Induction of Apoptosis with \textit{Kigelia africana} fruits in HCT116 Human Colon Cancer Cells via MAPKs Signaling Pathway

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Abstract – \textit{Kigelia africana} (Lam.) Benth. (Bignoniaceae) is a flowering plants in South, Central and West Africa and commonly known as the sausage tree (Eng.); worsboom (Afr.); umVunguta, umFongothi (Zulu); Modukguhlu (North Sotho); Muvevha (Venda). The dried, powdered fruits are used as dressing for wounds and ulcers, haemorrhoids, rheumatism, purgative, skin-firming, lactation in breast-feeding mothers. The aim of this study is to investigate the cytotoxic and apoptotic potentials of 70\% ethanolic extracts of \textit{Kigelia africana} fruits in HCT116 human colon cancer cells. Treatment of \textit{Kigelia africana} fruits with various concentrations resulted in a sequence of characteristic of apoptosis, including loss of cell viability and morphological changes. Flow cytometry analysis showed \textit{Kigelia africana} fruits increased the sub-G1 phase (apoptosis) population. Apoptosis confirmed by annexin V-fluorescein isothiocyanate and propidium iodide double staining in HCT116 human colon cancer cell lines. Moreover, analysis of the mechanism indicated that \textit{Kigelia africana} fruits showed an increased Bax and Bcl-2 expressions in a dose-dependent manner, resulting in activation of hallmarks of apoptotic events, caspase-3, caspase-9 and cleaved poly-ADP-ribose polymerase. This is the first report to demonstrate the cytotoxicity of \textit{Kigelia africana} fruits on HCT116 human colon cancer cells.

Keywords – Cytotoxicity, MAPK signaling pathway, Hallmarks of apoptosis

Introduction

Colorectal cancer remains the second leading cancer diagnostics and a high mortality and important global health problem.\textsuperscript{1} These therapeutic strategies such as many surgical and chemotherapy are used to treat cancer. However, there are bothersome side effects of chemotherapy, and surgery is associated with high mortality and recurrence.\textsuperscript{2} One kind of a promising approach has the biologically active ingredients of inhibiting the proliferation of cancer cells involves the administration of a natural biomolecule. Natural products are the majority of new anti-cancer drugs such as Taxol and Cisplatin provides a major source of drug development for centuries, is derived from natural products.\textsuperscript{3}

Apoptosis is the process of programmed cell death, is recognized as an important process for the preservation of tissue homeostasis, development and control over the current highly evolved. Many studies are efficacious in the treatment of various cancer cells that are recognized as weapons for the management of cancer it reported associations between cell death by apoptosis-inducing agents of cancer.\textsuperscript{4}

Two major apoptosis pathways have been identified: (a) extrinsic or death receptor pathways and (b) intrinsic or mitochondrial-related pathways.\textsuperscript{5} Pathway is controlled by the apoptosis inhibition of pro-apoptotic Bel-2 family members, which intrinsic pathway.\textsuperscript{6} It is characterized by translocation results permeability of the outer mitochondrial membrane cytochrome C from the mitochondria to the cytosol.\textsuperscript{7} This leads to the activation of caspase.\textsuperscript{8} The activated form of caspase-3 cleavage is one of many proteins, poly ADP-ribosepolymerase (PARP) because it contains the key executioner caspase.\textsuperscript{9}

\textit{Kigelia africana} (Lam.) Benth. (Bignoniaceae) known as sausage tree, are found in the Southern, Central and Western Africa. This plant has a long history as a medicinal plant used by many rural and African countries. It is used to treat fungal infections, boils, psoriasis and eczema, leprosy, syphilis, skin diseases, including cancer. Internally, it dysentery, ringworm, tape worm, postpartum hemorrhage, malaria, diabetes, pneumonia, and is used for the treatment of dental pain.\textsuperscript{10,11} The roots, wood and leaves have been found to contain kigelinone, vernolic

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acid, kigelin, iridoids, luteolin, and 6-hydroxyluteolin.12-13

In this study, we investigated the effects of 70% ethanolic extracts of Kigelia africana fruits on cell proliferation and apoptosis in HCT116 human colon cancer cells. Cell viability significantly decreased in a dose-dependent manner, after 72 hr of incubation with various concentrations of Kigelia africana fruits, which, prompted caspase-dependent signals.

Experimental

Plant material and preparation of the extract – The fruits of Kigelia africana were collected at Dar es Salaam, Tanzania, on September 2013. The botanical identification was made by Prof. Henry Joseph Ndangalasi, Department of Botany, University of Dar es Salaam, Dar es Salaam, Tanzania. The dried fruits of Kigelia africana (13.0 g) was soaked in 70% ethanol and sonicated for 3 hr at room temperature. The extracts were evaporated in a dry oven at 60 °C and stored at −20 °C until used for in vitro assay (yield: 0.2794 g).

Chemical reagents – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) and propidium iodide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against β-actin, Bcl-2, and Bax were purchased from Santa Cruz (Santa Cruz, CA, USA). The primary caspase-9, caspase-3, JNK, p-JNK, ERK, p-ERK, p38, and p-p38 antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA). All other chemicals and reagents were of the highest analytical grade.

Cell cultures – The human colon cancer cell lines, HT-29 and HCT116 were obtained from the Korean Cell Line Bank (KCLB, Seoul, Republic of Korea). The cells were maintained in Roswell Park Memorial Institute Media 1640 (RPMI 1640), 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% CO2 atmosphere. Cell counts were performed using a hemocytometer from Hauser Scientific (Horsham, PA, USA).

Cell viability assay – The cytotoxic effects of Kigelia africana (KAF) against the HCT116 cell lines were estimated colorimetrically using the MTT method, which is based on the reduction of tetrazolium salt by mitochondrial dehydrogenase in viable cells.14 Briefly, cells were seeded (2 × 10⁶ cells/mL) in a 96-well plate and were then treated with KAF at final concentrations of 0, 0.6 and 0.8 mg/mL. 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 dimethyl sulfoxide (DMSO) to dissolve the formazan product. The absorbance at 540 nm was then read using a spectrophotometric plate reader. 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. The viable cells were stained homogeneously, whereas apoptotic cells which had undergone chromatin condensation and/or nuclear fragmentation were not stained.15 The HCT116 cells were treated with KAF at different concentrations. Cells were then fixed for 30 min in 100% methanol, washed with PBS, and stained with Hoechst 33258 (2 μg/mL). The cells were observed under a fluorescence microscope (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 ApoScanTM Annexin V-FITC apoptosis detection Kit (BioBud, Seoul, Republic of Korea) in KAF-treated HCT116 cells. The cells were trypsinized, harvested, and washed with PBS. The cells were resuspended in 1 × binding buffer (500 μL) and incubated with 1.25 μL of Annexin V-FITC (200 μg/mL) at room temperature for 15 min. The supernatant was then removed after centrifugation. The cells were resuspended in 500 μL of 1 × 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 FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA, USA). The amount of early apoptosis and late apoptosis were determined as the percentage of Annexin V+/PI−, or Annexin V+/PI+ cells, respectively.

Cell cycle analysis – Cell cycle analysis was carried out to determine the proportion of apoptotic sub-G1 hypodiploid cells.16 HCT116 cells were plated in six-well plates and incubated for 24 hr. The cells were treated with KAF and incubated for 72 hr. The cells were trypsinized, harvested, and washed with PBS. The pellet was fixed using cold 70% ethanol for 30 min at 4 °C. The cells then washed once with PBS and resuspended in cold propidium iodide (PI) solution (50 μg/mL) containing RNase A (50 μg/mL) in PBS (pH 7.4) for 30 min in the dark. Fluorescence emitted from the PI-DNA complex was quantified using FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA, USA).

Western blotting analysis – Western blotting analyses were performed as previously described.17 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 × g for 15 min. Lysates from each sample were mixed with 5 × sample buffer (0.375 M Tris-HCl, 5% SDS, 5% β-mercaptoethanol, 50% glycerol, 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 were transferred onto a nitrocellulose membrane. The membranes were then 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 (SD). The results were subjected to analysis of variance (ANOVA) 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.

**Result and Discussion**

Colorectal cancer is the second leading cause of cancer-related death in the Western world. It 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. However, many people will have the toxicity to normal cells. Therefore, 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. Cancer is an abnormal growth of cells caused by uncontrolled proliferation. Apoptosis is a very important phenomenon due to its maintenance of cellular homeostasis by regulating cell division and cell death. Therefore, apoptotic and the control pathway are maintenance in the cell death induced by cytotoxic drugs in tumor cells, is important.

In the present study, we examined the effects of KAF on the growth of HT-29 and HCT116 human colon cancer cells using the MTT assay. Cells were exposed to various concentrations (0, 0.2, 0.4, 0.8 and 1.0 mg/mL) of 70% ethanolic extracts of *Kigelia africana* fruits (KAF) for 72 hr and their viability was determined after exposure. Cytotoxicity was determined as the percentage of viable KAF-treated cells in comparison with viable cells of untreated control cells. As shown in Fig. 1, KAF inhibited the proliferation of HCT116 human colon cancer cells in a dose-dependent manner, significantly. After 72 hr of exposure, KAF induced 34.1% growth inhibition at 0.4 mg/mL, and 73.9% at 0.8 mg/mL, respectively. Of note, KAF extract suppressed the growth of HCT116 cells in a dose-dependent manner, and the IC₅₀ value (50% inhibitory concentration) of cytotoxicity was determined to be 0.67 mg/mL. For these reasons, we selected KAF for subsequent experiments to identify the apoptotic mechanisms.

Nuclear Hoechst 33258 staining was performed in order to determine whether the anti-proliferative effect of
KAF was due to apoptosis. As shown in Fig. 2, HCT116 human colon cancer cells which were treated with KAF showed a number of morphological changes, including cell shrinkage, condensed chromatin, and a higher density of apoptotic bodies, compared with the untreated control cells. The number of apoptotic cells was increased in a dose-dependent fashion. To clarify the extent of apoptotic cells, HCT116 cells were subsequently subjected to staining with Annexin V and PI double staining, and were analyzed by flow cytometry. The Annexin V−/PI−-population was considered to represent unaffected cells, Annexin V+/PI− as early apoptosis, Annexin V+/PI+ as late apoptosis, and Annexin V−/PI+ as necrosis. The results showed that treatment of cells with KAF significantly increased the percentage of apoptotic cells, compared with untreated control cells (Fig. 3). KAF-treated HCT116 cells showed that early apoptotic cell populations increased by 12.3% at 0.8 mg/mL, compared with 5.0% for the control. The late apoptotic cell populations also increased by 4.3% and 7.4% at 0.6 and 0.8 mg/mL of KAF, respectively, compared with 1.2% for the control. The total apoptotic cell populations increased to 13.7% and 19.7% at 0.6 and 0.8 mg/mL of KAF, respectively, compared with 6.2% for the control. The data indicated that the percentage of late apoptotic cells was much greater than early apoptotic cells. On exposure of HCT116 cells to KAF, the total number of apoptotic cells increased in a dose-dependent fashion. Additionally, the population of late apoptotic cells was much greater than that of early apoptotic cells. These results indicated that KAF effectively induced apoptosis in HCT116 human colon cancer cells.

To investigate the distribution of HCT116 cell cycle progression, flow cytometry was performed on cells which were treated with 0.6 and 0.8 mg/mL of KAF. Accumulation of the sub-G1 population indicated characteristics of apoptosis. As shown in Fig. 4, untreated control cells displayed 1.9% of the sub-G1 phase, whereas KAF-treated cells displayed increased sub-G1 phases of 22.0% and 24.0% at 0.6 and 0.8 mg/mL, respectively. This was accompanied by a significant decrease in the G1 phase in a dose-dependent manner. The quantitative data of cell cycle phases are presented in Fig. 4B. These results suggest that KAF can induce apoptosis in HCT116 cells.

The regulation of the cell cycle is one of the features of tumorigenesis and involved in the uncontrolled proliferation in human cancer. The phases of the cell cycle can be divided into four in which the periods of DNA synthesis (S phase) and mitosis (M phase) is separated by a difference of G1 and G2. In present study, HCT116 cells treated with KAF significantly accumulated in the

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Fig. 2. Induction of apoptosis by KAF in HCT116 human colon cancer cells. The formation of apoptotic bodies (arrows) in Hoechst-33258-stained cells observed by fluorescent microscopy.

Fig. 3. Effects of apoptosis by KAF in HCT116 human colon cancer cells. (A) Flow cytometric analysis of HCT116 human colon cancer cells incubated with KAF for 72 h. 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). (B) Statistical analysis of apoptosis. *p < 0.05, significantly different from control cells.
sub-G1 phase (apoptotic cell population), whereas the S phase and G2/M populations were decreased, leading to apoptosis. In contrast, our results exhibited that KAF exerted apoptotic effect on HCT116 cells via sub-G1 increment and caspase activation.

To study the apoptotic effects of KAF on HCT116 cells, we examined the expression levels of a number of apoptosis regulatory proteins, including Bcl-2, Bax, caspase-3, caspase-9 and PARP. The mitochondrial pathway is an important apoptosis pathway as it regulates the apoptotic cascade via a convergence of signaling at the mitochondria. Bcl-2 interacts with the mitochondrial plasma membrane and prevents mitochondrial membrane pores, and blocking the signals of apoptotic factors during apoptosis. As a result, KAF increased Bax expression but decreased the expression of Bcl-2, each in a dose-dependent manner. The mitochondrial plasma membrane disruption by KAF was followed by the activation of caspase-9, caspase-3, and its target, PARP (Fig. 5). These results suggested that KAF can induce apoptosis through the regulation of apoptosis-related protein expression in HCT116 cells.

Apoptotic signals involve two main pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial-mediated pathway. The mitochondria-related pathway is regulated by the anti-apoptotic (Bcl-2, Bcl-x, and Bcl-XL) and Bcl-2 family of pro-apoptotic members (Bax, Bak and Bid). Wherein the mitochondrial outer membrane-apoptotic proteins via inhibition of apoptotic cell death in the presence of various stimuli, and maintains the integrity of the mitochondria in response to apoptotic stimuli, a pro-apoptotic protein is present in the cytoplasm, the mitochondria membrane film translocate to the mitochondria to induce pore formation. For these reasons, the balance between the expression levels of Bax and Bcl-2 is important to cell survival, as well as cellular death. Our data showed that KAF-induced apoptosis is associated with the up-regulation of Bax protein, as well as the down-regulation of Bcl-2 expression, each in a dose-dependent manner.

In addition, the Western blotting experiments indicated that caspase-9 and caspase-3 appear to be activated in KAF-induced HCT116 cells. In the cytosol, cytochrome C, caspase-9 which in turn activates the effector caspase activation including caspase-3. In addition to, KAF is the release of cytochrome C from mitochondria and then caused to increase caspase-3 activity. The partially or totally responsible for proteolytic cleavage of PARP as much
protein caspase-3 is one of the main executors of apoptosis. 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. The cleaved form of PARP was detected in KAF-treated HCT116 cells. Of these indicate that KAF induced apoptosis via the mitochondrial pathway.

The MAPK signaling pathway plays an important role in controlling the cell death induced by chemotherapeutic agents and a variety of cell stresses. However, the detailed mechanism of signal pathways in apoptosis remains elusive. We examined the phosphorylation expression levels of MAPKs to further determine whether MAPKs are involved in the KAF-induced HCT116 cytotoxicity. As shown in Fig. 6, KAF treatment for 24 hr significantly increased phosphorylation of ERK, and p38 MAPK. Phosphorylated levels of ERK, and p38 MAPK remained constant to 48 hr. These results indicated that ERK, and p38 MAPK might play an important role in apoptosis through regulating KAF-induced Bcl-2 family pathway in HCT116 cells.

In order to investigate the significance of MAPK activation, we treated KAF in the presence or absence of PD98059, and SB203580 in HCT116 cells. As shown in Fig. 7, the phosphorylated ERK, and P38 significantly blocked in response to co-treatment with KAF and PD98059 or SB203580 respectively. Taken together, these results indicated that ERK, and P38 might play an important role in the regulation of KAF-induced Bcl-2 family pathway in HCT116 cells.

MAPK, including c-Jun-N-terminal kinase (JNK), extracellular-regulated protein kinase (ERK), and p38 kinase, are associated with the initiation of apoptotic event in a various types of cells. Many antitumor compounds are demonstrated to induce apoptosis in cancer cells by activating p-38 MAPK and/or JNK signaling. As a group of MAPKs are found in all eukaryotic cells, cell growth and control of a series of physiological processes including differentiation, and apoptosis. Our study indicated KAF activated ERK, and p38 kinase within 24 hr of treatment. The levels of phosphorylated MAPK remained constant to 48 hr. This finding suggests that KAF induces apoptosis through MAPK-mediated pathways in HCT116 human colon cancer cells.

In conclusions, we have shown the mechanism for KAF-induced apoptosis in HCT116 cells. These results
suggest that HCT116 cells are highly sensitive to growth inhibition by KAF via the activation of apoptosis, as evidenced by activation of MAPK-mediated signaling as well as alteration in Bcl-2 family protein expression, and activation of caspase-3 and caspase-9. This is the first report to demonstrate the cytotoxic effects of KAF in HCT116 human colon cancer cells with a possible apoptotic mechanism to give a promising candidate with colon cancer therapy.

References