Induction of Apoptosis with *Moringa oleifera* Fruits in HCT116 Human Colon Cancer Cells Via Intrinsic Pathway

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Abstract – *Moringa oleifera* Lam (*M. oleifera*, Moringaceae) is a tree of the Moringaceae family that can reach a height of between 5 and 10 m. The current paper presents cytotoxic effect of *M. oleifera* fruits and its flavonoids **1** and **2**. The viability of HCT116 human colon cancer cells were 38.5% reduced by 150 µg/mL of ethanolic extracts in a concentration-dependent manner; in addition, we observed the apoptotic features of cell shrinkage and decreased cell size. Bcl-2 family proteins were regulated as determined by Western blotting analysis, suggesting that *M. oleifera* fruits and their flavonoids **1** and **2** induced apoptosis through an intrinsic pathway. Based on our findings, 70% ethanolic extracts of *M. oleifera* fruits and flavonoids **1** and **2** might be useful as cytotoxic agents in colorectal cancer therapy.

Keywords – *Moringa oleifera* fruits, Cytotoxicity, Intrinsic pathway

Introduction

Moringa oleifera Lam (*M. oleifera*, Moringaceae) is a feathery-looking, deciduous tree that can grow up to 10 m. It is commonly planted in well-drained soil at the coast and cultivated in tropical and subtropical climates.[1] The leaves, seeds, bark, roots, sap, and flowers of *M. oleifera* are widely used as a traditional medicine. Many previous reports have shown that aqueous, hydro alcohol, or alcohol extracts of *M. oleifera* leaves possess wide range of biological activities; these extracts have antioxidant, tissue-protective (liver, skins, kidneys, heart, testes, and lungs), analgesic, anti-ulcerative, antihypertensive, radio-protective, and inflammatory and immune-modulatory actions. A wide variety of polyphenols, phenolic acids, flavonoids, glucosinolates, and alkaloids are believed to be responsible for the observed biological activities from *M. oleifera*.[2-7]

Colorectal cancer accounts for about 9% of the global cancer incidence rate and is the fourth most common cancer.[8] The main causes of colorectal cancer include diets high in animal fat and meat, but low in vegetables and fruits, as seen in Westernized diets. This creates less stool and causes constipation, as it takes a long time for stool to pass through the large intestine.[9-10] Surgery and chemotherapy are commonly used to treat cancer, but have many side effects. Therefore, many studies have been conducted to discover safe and efficient natural substances that inhibit the growth of colon cancer cells.

Apoptosis, also called programmed cell death, is cell suicide by internal or external stimulus that is used to maintain bodily homeostasis.[11] Typical apoptotic phenomena include morphological features such as cell shrinkage, nuclear condensation, DNA fragmentation, membrane blebbing and cytoskeletal collapse.[12] A defect in the apoptosis process or an inappropriate signal causing dysregulated apoptosis can lead to abnormal cellular proliferation and eventually cancer.[13] Although many studies have been conducted on carcinogenesis, cancer progression, and related genes, appropriate restoration or induction of mechanism could be a promising method for cancer prevention and treatment. A framework of the apoptotic signal transduction pathway appears as follows; Various pro-apoptotic signals initially activate separate signaling pathways, which eventually converge upon a common mechanism driven by a unique family of cysteine proteases called caspases.[14] This mechanism is negatively regulated by several sets of genes, the best characterized of which is the Bcl-2 family.[15] In this study, we have investigated the cytotoxic activities of 70% ethyl alcohol extracts and flavonoids **1** and **2** of *M. oleifera* fruits in HCT116 human colon cancer cells.

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Experimental

Plant material and preparation of the extracts – *M. oleifera* fruits were collected in Dar es Saalam, Tanzania, in September 2013. The botanical identification was made by Prof. Henry Joseph Ndangalasi, Department of Botany, Dar es Salaam University, Dar es Salaam, Tanzania. Ground and dried *M. oleifera* fruits (13.0 g) were extracted three times with 70% ethyl alcohol and sonicated for 3 hr at room temperature. The 70% ethanolic extracts was evaporated in a dry oven at 60 °C and stored at −20 °C until they were used for *in vitro* assay (yield: 0.28 g).

Chemical reagents – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and 100 U/mL penicillin and 100 µg/mL streptomycin were purchased from Thermo Scientific Inc. (Waltham, MA, USA). The primary caspase-9, caspase-8, caspase-3, and PARP antibodies were purchased from Cell Signaling Technologies Inc. (Danvers, MA, USA). All other chemicals and reagents were of the highest analytical grade.

Instrumental analysis – Electron Impact mass (EI-MS) spectrometry spectra were obtained on a JMS 700 spectrometer (JEOL Ltd., Tokyo, Japan). The ultraviolet (UV)-visible and infrared (IR) spectra were recorded on a JP/U3010 Spectrophotometer (Hitachi Ltd., Tokyo, Japan) and a FT/IR-5300 spectrometer (JASCO International Co. Ltd., Tokyo, Japan), respectively. An Avance-500 spectrometer (Bruker Corporation, Billerica, MA) was used to record Nuclear Magnetic Resonance spectra (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) with tetramethylsilane and deuterium dimethyl sulfoxide (DMSO-d_6) as an internal standard and NMR solvent, respectively. The correlation spectroscopy and heteronuclear multiple bond correlation experiments were conducted using the two-dimensional (2D) NMR spectroscopic method. Thin layer chromatography (TLC) was performed on silica gel (Kieselgel 60 F$_{254}$) plates (0.25 mm layer thickness; Merck, Darmstadt, Germany). TLC plates were visualized using UV light, stained with FeCl$_3$ and 20% H$_2$SO$_4$, and then heated at 70~80 °C for 5 sec.

Extraction and column chromatography – The 70% ethanolic extracts of *Moringa oleifera* fruits (MOF) (16.4 g) were partitioned between ethyl acetate (EtOAc) and water. The EtOAc soluble fraction (5.2 g) was dried and separated on a Merck 60 A silica gel (230 - 400 mesh ASTM, Merck, Darmstadt, Germany) column (6.5 × 80 cm) using a CHCl$_3$:MeOH gradient (100:0~75:25) and

yielded eleven subfractions. Subfraction 7 (1.2 g) was further separated on a silica-gel vacuum column by elution with CHCl$_3$:MeOH (15:1~4:1) to produce a solid, yellow-colored material. This material was further purified into twelve sub-subfractions using Sephadex LH-20 (25~100 µm, Pharmacia Fine Chemicals, Piscataway, NJ) column chromatography. Sub-subfractions 4 (125.8 mg) and 9 (80.1 mg) were recrystallized with highly purified MeOH to yield pure forms of compounds **1** (12.7 mg) and **2** (8.3 mg) from the EtOAc soluble fraction of MOF.

Cell cultures – The human colon cancer cell lines, HCT116 were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were maintained in Dulbecco's modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin, and the cells were incubated at 37 °C in a humidified incubator in a 5% CO$_2$ atmosphere. Cell counts were performed using a hemocytometer from Hausser Scientific (Horsham, PA, USA).

Cell viability assay – The cytotoxic effect of MOF against the HCT116 cell lines were estimated colorimetrical using the MTT method, which is based on the reduction of a tetrazolium salt by a mitochondrial dehydrogenase in viable cells.[16] Briefly, HCT116 cells were seeded (2×10^6 cells/mL) in a 96-well plate and were then treated with MOF at the indicated concentrations. After 72 hr incubation, MTT solution was added to each well at a final concentration of 0.4 mg/mL. After 2 hr of incubation, the supernatants were aspirated and replaced with 150 µL of DMSO to dissolve the formazan product. The absorbance at 540 nm was then read using a spectrophotometric plate reader (Model 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Results were calculated as percentages of the unexposed control.

Nuclear staining with Hoechst 33258 – The nuclear morphology of the cells was observed using the DNA-specific blue fluorescent dye Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA). The viable cells were stained homogeneously, whereas apoptotic cells which had undergone chromatin condensation and/or nuclear fragmentation were not stained.[17] The HCT116 cells were treated with MOF at different concentrations. Cells were then fixed for 30 min in 100% methanol, washed with PBS, and stained with Hoechst 33258 (2 mg/mL). The cells were observed under a BX51 fluorescence microscope and images were captured with a DP70 camera (Olympus Optical Co., Tokyo, Japan).

Apoptosis analysis – Annexin V/PI double staining assay was carried out to further differentiate between early and late apoptosis stages. The assay was determined

using an *Apo*Scan[TM]AnnexinV-FITC apoptosis detection Kit (BioBud, Seoul, Republic of Korea) in MOF-treated HCT116 cells. The cells were trypsinized, harvested, and washed with PBS. The cells were then resuspended in $1 \times$ binding buffer (100 μL) and incubated with 10 μL of AnnexinV-FITC (200 μg/mL) at room temperature for 15 min. The supernatant was then removed after centrifugation. The cells were resuspended in 380 μL of $1 \times$ binding buffer and cell suspensions were then stained with 10 μL of PI (30 μg/mL) at 4 °C in the dark. Fluorescence was quantified using FACS Calibur flow cytometry (Becton Dickinson, San Jose, CA, USA). The amount of early and late apoptosis was quantified as the percentage of Annexin V+/PI-, or Annexin V+/PI+ cells, respectively.

Western blotting analysis – Western blotting analyses were performed as previously described.[18] The cells were cultured, harvested, and lysed on ice for 30 min in an appropriate lysis buffer (120 mM NaCl, 40 mM Tris (pH 8.0), and 0.1% NP 40), and were then centrifuged at $13,000 \times g$ for 15 min. Lysates from each sample were mixed with $5 \times$ sample buffer (0.375 M Tris-HCl, 5% SDS, 5% β-mercaptoethanol, 50% glycerol, and 0.05% bromophenol blue, pH 6.8) and were then heated to 95 °C for 5 min. Equal amounts of protein were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membranes were washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl) containing 0.05% Tween-20 (TBST) and were then blocked in TBST containing 5% nonfat dried milk. The membranes were then incubated with their respective specific primary antibodies overnight at 4 °C. After three washes in TBST, membranes were incubated with the appropriate secondary antibodies coupled to horseradish peroxidase (HRP) for 1 hr at room temperature. The membranes were then washed again, and detection was carried out using an enhanced Chemiluminescence Western blotting detection kit. Data of specific protein levels are presented as multiples relative to the control.

Statistical analysis – All measurements were made in triplicate, and all values are given as the mean ± the standard deviation. The results were subjected to analysis of variance followed by the Tukey range test to analyze differences between conditions. In each case, p-value of < 0.05 was considered to be statistically significant.

Results and Discussion

Colorectal cancer is thought to occur as a result of changes in the normal colon epithelial cells as adenomatous colorectal polyps. Inhibition of cancer cells has led to the development of new anticancer drugs and related objectives in many natural remedies. New agents are, in particular, intended for cancer, with low toxicity for normal colon epithelial cells, and constantly hold a great efficacy in the study to find natural resources.[18] *M. oleifera* has been used as traditional medicine in Asia, Arabia and Africa. Many numbers of research have been conducted as to *M. oleifera*, many publications presented biological activity and components from the leaves of *M. oleifera*.[4,5,19-26] In the present study, we examined the effects of *Moringa oleifera* fruits and its main flavonoids on the growth of HCT116 human colon cancer cells using MTT, Nuclear staining with Hoechst 33258 and Western blotting analysis.

Compounds **1** and **2** were isolated through an activity-guided fractionation and isolation method using silica gel and Sephadex LH-20 column chromatographic separation. The dried *M. oleifera* fruits was extracted with 70% ethanol to give ethanolic extracts (MOF), and then concentrated and partitioned with EtOAc and water. The EtOAc soluble extracts of MOF were inhibited HCT116 cell viability as shown with an MTT assay (IC_{50} value of 32.8 mg/mL). The EtOAc soluble fraction (5.2 g) was dried and separated on a silica gel column using a $CHCl_3$: MeOH gradient to yield eleven subfractions. Subfraction 7 (IC_{50} value of 40.6 mg/mL) was further separated on a silica-gel vacuum column by elution with $CHCl_3$:MeOH to produce a yellow solid material. Of the twelve sub-subfractions, sub-subfractions 4 (IC_{50} value of 16.7 mg/mL) and 9 (IC_{50} value of 40.1 mg/mL) were recrystallized with highly purified MeOH to yield pure compounds **1** (12.7 mg) and **2** (8.3 mg), respectively. Compounds **1** and **2** were assigned molecular formulas of $C_{27}H_{30}O_{16}$ and $C_{15}H_{10}O_7$ from their HR-EIMS (*m/z* 610.2504 and 302.2341, respectively). Compounds **1** and **2** were contained five OH groups at δ_C 115.1, 144.6, 133.2, 156.3, 163.2, and δ_C 113.2, 145.2, 160.1, 161.4, 162.9, respectively. The relative positions of the functional groups in compounds **1** and **2** were established from the following observations. The ^{1}H NMR spectrum of compounds **1** and **2** suggested that the A ring was double substituted based on the appearance of doublets at δ_H 6.18, 6.40 and 6.22, 6.43, respectively. An aromatic proton integration for three protons at δ_H 6.88, 7.53, 7.67 and 6.64, 7.56, 7.66, respectively, was assigned for H-5', H-6' and H-2' in the B ring. Compound **1** also showed an anomeric proton at δ_H 5.31 that was conjugated with one molecule of glucose and a rhamnose moiety at δ_C 155.2 in the C ring. The chemical structure of compounds **1** and **2** was characterized

based on published data,[27] Physicochemical methods, and co-TLC, EI-MS, UV, IR, and NMR data. We found that compound **1** was rutin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranosyloxy]-4*H*-chromen-4-one) and compound **2** was quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one) (Fig. 1).

As shown in Fig. 2, MOF inhibited the proliferation of HCT116 colon cancer cells in a dose-dependent manner. Cell viability of MOF-treated HCT116 cells was decreased by 85.5%, 63.3%, and 38.5% at concentrations of 80, 120, and 150 μg/mL, respectively. Compounds **1** and **2** have been shown potent cell inhibition as IC$_{50}$ 52.3 and 62.1 μM, respectively. As a result, it is found MOF and main flavonoids, rutin and quercetin significantly inhibited proliferation of HCT116 human colon cancer cells.

Apoptosis is characterized by a series of morphologic changes and chromatin condensation of the cell nucleus by distinct mechanisms. In order to determine whether the growth inhibition induced by MOF and compounds **1** and **2** was associated with the induction of apoptosis in HCT116 cells, we performed DNA-binding dye Hoechst 33258 staining. As shown in Fig. 3, the nuclei were homogeneously stained, and they showed an intact structure in the control cells. After treatment with MOF and compounds **1** and **2**, there was slight cell shrinkage and a reduction of cell numbers. These results suggest that MOF and compounds **1** and **2** inhibited cell proliferation in a dose-dependent manner, which is consistent with the results of the MTT assay.

To further investigate the extent of apoptosis, Annexin V and PI double staining was used and were analyzed by flow cytometry. Annexin V+/PI staining represents early apoptotic cells, due to the high affinity of Annexin V-FITC with phosphatidylserine (PS). One of the characteristic
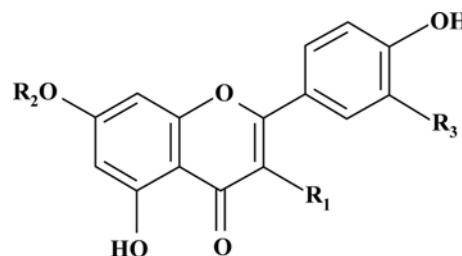
1: R$_1$=O-Glc-O-Rha; R$_2$=H; R$_3$=OH

2: R$_1$=OH; R$_2$=H; R$_3$=OH

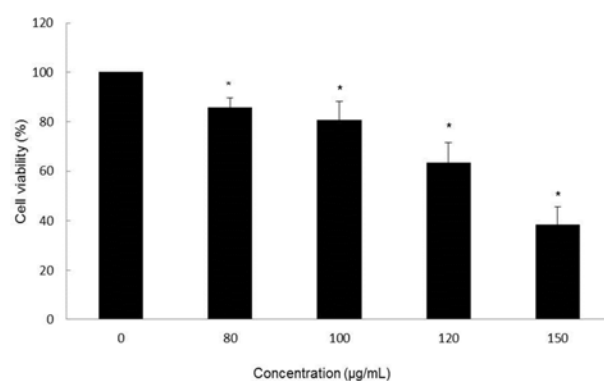**Fig. 1.** Chemical structure of compounds **1** and **2**.

Fig. 2. Cytotoxic effect of MOF in HCT116 cells. Cell viability at the indicated concentrations was assessed for 72 hr by MTT assay. *$p < 0.05$, significantly different from control cells.
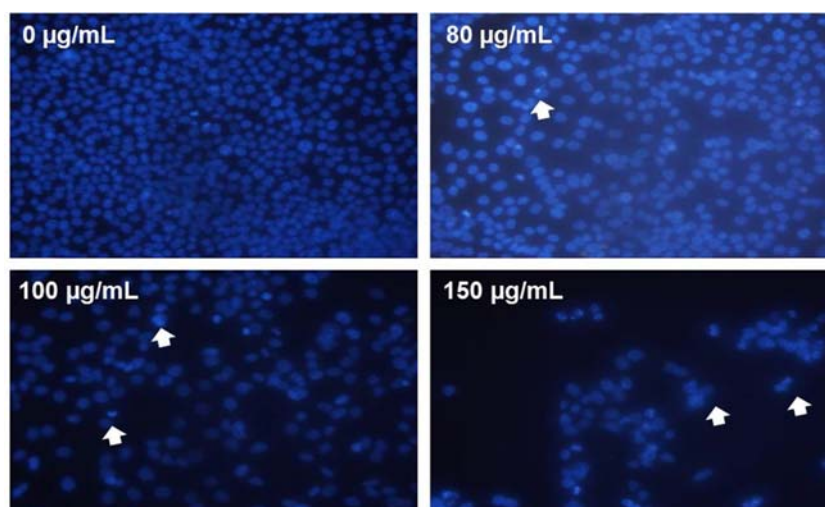

Fig. 3. Induction of apoptosis by MOF in HCT116 cells. The formation of apoptotic bodies (arrows) in Hoechst-33258-stained cells observed by fluorescent microscopy.
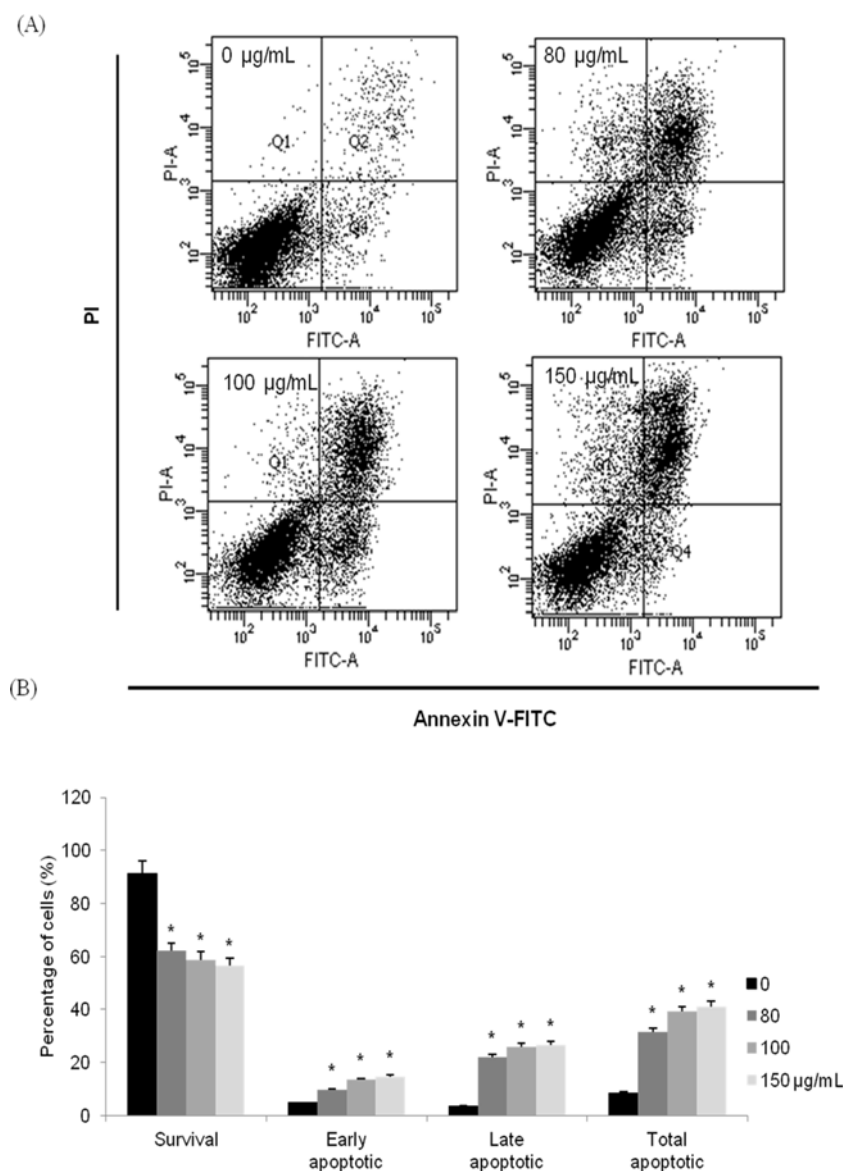
Fig. 4. Induction of apoptosis by MOF in HCT116 cells. Flow cytometric analysis of HCT116 cells incubated with MOF for 72 hr. The right bottom quadrant represents Annexin V-stained cells (early-phase apoptotic cells). The top right quadrant represents PI- and Annexin V-stained cells (late-phase apoptotic cells). *p < 0.05, significantly different from control cells.

features of early apoptotic cells is plasma membrane alteration, which involves translocation of PS from the inner side of the plasma membrane to the outer layer. Annexin V/PI+ staining represents necrotic cells due to the use of a membrane-impermeable DNA binding dye that cannot penetrate intact cell membranes. The Annexin V-/PI-population represents viable cells, and Annexin V+/PI+ represent late apoptotic cells. Treatment of the cells with MOF significantly increased apoptotic cell populations compared with untreated controls (Fig. 4). MOF-treated HCT116 cells showed an increased total percentage of apoptotic cells from 8.3% in control cells to 31.4%,

39.1%, and 40.9% at 80, 100 and 150 µg/mL, respectively. In addition, the percentage of late apoptotic cells was increased more than the percentage of early apoptotic cells at 80 µg/mL. These results indicate that the anti-proliferative effects of MOF against HCT116 cells were caused by apoptosis induction in a dose-dependent fashion. According to flow cytometry data, the proportion of apoptotic cells significantly increased following MOF treatment. All of these results indicate that MOF induces apoptosis in HCT116 human colon cancer cells.

To study whether MOF and compounds **1** and **2** induced apoptosis through the cascade-dependent pathway,
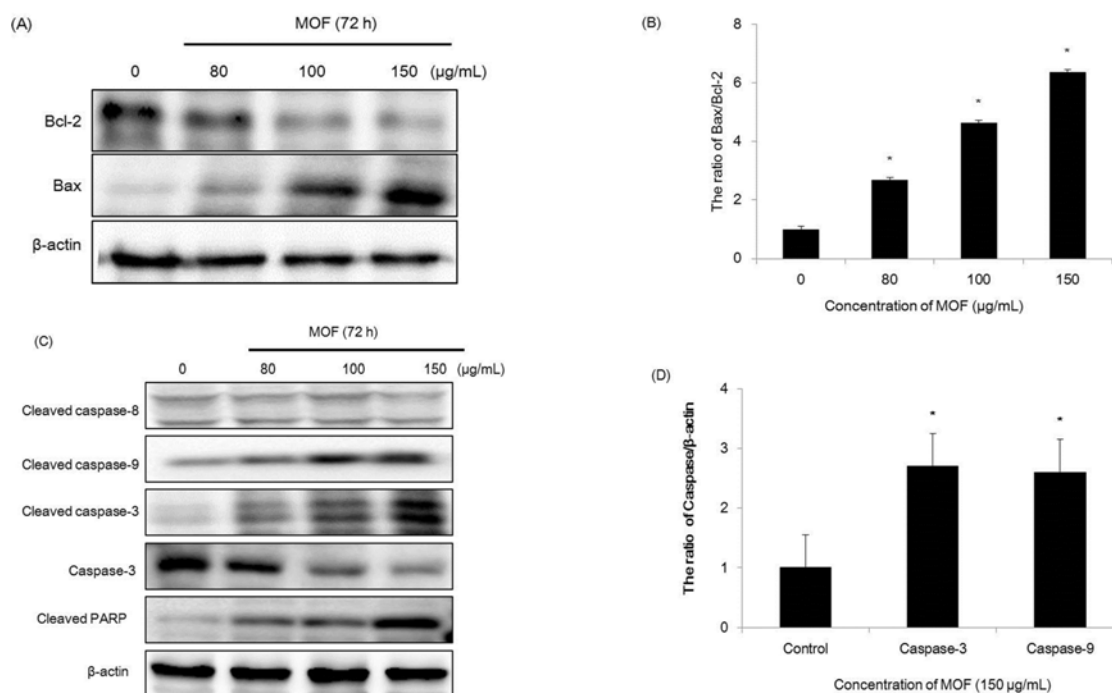
Fig. 5. Regulation of Bcl-2 family in MOF-treated HCT116 cells. Equal amounts of cell lysates were electrophoresed, and Bax and Bcl-2 expression form were detected by Western blotting analysis with corresponding antibodies. *$p < 0.05$, significantly different from control cells.
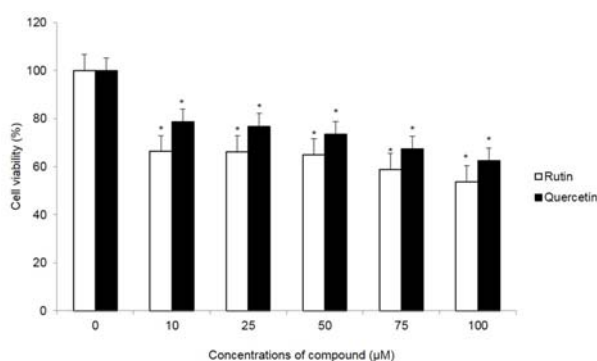

Fig. 6. Cytotoxic effect of compounds **1** and **2** in HCT116 cells. Cell viability at the indicated concentrations was assessed for 72 hr by MTT assay. *$p < 0.05$, significantly different from control cells.

we performed western blots on HCT116 cells. As shown in Fig. 5, it is observed a concentration-dependent reduction in the level of Bcl-2 protein with a concomitant increase in the level of Bax. With this change in Bcl-2 family proteins, it is also examined the expression levels of caspase-9, caspase-3 and poly ADP-ribose polymerase (PARP). The mitochondrial pathway is an important apoptosis pathway as it regulates the apoptotic cascade via convergence of signaling at the mitochondria.[28,29] It is shown mitochondrial plasma membrane was disrupted with MOF as well as compounds **1** and **2**, followed by the

activation of caspase-9, caspase-3, and its target, PARP. Mitochondria, organelles essential to the intrinsic apoptosis pathway, regulate the expression of the Bcl-2 family. The Bcl-2 family consists of pro-apoptotic (Bax, Bak, Bad, Bcl-Xs, Bid, Bik and Bim) and anti-apoptotic (Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1) proteins. It is observed a reduction in Bcl-2 expression accompanied by activation of caspase-9 and caspase-3 in HCT116 cells treated with MOF and compounds **1** and **2** (Figs 7, 8). The PARP is an important element to allow the cells to maintain viability, the cell is decomposed to promote the division and caspase-dependent functions as a major marker for apoptosis.[30] The cleaved form of PAPR was detected in MOF-treated HCT116 cells. Of these indicate that MOF and compounds **1** and **2** induced apoptosis via the mitochondrial pathway.

In conclusions, we have shown the mechanism for MOF-induced apoptosis in HCT116 cells. These results suggest that HCT116 cells are highly sensitive to growth inhibition by MOF via the activation of apoptosis, as evidenced by alteration in Bcl-2 family protein expression, and activation of caspase-3, caspase-9 and PARP. This is to demonstrate the cytotoxic effects of MOF and its main flavonoids **1** and **2** in HCT116 human colon cancer cells with a possible apoptotic mechanism to give a promising candidate with colon cancer therapy.
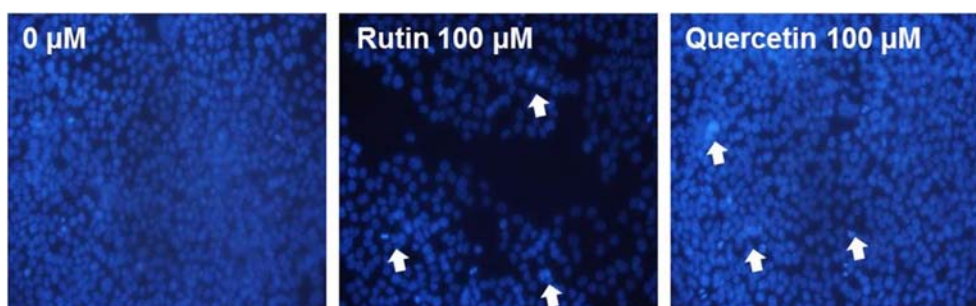
Fig. 7. Induction of apoptosis by compounds **1** and **2** in HCT116 cells. The formation of apoptotic bodies (arrows) in Hoechst-33258-stained cells observed by fluorescent microscopy.
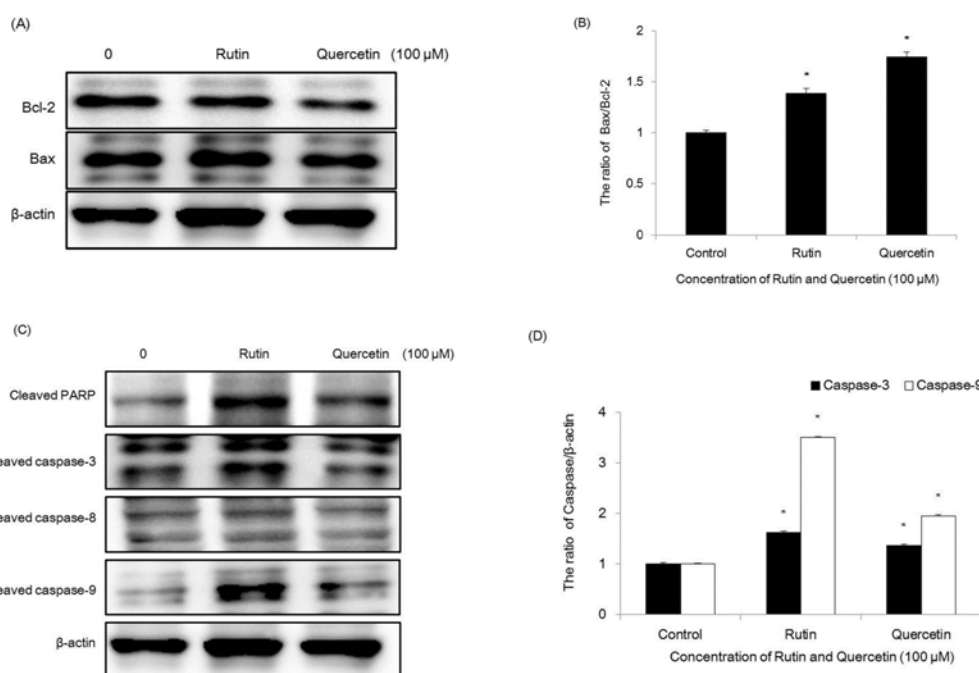

Fig. 8. Regulation of Bcl-2 family on compounds **1** and **2**-treated HCT116 cells. Equal amounts of cell lysates were electrophoresed, and Bax and Bcl-2 expression form were detected by Western blotting analysis with corresponding antibodies. $*p < 0.05$, significantly different from control cells.

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