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Cytotoxicity, Antioxidant and Apoptosis Studies of Quercetin-3-O Glucoside and 4-(β-D-Glucopyranosyl-1→4-α-L-Rhamnopyranosyloxy)-Benzyl Isothiocyanate from *Moringa oleifera*

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M. Singh

Abstract: *Moringa oleifera,* from the family Moringaceae, is used as a source of vegetable and herbal medicine and in the treatment of various cancers in many African countries, including Kenya. The present study involved the phytochemical analyses of the crude extracts of *M.oleifera* and biological activities (antioxidant, cytotoxicity and induction of apoptosis *in-vitro*) of selected isolated compounds. The compounds isolated from the leaves and seeds of the plant were quercetin-3-O-glucoside (**1**), 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate (**2**), lutein **(3),** and sitosterol (**4**). Antioxidant activity of compound **1** was significant when compared to that of the control, while compound **2** showed moderate activity. The cytotoxicity of compounds **1** and **2** were tested in three cell lines, viz. liver hepatocellular carcinoma (HepG2), colon carcinoma (Caco-2) and a non-cancer cell line Human Embryonic Kidney (HEK293), using the MTT cell viability assay and compared against a standard anticancer drug, 5-fluorouracil. Apoptosis studies were carried out using the acridine orange/ethidium bromide dual staining method. The isolated compounds showed selective *in vitro* cytotoxic and apoptotic activity against human cancer and non-cancer cell lines, respectively. Compound **1** showed significant cytotoxicity against the Caco-2 cell line with an IC_{50} of 79 μg mL^{-1} and moderate cytotoxicity against the HepG2 cell line with an IC_{50} of 150 μg mL^{-1}, while compound **2** showed significant cytotoxicity against the Caco-2 and HepG2 cell lines with an IC_{50} of 45 μg mL^{-1} and 60 μg mL^{-1}, respectively. Comparatively both compounds showed much lower cytotoxicity against the HEK293 cell line with IC_{50} values of 186 μg mL^{-1} and 224 μg mL^{-1}, respectively.

Keywords: Apoptosis induction, cytotoxicity, 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate, *Moringa oleifera*, quercetin-3-O-glucoside.

1. INTRODUCTION

Cancer is a leading cause of mortality worldwide with over 7.6 million deaths being reported in 2008 alone [1], with 7 out of 10 deaths occurring in low and middle-income countries. Medicinal plants have been in use in many cultures around the world for thousands of years, to cure and prevent several diseases, including cancer. In Kenya, the majority of the population in rural and urban areas use traditional medicine to treat illnesses ranging from stomach ache, HIV/AIDS to cancer [2]. Plants possess activity against several known diseases, mainly due to the bioactive compounds present. A large number of bioactive plant compounds have been isolated and are commercially available, with over 60% of them being employed as anti-cancer drugs. These include Vincristine and Vinblastine isolated from *Catharanthus roseus* and paclitaxel (Taxol) isolated from *Taxus brevifolia* [3].

Moringa oleifera, from the family Moringaceae, is a fast growing tree native to India, and is believed to have been introduced to Kenya in the early 1900s by the Asians [4]. In Kenya, the *Moringa* tree is mainly used as a source of vegetable and herbal medicine and is often referred to as the miracle tree because of its versatility. The plant's nutritional and medicinal value is largely due to the presence of essential phytochemicals, minerals, protein and vitamins in the plant. All parts of this tree edible and are consumed in different ways. The plant is a rich source of nutrients, containing high concentrations of vitamins A and C, calcium and iron. The leaves are used as animal feed, in biogas production, as manure and also as a biopesticide due to its antifungal activity [5, 6]. The seeds are used as fertilizer, and in water purification and treatment owing to the active coagulating agent found therein and its antimicrobial activity [7, 8]. The *Moringa* tree is the main source of medicine for some rural communities in Kenya. All parts of the plant are used in traditional medicine to treat a plethora of diseases, including abdominal pains, eye, throat and skin infections, sexually transmitted diseases and cancer [4].

Crude extracts of the leaves and seed have been shown to possess cytotoxic and antioxidant activity [5, 6, 9]. Compounds previously isolated from this plant include phenolic compounds, nitrile glycosides, sterols and alkaloids. Apoptosis is a term that refers to programmed cell death [10]. This process is defective in most cancers and is a valuable drug target in cancer research. Sreelatha and co-workers [11] studied the anti-proliferative activity of the *Moringa* leaf extract on the human nasopharyngeal carcinoma (KB) cell line and reported apoptosis as a possible cause of cell death. The antioxidant activity of the leaf extracts of this plant were reported to be comparable to those of reference antioxidants [5]. This is attributed to the presence of a high quantity of flavonoids and other polyphenols in the leaves. Antioxidants are important scavengers of free radicals that are the main cause of oxidative stress in the body. Oxidative stress is believed to contribute significantly to the causation of a number of diseases including cancer mainly through DNA damage [12] and hence plays a major role in the pathophysiology of carcinomas [13]. Flavonoids are polyphenolic compounds with potent free radical scavenging ability and their biological activity has been related to this activity [14]. Strong antioxidants have been reported to cause cell death *in vitro* and enhance activity of chemotherapy drugs [15],

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causing improved patient remissions by reducing oxidative stress caused by conventional chemotherapeutic drugs.

Novel approaches for cancer chemo-prevention and therapy are of great importance to overcome the challenges associated with modern chemotherapy. In this study, we isolated and identified compounds using chromatographic and spectroscopic techniques. Compounds of interest were tested for antioxidant, cytotoxic and apoptosis induction in cancer and non-cancer mammalian cell lines. Our research sought to identify compounds from *Moringa oleifera* with possible anticancer activity that could lead to the production of potential drug candidates.

2. MATERIALS AND METHODS

2.1. General Experimental Procedures

The solvents used for extraction and chromatography were of analytical grade and supplied by either Sigma or Merck chemical companies. Silica gel was used for column chromatography and pre-coated aluminium plates for thin layer chromatography (TLC). Both procedures were conducted at room temperature. Compounds were visualized on TLC under UV light using an H_2SO_4 (MeOH) spray reagent. 1D (^1H and ^{13}C) and 2D (HMBC, HSQC and COSY) NMR spectra were recorded at 400 MHz or 600 MHz on a Bruker Avance spectrometer with tetramethylsilane (TMS) as the internal standard. The IR spectra for identification of functional groups in the compound were obtained on a Perkin Elmer spectrum 100 FT-IR spectrometer with a universal ATR sampling accessory. UV spectra were obtained on a UV-Vis-NIR Shimadzu UV 3600 spectrophotometer.

The cell lines used were human cancer cell lines HepG2 (human hepatocellular carcinoma) and Caco2 (human colon carcinoma) and HEK293 (human embryonic kidney) cells as the control. The cell lines were originally obtained from the American Tissue Culture Collection (ATCC). The standard anticancer drug, 5-fluorouracil was purchased from Sigma, St Louis, MO, USA.

2.2. Plant Material

Fresh leaves, stem, bark and seeds were collected in Maseno, Kisumu county, Kenya. The plant was identified at the Kenyan Forestry Research Institute (KEFRI). The plant material was air-dried in the shade for 6 weeks and then ground into a fine powder.

2.3. Extraction and Purification

Dried and ground samples of the leaves (643.24 g), bark (580.60 g) and seeds (372.89 g) were sequentially extracted with hexane, dichloromethane (DCM) and MeOH for 48 hours on an orbital shaker at room temperature. The collected extracts were concentrated in a rotary evaporator to obtain the crude extracts which were then subjected to column chromatography using silica gel (Merck Kieselgel 60, 0.063-0.200 mm, 70-230 mesh ASTM) as the stationary phase and different solvent systems (stated below) as the mobile phase. Collected fractions were monitored by thin layer chromatography (TLC). Prior to column chromatography, the aqueous MeOH extract (139.64 g) was further partitioned in triplicate with equal volumes (200 mL) of DCM and then ethyl acetate to produce a DCM fraction (21.87 g) and ethyl acetate fraction (9.12 g).

The DCM extract (3.67 g) was subjected to column chromatography and the compounds were eluted with a hexane: ethyl acetate (v/v) solvent system, starting with 100% hexane, stepped with ethyl acetate, until 100% ethyl acetate was reached. Fractions (100 mL) were collected and fraction 75, collected at 70% hexane: 30% ethyl acetate was further purified in a 1 cm column resulting in the isolation of compound **3** (73.45 mg) from fraction 30 which eluted at 80% hexane: 20% ethylacetate. Compound **4** (29.14 mg) obtained from the DCM fraction of the MeOH extract, (21.87 g) eluted at a solvent system of 60% hexane: 40% ethyacetate in fractions 55-63 after collecting 50 mL fractions. The combined fractions (1.80 g) were further purified to yield the pure compound as fraction 22 (20 mL) using at 50:50 hexane: ethyl acetate mobile phase. Fractions (123-142) (50 mL each) obtained from the ethyl acetate fraction of the partitioned methanol extract (9.12 g) after using a 95% ethyl acetate: 5% MeOH mobile phase was dried and further purified using a 1 cm sephadex LH 20 column. After collecting 2 mL fractions using 100% MeOH, compound **1** (Figs. **1**, **2**) (293.46 mg) was detected in fractions 20-28. Crude fractions (50-55) (50 mL) from the ethyl acetate fraction (1.86 g) of the partitioned methanol seed extract (4.01 g) obtained at 70:30 ethyl acetate: methanol, were combined. Further purification in sephadex LH 20 by collecting 2 mL fractions yielded compound **2** (Figs. **1**, **3**) (22.56 mg).

2.3.1. Compounds Isolated

Quercetin-3-O-glucoside (**1**): yellow powder; $C_{21}H_{20}O_{12}$; UV λ_{max} nm MeOH: 266, 361: ^{1}H-NMR (MeOH, 400 MHz): δ ppm 7.70 (1H, d, *J*=2.16 Hz H-2'), 7.58 (1H, dd, *J*=8.53 Hz *J*=2.26 Hz, H-6'), 6.87 (1H, d, *J*=8.40 Hz, H-5'), 6.38 (1H, d, H, *J*=2.14 Hz, H-8), 6.19 (1H, d, *J*=2.11 Hz, H-6), 5.24 (1H, d, *J*=7.44 Hz, H-1''), 3.72-3.34 (Glucoside). ^{13}C NMR (MeOH, 400 MHz): δ 179.4 (C-4), 166.0 (C-7), 163.0 (C-5), 159.0 (C-2), 158.4 (C-9), 149.8 (C-4'), 145.9 (C-3'), 135.6 (C-3), 123.0 (C-1'), 123.2 (C-6'), 117.6 (C-2'), 116.0 (C-5'), 104.3 (C-1''), 99.9 (C-6), 94.7 (C-8), 75.7 (C-2''), 78.3 (C-3''), 78.1 (C-5''), 71.2 (C-4''), 62.5 (C-6'').

4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate (**2**): light brown oily solid; $C_{20}H_{27}NO_{10}S$; UV λ_{max} nm MeOH: 222, 270: ^{1}H-NMR (MeOH), 600 MHz): δ ppm 7.38 (2H, d, *J*=8.28 Hz), 7.08 (2H, d, *J*=8.57 Hz), α-rhamnose; 5.42 (1H, d, *J*=3.7 Hz, H-1'), 4.13(1H, m, H-2'), 4.05(1H, m, H-3'), 3.66(1H, m, H-5'), 3.40(1H, m, H-4'), 1.26(3H, d, *J*=6.0 Hz, H-6'), Glucose; 5.42 (1H, d, *J*=2.3 Hz, H-1''), 4.03-4.02(1H, m, H-3''), 3.84-3.80(1H, m, H-5''), 3.75(1H, m, H-2''), 3.73-3.68(1H, m, H-6''), 3.48-3.46(1H, m, H-4''). ^{13}C-NMR (MeOH, 600 MHz): δ 155.7 (C-1), 131.1 (N=C=S), 129.9 (C-4), 129.0 (C-5), 129.0 (C-3), 116.5 (C-6), 116.5 (C-2), 98.5 (C-1''), 92.2 (C-1'), 82.3 (C-2''), 81.0 (C-4'), 77.9 (C-2'), 72.9 (C-3''), 71.8 (C-4''), 71.8 (C-3'), 70.8 (C-5''), 68.5 (C-5'), 61.9 (C-6''), 60.8 (C-7), 16.6 (C-6').

Sitosterol (**3**): white powder: $C_{35}H_{61}O_6$; UV λ_{max} nm MeOH: 234: ^{1}H-NMR ($CDCl_3$, 400 MHz): δ ppm 5.33-5.32 (1H, d, *J*=5.06 Hz, H-6), 3.53-3.47 (1H, m, H-3), 2.14 (2H, s, H-4), 1.97 (2H, s, H-7), 1.84 (2H, s, H-2), 1.83 (2H, s, H-2β), 1.81 (H, s, H-16α),1.79 (H, s, H-1α),1.55 (2H, s, H-7), 1.23 (H, s, H-16β), 0.98 (H, s, H-1β), 0.90 (3H, d, *J*=6.5 Hz, H-21), 0.89 (3H, s, H-21), 0.84 (3H, s, H-29), 0.82 (3H, s, H-26), 0.80 (3H, d, *J*=1.5 Hz, H-27), 0.65 (3H, s, H-18, H-19). ^{13} C NMR ($CDCl_3$, 400 MHz): δ 140.6 (C-5), 121.6 (C-6), 71.7 (C-3), 56.6(C-14), 55.9(C-17), 50.6 (C-9), 45.7 (C-24), 42.2 (C-13), 42.2 (C-4), 39.6 (C-12), 37.1 (C-1), 36.4 (C-10), 36.0 (C-20), 33.8 (C-22), 31.8 (C-8), 31.5 (C-7), 31.5 (C-2), 29.0 (C-25), 28.9 (C-16), 25.9 (C-23), 24.2 (C-15), 22.9 (C-28), 20.9 (C-11), 19.7 (C-27), 19.2 (C-19), 18.9 (C-26), 18.6 (C-21), 11.8 (C-29), 11.7 (C-18).

Lutein (**4**): reddish solid: $C_{40}H_{56}O_2$: UV λ_{max} nm MeOH: ^{1}H-NMR ($CDCl_3$ 400 MHz): δ ppm 6.62-6.58 (m, H-11, H-11', H-15, H-15'), 6.35-6.31 (d, *J*=14.92 Hz, H-12, H-12'), 6.24-6.21 (m, H-14, H-14'), 6.17 (m, H-8, H-10, H-10'), 6.13 (s, H-7, H-8), 5.52 (1H, s, H-11), 5.43-5.37 (dd, *J*=25.59, *J*=10.04, H-7'), 4.22 (1H, s, H-3'), 4.00-3.95 (1H, m, H-3), 2.39-2.37 (1H, m, H-6'), 2.03-2.01 (1H, m, H-4), 1.94 (s, H-19, H-20, H-20'), 1.94 (s, H-19'), 1.60-1.57 (d, H-18, H-18'), 1.05 (s, H-16, H-17), 0.97 (s, H-16'), 0.82 (s, H-17'). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 138.5 (C-8), 137.9 (C-6),

137.7 (C-12), 137.7 (C-5'), 137.7 (C-8'), 137.5 (C-12'), 136.4 (C-13), 136.4 (C-13'), 135.6 (C-9), 135.0 (C-9'), 132.5 (C-14), 132.5 (C-14'), 131.3 (C-10), 130.8 (C-10'), 130.0 (C-15), 130.0 (C-15'), 128.7 (C-7'), 126.1 (C-5), 125.5 (C-7), 124.9 (C-11), 124.8 (C-4'), 124.4 (C-11'), 65.9 (C-3'), 65.0 (C-3), 54.9 (C-6'), 48.4 (C-2), 44.6 (C-2'), 42.5 (C-4), 37.1 (C-1), 34.0 (C-1'), 29.5 (C-16), 28.7 (C-16'), 24.2 (C-17), 24.2 (C-17'), 22.8 (C-18'), 21.6 (C-18), 16.0 (C-19), 13.1 (C-19'), 12.8 (C-20), 12.7 (C-20').

2.4. Cell Culture

Cells used were originally obtained from the American tissue culture collection (ATCC) and propagated in the laboratory. The cells were grown to 100% confluency in 25 cm^2 tissue culture flasks in MEM + Glutmax supplemented with 10% fetal bovine serum and antibiotics (100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin). Cells at a seeding cell density of 1.8 x 10^5 cells per well were plated into a 48 well plate containing 200 µL of medium. The cells were then incubated overnight at 37 °C in a humidified atmosphere of 5% CO_2, after which the medium was removed and 200 µL fresh medium was added. The compounds were first dissolved in dimethylsulfoxide (DMSO) and further diluted with phosphate buffered saline (PBS) for the cytotoxicity assay. Compounds **1, 2** and the control 5-Fluorouracil (5-FU), at concentrations of 20 µg mL^{-1}, 40 µg mL^{-1}, 60 µg mL^{-1} , and 80 µg mL^{-1}, were then added in triplicate to the cells and incubated for 48 hours at 37 °C.

2.5. Cytotoxicity Test

2.5.1. MTT Assay

This assay measures the metabolic activity of cells and their ability to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan using the succinate-tetrazolium reductase system [16]. After 48 hours of incubation with the standard and test compounds, the old medium was discarded and fresh medium added to the wells. Cells with no test compounds were used as positive controls (100% viability). Approximately, 200 µL of MTT (5 mg mL^{-1} in PBS) was added to each well. The cells were incubated with the MTT solution for 4 hours at 37 °C, after which, the medium and MTT was removed from the wells, and 200 µL of DMSO was added to each well to dissolve the formazan salt. The presence of the formazan salt is indicative of cell viability and upon addition of DMSO produces a purple solution, the absorbance of which was read in a Mindray 96A microplate reader at 570 nm. The absorbance is proportional to the number of viable cells in each well. The tests were carried out in triplicate and graphs that were generated were used to calculate the concentration at which 50% cell death was achieved (IC_{50}) using Microsoft Excel 2010 ™.

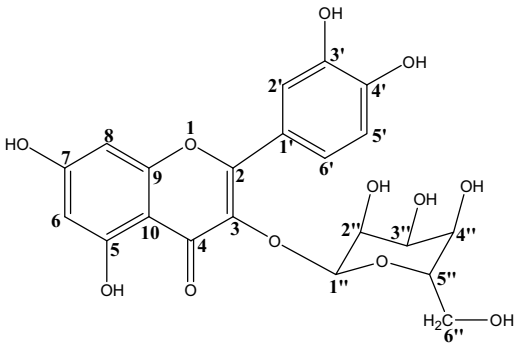
2.6. Apoptosis Assay

The Acridine Orange/Ethidium Bromide (AO/EB) dual staining method is a simple, inexpensive qualitative and quantitative method of studying cell apoptosis. Acridine orange is taken up by both viable and non-viable cells. It intercalates into the DNA making viable cells appear green under a fluorescent microscope. Conversely, ethidium bromide is only taken up by non-viable cells whose membrane integrity has been compromised making their nucleus to fluoresce bright orange [17]. Cells were seeded at a density of 1.2 x 10^5/well into a 24-well plate and incubated overnight to allow the cells to attach. Thereafter, compounds **1** and **2** were added to the cells at a concentration of 20 µg mL^{-1} and incubated for 48 hours. The cells were then washed with PBS, and 10 µL of AO/EB dye (100 µg mL^{-1} acridine orange and 100 µg mL^{-1} Ethidium bromide in PBS was added and cells left at room temperature for 5 minutes. The cells were then viewed under a fluorescent microscope (OLYMPUS) at X200 magnification and examined for morphological changes due to apoptosis. The apoptotic index was calculated as shown below:

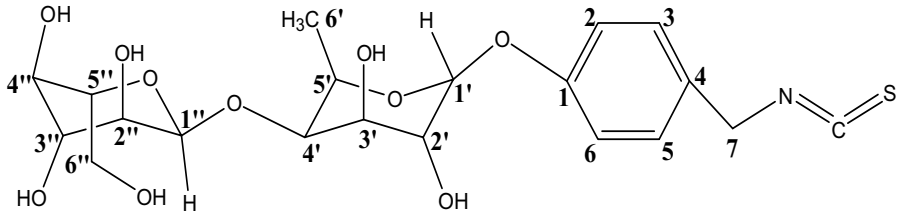
Apoptotic index = number of apoptotic cells/ number of total cells counted

2.7. Antioxidant Activity

The antioxidant activity of compounds **1** and **2** was determined using the DPPH assay. This assay measures the reducing ability of



Quercetin-3-O-glucoside (**1**)



4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate (**2**)

Fig. (1). Compounds **1** and **2** isolated from *Moringa oleifera* and tested for cytotoxic, antioxidant and apoptotic activity.

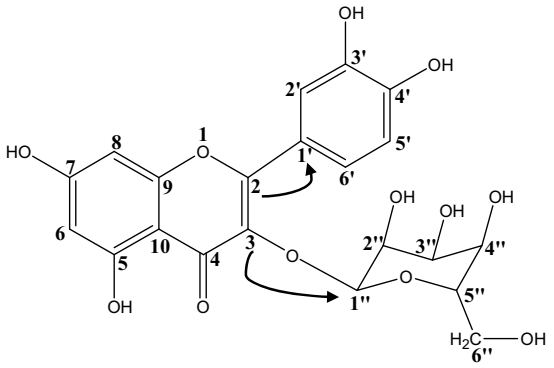


Fig. (2). Important HMBC correlations of compound **1**.

a compound on the stable free radical DPPH. Antioxidants reduce DPPH to DPPH-H by donating hydrogen atoms. The antioxidant activities of the compounds were compared to that of ascorbic acid as the standard.

Compounds **1** and **2** and standard ascorbic acid were prepared at concentrations of 20 µg mL^{-1}, 50 µg mL^{-1}, 100 µg mL^{-1}, 250 µg mL^{-1}, and 500 µg mL^{-1}. DPPH (0.01 M in MeOH) was added to the compounds in a 3:1 ratio. Methanol only was used as the blank. The solution was left in the dark for 30 minutes since DPPH decays in the presence of light. Absorbance was then read on a Biomate UV-Vis spectrometer at 517 nm. IC_{50} values were calculated on Microsoft Excel 2010™. The IC_{50} value in this assay represented the concentration of sample required to scavenge 50% of the DPPH radical.

3. RESULTS AND DISCUSSION

In the present study phytochemical analysis of the plant led to isolation and purification of four compounds, two of which were tested further for their antioxidant, cytotoxic and apoptotic activities in the HepG2, Caco-2 and HEK293 cell lines.

3.1. Phytochemistry

3.1.1. Isolation of Compound 1

Column chromatography of the methanol extract of the leaves yielded compound **1** which was isolated as a yellow powder (293.46 mg). The ^{1}H-NMR and ^{13}C-NMR spectra showed characteristic resonances for flavonols namely quercetin. From the ^{1}H-NMR spectrum, resonances for the A-ring protons at δ_H 6.1 (H-6) and at δ_H 6.3 (H-8) that were meta-coupled were observed. The B-ring proton that resonated at δ_H 7.5 (1H, dd, *J*=8.53 Hz, *J*=2.26 Hz, H-6') was ortho coupled to the proton at δ_H 6.8 ppm (1H, d, *J*=8.40 Hz, H-5') and meta coupled to the proton at δ_H 7.7 (1H, d, *J*=2.14 Hz, H-2'). The presence of the sugar moiety was confirmed by the presence of the anomeric proton resonating at δ_H 5.2 (1H, d, *J*=7.44 Hz, H-1") and resonances between δ_H 3.32-4.24. In the DEPT experiment, the resonance at δ_C 62.5 for C-6" was shown to be a methylene indicating that the sugar is a glucose. The $[M]^+$ ion at *m/z* 465 is in agreement with the molecular formula of $C_{21}H_{20}O_{12}$ for quercetin. This correlated to the HRMS. The IR spectrum showed characteristic absorption bands for the O-H group (3400–3100 cm^{-1}), C=C group (1615 cm^{-1}), and C-O group (1150–1010 cm^{-1}). The UV-Vis spectrum showed two absorption bands, a strong one at 238 nm and a weaker one at 281 nm originating from the A and B rings. The physical and spectroscopic data for compound **1** matched those published in literature [18, 19] therefore compound **1** was identified as quercetin-3-O-glucoside.

3.1.2. Isolation of Compound 2

Compound **2** was isolated as light brown and oily with a mass of 22.56 mg. The ^{1}H-NMR spectrum of compound **2** showed resonances for two ortho coupled aromatic protons at δ_H 7.08 (2H, d, *J*=8.57 Hz, H-2,H-6) and δ_H 7.38 (2H, d, *J*=8.28 Hz, H-3,H-5) indicating a para-substituted benzene ring. This was confirmed by the ^{13}C NMR data which showed resonances at δ_C 116.5, 129.0 for (C-2, C-6) and (C-3, C-5), respectively. Anomeric proton resonances at δ_H 5.43 (H, d, *J*=3.75 Hz, H-1') correlated with C-1' (δ_C 92.2) and δ_H 5.42 (H, d, *J*=2.3 Hz, H-1'') correlated with C-1'' in the HSQC spectrum indicating the presence of 2 sugars. Methyl doublets at δ_H 1.26 (3H, d, *J*=6.0 Hz, H-6') was assigned to the proton at C6' and confirmed one of the sugars to be a rhamnose. The DEPT experiments showed the presence of three quartenary carbons resonating δ_C 155.7, 129.1, and 131.1 which were assigned to C-1, C-4 and C=N respectively. Two methylene carbons at δ_C 61.99 and δ_C 60.80 was assigned to C-6'' and C-7, respectively. HMBC correlations of H-1' and C-1 showed that the rhamnose is attached at C-1 and further indicated that the glycosidic linkage between glucose and rhamnose is at 4' and 1''.

The IR spectrum showed characteristic bands at 1630-1510 cm^{-1} indicating the presence of the C=S and C=N bonds of the thiocarbonate linkage attached to C7. A broad absorption band at 3307 cm^{-1} indicated the presence of hydroxyl groups (-OH) in the molecule. The $[M+ Na]^+$ ion at *m/z* 494 is in agreement with the molecular formula of $C_{20}H_{27}NO_{10}S$ for 4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate and further confirmed with HRMS. The UV-Vis spectrum showed two absorption bands, a strong one at 222 nm and a weaker one at 270 nm. This information was consistent with published data [20, 21] and led to the identification of compound **2** as 4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate.

The DCM extract of the leaves yielded compound **3** which was isolated from the leaves, stem, bark and seeds of the plant and its structure was further confirmed to be β-sitosterol, a ubiquitous plant sterol, by comparing spectroscopic data with that in published literature [22]. Compound **4** was obtained from the leaves of the plant and the structure was confirmed to be lutein by comparing spectroscopic data with that in literature [22-24].

3.2. Antioxidant Activity

The antioxidant test showed that the compound with the highest antioxidant activity was compound **1**, quercetin-3-O-glucoside with an IC_{50} of 26.73 µg mL^{-1}, which was slightly higher than that of the control (31.68 µg mL^{-1}). Quercetin is a strong antioxidant and is commonly used as a standard in antioxidant assays. Compound **2**,

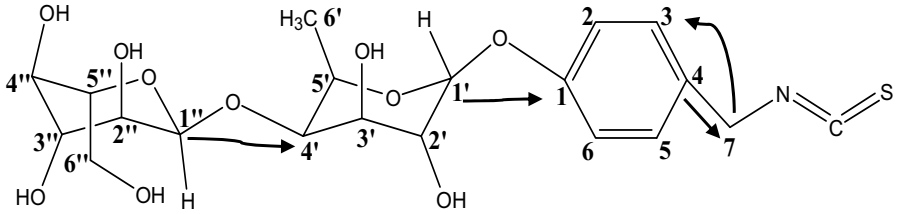


Fig. (3). Important HMBC correlations of compound **2**.

4-(β-d-glucopyranosyl-1→4-α-l-rhamnopyranosyloxy)-benzyl isothiocyanate, showed moderate antioxidant activity with an IC_{50} value of 78.96 μg mL^{-1} (Table **1**) (Fig. **4**).

Table 1. Radical scavenging activity and related IC_{50} values of compounds 1 and 2 against the standard, ascorbic acid. Data represented as mean ± SD (n=3).

Concentration in μg mL^{-1}	% Scavenging Activity		
	Ascorbic Acid	1	2
500	98.99±0.002 0.002887	93.81±0.005	61.20±0.003
250	97.74±0.005	93.01±0.001	60.32±0.004
100	65.15±0.004	68.21±0.006	50.81±0.020
50	59.92±0.004	53.80±0.001	52.90±0.090
20	41.90±0.020	49.53±0.060	35.38±0.500

3.3 Cytotoxicity tests

The cytotoxicity of compounds **1** and **2** and 5-fluorouracil (5-FU) on the three cell lines, Caco-2, HepG2 and HEK293 was carried out using the MTT assay. The results (Fig. **5**, Table **2**) indicate that compound **1** showed selective but moderate cytotoxicity across all three cell lines. It was more cytotoxic to the Caco-2 cell line with an IC_{50} of 79 μg mL^{-1} when compared to the HepG2 cell line (IC_{50} of 150 μg mL^{-1}), but showed lower cytotoxicity in the control HEK293 (IC_{50} of 186 μg mL^{-1}). Compound **2** displayed the highest cytotoxicity in the Caco-2 cell line (IC_{50} of 45 μg mL^{-1}), but lower cytotoxic levels in the HepG2 cell line (IC_{50} of 60 μg mL^{-1}). From these cytotoxicity studies it can be deduced that compound **2** is more to cancer cells selective, considering the low cytotoxicity observed in the HEK293 non-cancer control cells (IC_{50} of 224 μg mL^{-1}). The cytotoxicity to 5-FU was dose dependent and cell specific with IC_{50} values of 108 μg mL^{-1} , 62 μg mL^{-1} and 46 μg mL^{-1} in the HEK293, HepG2 and Caco-2 cell lines respectively. The greatest degree of cytotoxicity was observed in the Caco-2 cells with cell death of up to 70% at a concentration of 80 μg mL^{-1}. This was closely followed by the HepG2 cells and the non-cancer cell line, HEK293 showing survival above 63% at 80 μg mL^{-1}. Both compounds 1 and 2 seemed to be better tolerated by the HEK293 cells in comparison to the standard 5-FU.

Table 2. IC_{50} Values of compound 1, 2 and 5-Fluorouracil (5-FU) on HEK293, HepG2 and Caco-2 cell lines.

Cell Lines	IC_{50} Values (μg mL^{-1})		
	1	2	5-FU
HEK293	186	224	108
HepG2	150	60	62
Caco-2	79	45	46

3.4. Apoptosis Assay

Apoptosis studies were carried out to determine whether the isolated compounds induced apoptosis in the selected cell lines. The double staining method using acridine orange and ethidium bromide was employed to detect the characteristic morphological changes of the cells due to apoptosis under a fluorescent microscope (Olympus). These changes include cell shrinkage, chromatin condensation, fragmentation of the nucleus and cell membrane blebbing. Viable cells fluoresce green under the microscope; early apoptotic cells a brighter green, late apoptotic cells orange with a condensed chromatin and necrotic cells orange without condensed chromatin [25]. Results of exposure of the cells lines to the compounds for 48 hours at a concentration of 20 μg mL^{-1} are shown in Fig. **6**. Compounds **1** and **2** were seen to induce apoptosis in all cell lines. Apoptotic features were clearly observed in these cells. Apoptosis induction was selective as evidenced by the high apoptotic index in the cancer cell lines compared to the non-cancer cell line. The apoptotic index was much lower in all the control cells compared to cells treated with compounds **1** and **2** as shown in Table **3** below.

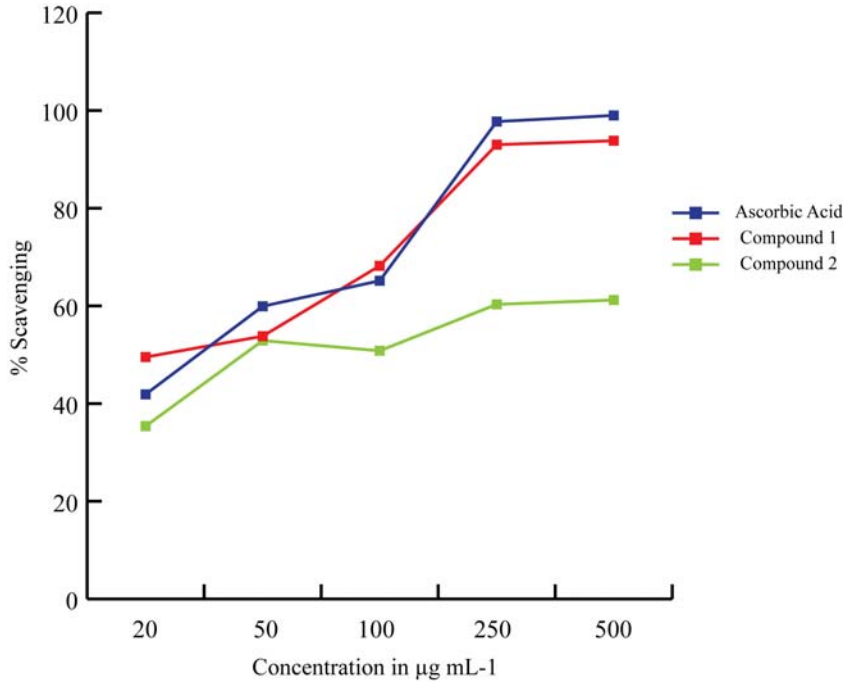


Fig. (4). Antioxidant activity of compounds 1,2 and Ascorbic Acid as standard in the radical scavenging activity essay.

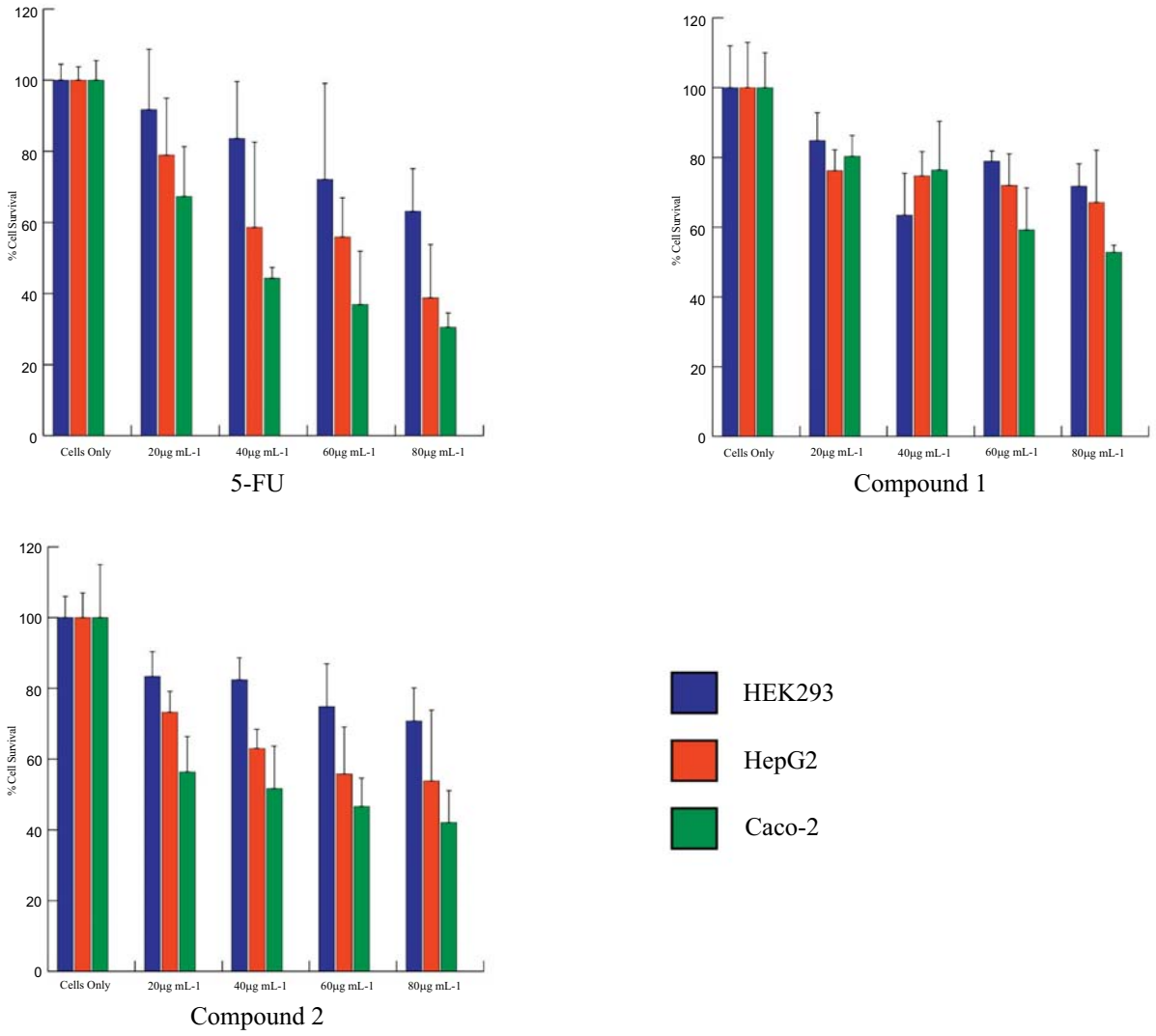


Fig. (5). MTT cell viability assay of compounds 1,2 and a control 5-Fluorouracil in HEK293, HepG2 and Caco-2 cells. Data represented as mean ±SD (n=3). Compound 2 showed greater cytptoxocity against cancer cell lines HepG2 and Caco-2 similar to the control drug 5-Fluorouracil as compared to compound 1.

Table 3. Apoptotic index values of compounds 1 and 2 on HEK293, HepG2 and Caco-2 cell lines.

Cell Lines	Apoptotic Index	
	Compound 1	**Compound 2**
HEK293	0.057	0.03
HepG2	0.37	0.132
Caco-2	0.5	0.6

Some synthetic drugs have their structure based on natural compounds or are synthetic derivatives of these compounds e.g. flavopiridol [26]. *In vitro* cytotoxicity testing is a valuable tool for screening compounds with potential anti-cancer activity. In this study, we have correlated growth inhibition with induction of apoptosis. Crude extracts of the *Moringa* tree have been shown to possess cytotoxic and antioxidant activity. The high antioxidant activity of compound **1** favours the elimination of reactive oxygen species from the body, thereby preventing DNA damage, resulting in lowering of mutation rates and occurrence of tumour cells. However, when quercetin is used as an antioxidant it is broken down and oxidised to quinone-type metabolites which have pro-oxidant action that binds and fragments DNA [27], which would explain its apoptosis induction activity (Fig. **3**). Flavonoids have been reported to cause cell death and apoptosis in A549 (human lung adenocarcinoma) cells by inducing transcription of *p21,* a tumour suppressor gene that is only activated in the presence of *p53* which is inactivated in most tumours [28, 29]. Quercetin has been proposed to induce apoptosis *via* the intrinsic/mitochondrial pathway through inhibition of protein kinase B phosphorylation and survivin which are responsible for activation of caspases and down regulation of anti-apoptotic proteins Bcl-2 and Mcl-1 [27, 30].

Compound **2** has been isolated once previously by Oluduro and co-workers [20], and here we report the first evidence of its cytotoxic and apoptotic activity on mammalian cell lines to the best of our knowledge. This group of compounds is unique to the *Moringa* family and similar compounds are reported to possess cytotoxic activity *in vitro* and *in vivo,* suggesting that the isothiocyano group is an essential part of this activity [22, 31]. Studies on isothiocyanates report that these compounds inhibit cancer cell growth and induce apoptosis through different targets. Srivastava and Singh [32] reported growth inhibition and apoptosis induction by benzyl isothiocyanate in animal models. They postulated that the apoptosis induction was due to down-regulation of Bcl-2 and up-regulation of Bax which are anti-apoptotic and pro-

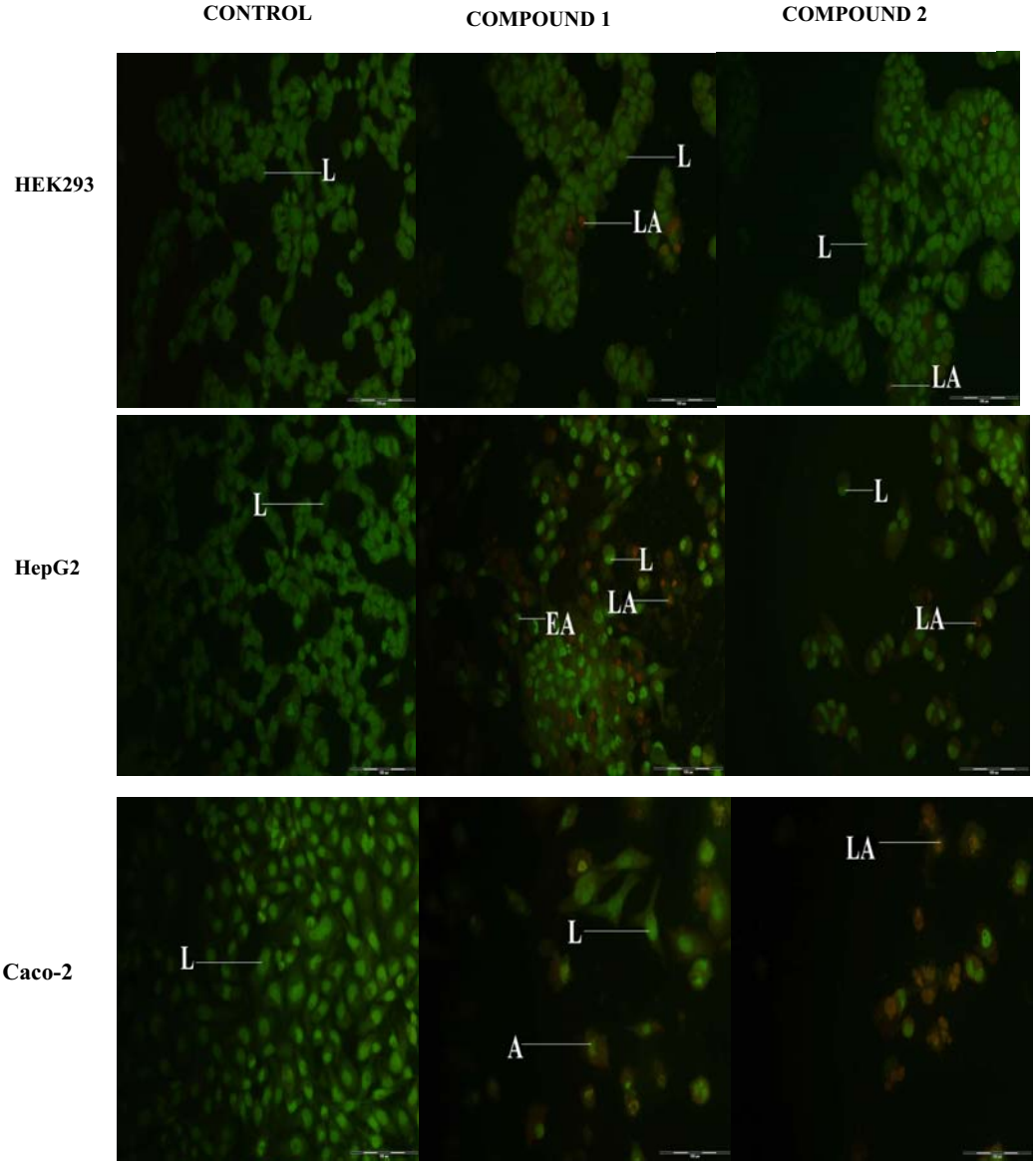


Fig. (6). Induction of apoptosis by compounds **1** and **2** on HEK293, HepG2 and Caco-2 at a concentration of 20 µg mL^{-1}. L=Live A=Apoptotic LA= Late apoptotic EA= Early apoptotic. Scale is given as 100 µm.

apoptotic proteins, respectively. Regulation of phase I and phase II enzymes implicated in cancer risk have also been proposed to be major mechanisms of action of isothiocyanates [33, 34]. The water extract of the *Moringa* leaf has been reported to induce apoptosis *in vitro*, and this was attributed to the presence of a high concentration of the flavonoids quercetin and kaempferol in the leaves [11].

The compounds in this study showed a concentration dependent cytotoxicity against both HepG2 and Caco-2 cell lines. They also displayed selective cytotoxicity on cancer cells versus normal cells with an IC_{50} of 186 µg mL^{-1} and 224 µg mL^{-1} respectively.. Compound **2** was the more active of the two compounds with an IC_{50} value of 45 µg mL^{-1} in the Caco-2 cell line. Both compounds were not well tolerated by the Caco-2 than HepG2 cell lines. Apoptosis induction is an important indicator of the ability of chemotherapeutic drugs to induce death of tumour cells. The results suggest that one of the causes of cell death upon exposure to these compounds is apoptosis. Lutein and β-sitosterol have been isolated previously from this plant and occur in a wide variety of plants. They have been widely studied for their anticancer, antioxidant as well as chemoprevention potential [35-39]. Hence they were not taken further in this study.

CONCLUSION

Four compounds were isolated from *M.oleifera* namely quercetin-3-O-glucoside (**1**), 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate (**2**), lutein **(3)** and sitosterol (**4**), and two compounds tested for antioxidant, cytotoxicity and apoptosis induction. Quercetin-3-O-glucoside and 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy)-benzyl isothiocyanate have demonstrated varying degrees of cytotoxicity against two mammalian cancer cell lines. Their cytotoxic selectivity to cancer cells *in vitro* warrants their future investigation as potential chemotherapeutic agents against cancer cells with fewer side effects than drugs currently in the market. Future studies need to be undertaken to understand the mechanism of action of these compounds and their specific target at the molecular level with regards to induction of apoptosis. *In vivo* studies also need to be conducted to investigate their effect on solid tumours and also to

evaluate the pharmacological and pharmacokinetic activities of these compounds in an animal model.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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