 10, from 22.57 μM (towards MCF-7 cells) to 61.28 μM (towards HepG2 cells) for 11, from 9.25 μM (against MCF-7 cells) to 47.53 μM (against A549 cells) for 13, and from 0.07 μM (towards SPC212 cells) to 1.01 μM (towards A549 cells) for doxorubicin. The six most active compounds (2, 4, 9, 10, and 13) were generally less toxic towards normal CRL2120 fibroblast than carcinoma cells, and the obtained selectivity indexes were above 1.49, 1.96, 2.51, 2.91 and 59, respectively for 2, 13, 10, 9 and 4. Nonetheless, 11 as well as doxorubicin were in many cases slightly more toxic on normal CRL2120 fibroblast than on cancer cells (Table 1). Two compounds having the lowest IC50 values, namely 4 (below or around 1 μM in all the six cancer cell lines) and 9 (lowest IC50 value of 2.27 μM towards SPC212 cells) as well as doxorubicin were tested for the effects on cell cycle distribution, caspases activity, MMP breakdown and ROS production in MCF-7 cells.

**Cell cycle analysis and apoptosis**

Naphthoquinone 4 and benzoquinone 9 were analyzed for their ability to alter the distribution of the cell cycle of MCF-7 breast cancer cells (Fig. 2). It was observed that the two compounds induced concentration-dependent cell cycle modifications with progressive increase of sub-G0/G1 phase cells. Compounds 4 and 9 induced cell cycle arrest between G0/G1 and S phases. MCF-7 cells treated with the compounds 4 and 9 progressively underwent apoptosis, with increase of sub-G0/G1 cells from 10.4% (¼ IC50) to 20.4% (IC50) for 4 and from 34.8% (¼ IC50) to 43.2% (IC50) for 9. The positive control, doxorubicin also caused up to 60% sub-G0/G1 phase with IC50 treatment in comparison to only 3.1% in non-treated cells.

**Caspases activities**

Upon treatment of MCF-7 cells with naphthoquinone 4 and benzoquinone 9 with equivalent (eq.) to the IC50 and 2-fold IC50 for 6 h, no modification of the activity of caspase 3/7 and caspase 9 was observed (data not shown).
MMP breakdown
Treatment of MCF-7 cells with compounds 4 and 9 with eq. to the 1/4 × IC₅₀, 1/2 × IC₅₀ and IC₅₀ values for 72 h induced concentration-dependent depletion of MMP (Fig. 3). More pronounced effect was observed with 9 with up to 88.1% depletion of MMP at eq. to IC₅₀ while 4 caused 12.2% MMP loss at IC₅₀. In similar experimental condition, doxorubicin caused 26% loss of MMP meanwhile only 4.3% was observed with non-treated control.

ROS production
After treatment of MCF-7 cells with naphthoquinone 4 and benzoquinone 9 at eq. to the 1/4 × IC₅₀, 1/2 × IC₅₀ and IC₅₀ values for 24 h, the production of ROS in cells was analyzed (Fig. 4). Naphthoquinone 4 induced increased ROS levels of more than 3-fold (at IC₅₀) as compared with non-treated cells meanwhile the increase was lesser (less than 2-fold) after treatment with benzoquinone 9. In similar experimental condition doxorubicin also induced more than 2-fold increase in ROS production in MCF-7 cells at eq. to IC₅₀.
Discussion

Neoplastic diseases are one of the leading causes of mortality worldwide and the number of cancer cases are increasing regularly [1]. In general, leukemia cells are clinically more sensitive to chemotherapy than tumors [18, 19]. In the present study we focused on carcinoma cells involved in lung, colon, breast and liver cancers. In regards of the broad diversity of phytochemicals, the search of anticancer agents from plants represents an attractive strategy [20]. Molecules having IC\(_{50}\) values around or below 4 μg/mL or 10 μM [3, 21, 22] have been recognized as potential cytotoxic substances. IC\(_{50}\) values below 10 μM were observed with naphthoquinone 4 in all the six cancer cell lines. Interestingly, IC\(_{50}\) values below 1 μM were obtained with this compound in 4 of the 6 cancer cell lines, highlighting its good cytotoxic potential. In addition, the IC\(_{50}\) values obtained with 4 towards Caco-2 cells and MCF-7 cells were lower than that of the reference compound, doxorubicin. Other compounds such as 8, 9 and 10 against SPC212 cells as well as 13 towards MCF-7 also displayed IC\(_{50}\) values below 10 μM, suggesting that they can be useful in the management of human carcinoma. Moreover, they were more toxic towards carcinoma cells than towards normal CRL2120 fibroblast (selectivity index > 1), indicating their good selectivity. The good activity obtained with naphthoquinone 4 is in accordance with previous studies. In fact, 2-acetylfuro-1,4-naphthoquinone previously displayed good cytotoxicity with IC\(_{50}\) values below 10 μM against a panel of cancer cell lines such as PF-382 leukemia T-cells, MiaPaCa-2 pancreatic cells, U87MG glioblastoma-astrocytoma cells, Colo-38 skin melanoma cells, HeLa and Caski cervical carcinoma cells [8]. Also, compound 4 is well known for its remarkable anticancer activities [6]. The present study therefore provides additional data on the anticancer potential of this compound and highlights the role of naphthoquinones as good cytotoxic compounds. The moderate cytotoxic effects of some anthraquinones such as damnacanthol, damnacanthol, 3-hydroxy-2-hydroxymethyl anthraquinone and schimperiquinone B on a panel of cancer cell lines was documented [9]. In the present study, the moderate to low activities obtained with anthraquinones 1–3 are also in accordance with such results. Induction of apoptosis is recognized as an efficient strategy for cancer chemotherapy and a useful indicator for cancer treatment and prevention. In the present study, it was found that compounds 4 and 9 induced apoptosis in MCF-7 cells (Fig. 2). Hence, further investigations of the mode of induction of apoptosis were performed. Caspases regulate apoptosis by cleaving cellular proteins at specific aspartate residues [23]. The activity of initiator caspase 9 and effector caspases 3/7 were investigated in MCF-7 cells treated with 4 and 9. However, it was found that caspase-dependent cell death may not be one of the pathways of induction of apoptosis by 4 and 9 in MCF-7 cells. Loss of MMP is also classical evidence for apoptosis, occurring during the early stage of apoptosis before the cell morphology changes. The disruption of MMP was suggested to be very strong at percentages above 50%, and strong between 20 and 50% [3]; Up to 88.1% (at IC\(_{50}\)) MMP depletion was obtained, when MCF-7 cells were treated with IC\(_{50}\) concentrations of 9, suggesting that MMP depletion is involved in apoptotic pathway induced by this compound. ROS levels between 20 and 50% are considered as high [3]; More than 3-fold increase in ROS production was also obtained as results of treatment of MCF-7 with compound 4. However, only 12.2% MMP depletion was obtained with this compound, suggesting that increase in ROS production the likely mode of apoptosis induced by naphthoquinone 4. The tetraprenylquinone, sargaquinoic acid was shown to

![Fig. 4 Induction of ROS in MCF-7 cells after treatment with plumbagin (4), rapanone (9), and doxorubicin for 24 h. IC\(_{50}\) values were 0.06 μM (4), 16.94 (9) and 0.35 μM (doxorubicin). Cells were treated with ¼ \times IC\(_{50}\) (C1), ½ \times IC\(_{50}\) (C2) and IC\(_{50}\) (C3) of each compound.](image)
induce cycle arrest in MDA-MB-231 cells and apoptosis via increase in the activities of caspases 3, 6, 8, 9 and 13 [7]. However, it was demonstrated in this study that the related compound 9 induced apoptosis mediated by MMP loss but did not induced increase in the activity of caspases 3 and 9. Herein, it was also shown that compound 4 induced MMP loss in MCF-7 cells. Nonetheless, the induction was moderate. Compound 4 was also reported to induce apoptosis in PC-3 and DU145 cells, mediated by MMP loss [24]; this corroborates the results obtained in this work.

Regarding the structure-activity relationship, it appears that the naphthoquinone 4 was more potent in all six tested cancer cell lines than anthraquinones (1–5) and benzoquinone (5–14). Within anthraquinones, the substitution of hydroxyl (–OH) group in C8 (1 and 2) by a methyl group (3) significantly reduced the cytotoxic activity meanwhile the presence of –OH group in both C8 and C6 (2) seems to increase the activity. Consequently, IC50 values were obtained with 2 on all tested cancer cells lines and 1 on 2/6 (Table 1). Within benzoquinones, the degree of activity seems to increase with the size of the lateral chain in C2, the best effects being obtained between n = 10 (11) to n = 14 (10). However, compound 9 with n = 12 displayed better cytotoxic effects than 10 and 11 in all tested cancer cell lines (Table 1), most probably because of its higher lipophilicity.

Conclusions

Finally, we demonstrated the cytotoxicity of naturally occurring quinones against human carcinoma cell lines. Naphthoquinone 4 as well as anthraquinone 2 and benzoquinones 9, 10, 11, and 13 displayed cytotoxic effects on all tested cancer cell lines. Compounds 4 and 9 induced apoptosis in MCF-7 cells mediated by increase ROS production and MMP loss repectively. The studied compounds and especially the most active ones deserve more investigations to develop novel cytotoxic drugs against cancers.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors’ contributions

VK, LKO, VRST, JOM, AMT and OK carried out the experiments; VK wrote the manuscript. VK and HS designed the experiments; HS supervised the work, provided the facilities for the study. All authors read the manuscript and approved the final version.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

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Ethics approval and consent to participate

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