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Kaempferia rotunda* tuberous rhizome lectin induces apoptosis and growth inhibition of colon cancer cells *in vitro

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Abstract

Kaempferia rotunda Linn. is a plant of ginger family and has many medicinal values in traditional applications, including as antimicrobial and anticancer agents. The present study aims to examine the anticancer activity of *Kaempferia rotunda* tuberous rhizome lectin (KRL, MW 29 ± 1.0 kDa) against colon cancer cells SW480 and SW48. KRL inhibited 67% and 59% of SW480 and SW48 cells growth respectively at the concentration of 1.0 mg/mL. The cells growth inhibition was a dose dependent manner. KRL treatments notably inhibited the colony formation capacity of the cancer cells. The surviving fractions of SW480 and SW48 cells treated with KRL significantly ($p < 0.001$) reduced compared to that of control cells. Significant increment of the apoptotic cells were noted following by G₀/G₁ or G₂/M cell cycle arrest in KRL treated SW480 and SW48 cells, respectively. Modulation of PARP1, p53, p21, Bax and Bcl2 proteins expression was observed in treated cells in comparison to that of untreated cells. Furthermore, activation of caspase-3 and caspase-9 was noted in KRL treated cells and caspase-3 and caspase-9 inhibitors pre-treated cells were shown insensitive to KRL treatment. The results implied that KRL prevents SW480 and SW48 cells proliferation by the induction of apoptosis in the mitochondrial intrinsic pathway.

Keywords: Colony formation; protein expression; apoptosis.

1. Introduction

A group of proteins found in all kinds of living organism *i.e.* plant, human, bacteria and fungi either in membrane-bound form or in soluble form that can agglutinate cells by binding to cell-surface glyco-conjugates and glycoproteins is known as lectins [1-4]. Recently, lectin increased lots of interest to the scientist due to their diverse biological actions, such as anti-fungal [1], antibacterial [2] and antiproliferative activities [3, 4]. Mannose and N-acetyl-d-glucosamine are generally distributed in micro-organisms, insects and animals, so among the several sugar binding lectins these two sugars-binding lectins are believed as biologically important defense proteins [5, 6].

Recently two different proteins were isolated from *Kaempferia rotunda* tuberous rhizome with the molecular weight of 29 and 21 kDa and designated as KRL and KRL-2, respectively [7, 8]. Methyl- α -D-mannopyranoside, Methyl- α -D-glucopyranoside and D-mannose were the most potent inhibitory sugars for KRL. N-terminal sequence of KRL was determined and the similarity of the first seven amino acids was found with Concanavalin A. Sugar content and the molecular weights of these two proteins were different [7]. KRL inhibited the growth of different pathogenic bacteria and exhibited cytotoxicity against brine shrimp nauplii. Antitumore activity of KRL was evaluated against Ehrlich ascites carcinoma cells *in vivo* in mice. 51 and 67% cells growth inhibition was observed when lectin was administered at 1.25 and 2.5 mg/kg/day doses, respectively for five consequent days. [7]. Antiproliferative study of this lectin against EAC cells revealed that the lectin inhibited EAC cells growth by the induction of apoptosis that was confirmed by cell morphological change, using caspase-3 inhibitor and apoptotic related genes expression. Different phases of cell cycle were determined by flowcytometry in the presence and absence of KRL. The lectin arrested G₀/G₁ phase of EAC cells [9]. Although the anticancer properties of the lectin

against mice related EAC cells were elucidated, however till now the effects of KRL against any human cancer cell lines was not reported. In the present study, antiproliferative activity of KRL was checked against human colorectal cancer cell lines (SW48 and SW480) and elucidated the mechanism of anticancer activity of KRL by cell morphological study, cell cycle analysis and apoptosis related protein expressions.

2. Materials and Methods

2.1. Chemicals and reagents

In this study analytical grade of chemicals and reagents were used those were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Sepharose-4B was purchased from Fluka (Sweden). Anti-mouse monoclonal PARP1, Bcl2, Bax and GAPDH antibodies were procured from Santa Cruz biotechnology (Dallas, Texas, USA). Anti-rabbit monoclonal p21, p53, caspase-3 and caspase-9 antibodies were procured from Abcam (Melbourne, VIC, Australia). By using 10 mM of phosphate buffer saline (PBS, pH 7.4) experimental solutions and reagents were prepared. PBS was used for the dilution of antibodies, proteins and cell extracts. *Kaempferia rotunda* tuberous rhizomes were collected from the local market (Rajshahi, Bangladesh).

2.2. Purification and characterization of *Kaempferia rotunda* tuberous rhizome lectin

Lectin (designated as KRL) was purified from *Kaempferia rotunda* tuberous rhizome followed by the protocol previously published by Kabir et al. [7]. Briefly, *Kaempferia rotunda* tuberous rhizomes were homogenized with Tris-HCl buffer (pH 8.2) and centrifuged at 8,200 g for 15 min. Then supernatant was incubated overnight with 10 mM of CaCl_2 and glucose-sepharose at 4 °C with occasional shaking. Column was prepared with 20 ml of glucose-sepharose and crude protein was loaded to the top of the column. Then column was

washed out with 10 mM Tris-HCl buffer saline (TBS, pH 8.2) containing 1 mM of CaCl_2 to eliminate the unbound proteins. After that non lectin fraction was washed out by the equal volume of TBS containing 0.6 M of glucose. Finally, lectin was eluted with 10 mM sodium acetate buffer saline, pH 4.6 and 5 mM EDTA (ethylenediaminetetraacetic acid). 1 M Tris-HCl buffer, pH 8.2 was added to the eluted fraction to adjust the pH ~ 7.0 . Finally, purity of the lectin was checked by SDS-PAGE in 16% (w/v) as observed in our previous study [7].

2.3. Cell culture

Two colon cancer cell (SW480 and SW48) lines were used in the present study. The cells were recruited from American type of culture collection (ATCC). They were cultured and maintained in Leibovitz's L-15 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C in a CO_2 incubator as previously described [10].

2.4. Cell proliferation assay

A cell counting kit-8 (CCK-8) (Sigma-Aldrich, St. Louis, Missouri, USA) used to examine the effects of KRL on proliferation of SW480 and SW48 cells following the previously published protocol [11]. Briefly, both SW480 and SW48 cells were first seeded in flat-bottom 96-well plates at 1×10^4 cells/well and were treated with KRL at different doses (0.25, 0.5 and 1.0 mg/mL) after 24 h of initial seeding (day 0). Then the proliferation rate was determined on day 1, day 2 and day 3 after the treatment using CCK-8 following the manufacturer's guidelines. The absorbance was taken at 450 nm using Polarstar Omega microplate reader (BMG Labtech, Ortenberg, Germany).

2.5. Colony formation assay

The effect of KRL on clonogenic/cytotoxic capacity of colon cancer was examined by colony formation assay as previously described [12]. For this, equal numbers of SW480 and SW48 cells were seeded in 6-well culture plates with complete medium. Then the cells were treated with KRL (1 mg/mL) after 24 h of initial seeding and plate was kept in the incubator at 37 °C in 5% CO₂ to grow and form the colonies. When colonies were visualized, cell growth was stopped by discarding the medium. Then phosphate buffered saline (PBS) was used to wash the plates and the cells were fixed with 70% cold ethanol for 15 min at room temperature. Afterwards 0.5% crystal violet was added and incubated for 2 h at room temperature to stain the clones and washed with tap water. Subsequently, the plates were air-dried and images of the plates were captured. Finally, using the following formula, clone formation rates and surviving fractions were calculated

$$\text{Clone formation rate or plating efficiency (PE)} = \frac{\text{No. of colony formation}}{\text{No. of cells seeded}} \times 100$$

$$\text{Surviving fractions} = \frac{\text{No. of colony formation}}{\text{No. of cells seeded}} \times \text{PE}$$

2.6. Apoptosis assay

Induction of apoptosis of SW480 and SW48 cells followed by KRL treatment and control cells were examined using an annexin V-FITC apoptosis detection kit (Invitrogen, Carlsbad, CA, USA) following the protocol previously published [13]. BDFACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA) was used to detect population of annexin V and propidium-iodide positive cells. The data were processed and analyzed with FlowJo v10 software (FlowJo LLC, Ashland, OR, USA).

In addition, apoptotic changes following by KRL treatment in SW480 and SW48 cells were assayed using nuclear stain, Hoechst 33342 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Briefly, KRL treated and non-treated cells were fixed in 4%

paraformaldehyde for 10 min and permeabilised with 0.1% Triton-X-100 for 5 min at room temperature. Then cells were incubated with Hoechst 33342 (10 µg/ml) for 30 min at 37 °C and washed with PBS. Finally, fluorescence microscope (Olympus, Shinjuku, Tokyo, Japan) was used to observe the cells and the percentages of apoptotic cells were recorded in three independent experiments from different plates [12].

2.7. Cell cycle analysis

The effects of cell cycle kinetics of KRL treatment on SW480 and SW48 cells, treated and non-treated cells were fixed with cold 70% ethanol for 1 h as previously described [14]. Then cells were washed by cold PBS and 5 µl of RNase A (10 mg/ml) was added and incubated for 1 h at 37 °C. After that, 10 µl of propidium iodide (PI) solution (1 mg/ml) was added to the cell suspension and incubated for 2-5 min. Finally, analysis was performed with flow cytometry (BD FACSCalibur, BD Biosciences).

2.8. Western blot analysis

To investigate the expression of proteins related to cell growth, proliferation, cell cycle progression and apoptosis (*e. g.* PARP1, p53, p21, BCL2, BAX, caspase-3, caspase-9) followed by KRL treatment were examined by western blot analysis [15]. In short, total proteins were extracted from KRL treated/untreated cells with lysis buffer (Bio-Rad, Gladesville, NSW, Australia). Subsequently proteins were quantitated by absorbance spectrometry [12]. Then, 30 µg of total protein was separated by 15% SDS-PAGE (Bio-Rad) and transferred to nitrocellulose membranes (Bio-Rad). Afterwards, the membrane was blocked with 5% non-fat milk powder for 2 h at room temperature and the membrane was then incubated with anti-mouse monoclonal PARP1 and GAPDH, BCL2, BAX antibodies (Santa Cruz) at 1:500 and anti-rabbit monoclonal p53, p21, caspase-9 and casepase-3

antibodies from Abcam (Melbourne, VIC, Australia) (1:1000) overnight at 4 °C. After that, the membranes were washed three times with PBS-T and incubated at room temperature for 2 h with the mouse and rabbit secondary antibodies, respectively (Santa Cruz) (1:5000). Finally, a chemiluminescence HRP detection kit (Bio-Rad, USA) was used for developing and detecting the protein bands and images were captured by ChemiDoc MP Imaging system (Bio-rad).

2.9. Effect of caspase-3, -8 and -9 inhibitors on KRL-induced cytotoxicity in cancer cells

SW480 and SW48 cells were treated with caspase-3 (z-DEVD-fmk, 2 µmol/mL) caspase-8 (z-IETN-fmk, 2 µmol/mL) and caspase-9 inhibitor (z-LEHD-fmk, 2 µmol/mL) and incubated for 2 h at 37°C. Then, the cells were treated with KRL 1.0 mg/mL and incubated for another 24 h at 37°C and subsequently the cytotoxicity was determined using CCK-8 assay kits [12, 16].

3. Results

3.1. Purification and characterization of KRL

The lectin was purified from *Kaempferia rotunda* tuberous rhizomes using affinity chromatography on glucose-sepharose column. The KRL was eluted by sodium acetate buffer and migrated on SDS-PAGE as a single band with an apparent molecular mass of 29.0 ± 1.0 kDa. Approximately 15 mg of KRL was isolated from 500 g of *Kaempferia rotunda* rhizomes. The minimum haemagglutination activity of KRL was noted 3.0 µg/mL and 6 µg/mL for rat and human erythrocytes, respectively.

3.2. KRL inhibited proliferation of colon cancer cells

Colon cancer cells treated with KRL (SW480^{+KRL} and SW48^{+KRL}) exhibited significant ($p < 0.001$) reduction of cell proliferation in comparison to that of non-treated

control cells (SW480^{control} and SW48^{control}). Importantly, KRL inhibited the growth of SW480 and SW48 cells in a dose dependent manner (Figure 1A-1B). It was noted that SW480^{+KRL} cells showed approximately 42, 54 and 67% cell growth inhibition at the doses of 0.25, 0.5 and 1.0 mg/mL, respectively in comparison to that of SW480^{control} cells. Similarly, ~35, 49 and 59% cell growth inhibition was noted in SW48^{+KRL} cells at the doses of 0.25, 0.5 and 1.0 mg/mL, respectively when compared to that of SW48^{control} cells. In addition, it was observed that KRL could inhibited SW480^{+KRL} and SW48^{+KRL} cells proliferation up to three days of the treatment (Figure 1A-1B).

3.3. KRL inhibited colony formation and surviving fraction of colon cancer cells

KRL inhibited the colony formation capacity of SW480^{+KRL} and SW48^{+KRL} cells significantly ($p < 0.001$). SW480^{+KRL} and SW48^{+KRL} cells showed remarkably reduced colonies in comparison to that of SW480^{control} & SW48^{control} cells (Figure 2A). The numbers of colonies generated by SW480^{+KRL} cells were significantly lower than that of SW480^{control} cells (1471 ± 25 versus 464 ± 21) (Figure 2B). Similarly, 1347 ± 43 and 512 ± 18 colonies were noted in SW48^{+KRL} and SW48^{control} cells, respectively (Figure 2B). In addition, SW480 and SW48 cells treated with KRL (1.0 mg/mL) reduced the surviving fractions significantly. It was noted that approximately 9.56% and 11.65% surviving fractions were noted in SW480^{+KRL} and SW48^{+KRL} cells, respectively, whereas ~88% and 80.64% surviving fractions were observed in SW480^{control} & SW48^{control} cells, respectively (Figure 2C).

3.4. KRL enhances the apoptosis of colon cancer cells

A significant increase in the apoptotic populations of SW480^{+KRL} and SW48^{+KRL} cells were noted when compared to that of control cells (Figure 3A). In SW480 cells, 8.5 % versus 4.8% apoptotic cells were noted in SW480^{+KRL} and SW480^{control} cells, respectively

(Figure 3B). Similarly, enhancement of apoptotic cells were noted in SW48^{+KRL} in comparison to that of SW48^{control} (10.91 versus 4.1) cells followed by of KRL treatment (Figure 3B). Moreover, SW480 and SW48 cells stained with Hoechst 33342 dye followed by KRL treatment showed notable nuclear morphological alterations compared to control cells (Figure 3C-3D). Increased number of cells treated with KRL showed apoptotic phenotypes when visualized under fluorescence microscope in comparison to that of control cells (Figure 3C). In addition, phase-contrast microscopic analysis of KRL treated and non-treated cells showed that KRL treatment induced increased number of cell death by apoptosis (indicated by the formation of apoptotic bodies) (Figure 3D).

3.5. KRL treatment alters cell cycle kinetics in colon cancer cells

Colon cancer cells treated with KRL (1.0 mg/mL) showed G₀/G₁, G₂/M arrest and accumulation, thereby complemented with the reduction of cells in S phase (Figure 4). The histograms of control SW480 and SW48 cells and after treatment with KRL presented in Figure 4A. In the case of SW480 cells, the percentage of cells in treated and non-treated groups in G₀/G₁ phase were $32 \pm 1.4\%$ versus $25.2 \pm 2.5\%$, while cells in S phase were $54 \pm 1.3\%$ versus $48 \pm 2.0\%$, respectively (Figure 4B). Similarly, KRL induced accumulation of SW48 cells in G₂/M phase ($17 \pm 1.1\%$ versus $12.1 \pm 0.9\%$) and suppressed the population of cells in S phase ($43.2 \pm 2.2\%$ versus $50.1 \pm 1.8\%$) when compared to that of control cells (Figure 4C). Thus, the results implied that KRL inhibited SW480 and SW48 colon cancer cells by arresting them at G₀/G₁ & G₂/M phase along with suppressing the population of cells in S phase of the cell cycle.

3.6. KRL modulated the expression of cell growth and apoptosis specific proteins

Activation of tumour inhibitors/suppressors or pro-apoptotic and inactivation of tumour promoting proteins in SW480 and SW48 cells were noted followed by KRL treatment

(1.0 mg/mL) in the present study (Figure 5). KRL treatment deactivated tumour promoter PARP1 and significantly reduced the expression in both cells when compared to that of control cells (Figure 5). Whereas KRL treatment induced activation of growth and apoptotic inhibitors such as p53, p21 BCL2, caspase-3, & -9. As illustrated in figure 5, KRL treated cells showed increased expression of p53, p21 BCL2 protein when compared to that of control cells. In addition, a significant ($p<0.001$) increment of BAX expression was noted in both cell followed by KRL treatment (Figure 5). Also, KRL treated cells exhibited significant higher expression of activated (cleaved) caspase-3 and caspase-9 in comparison to that of control cells (Figure 5).

3.7. KRL induced activation mitochondrial intrinsic apoptotic pathway in colon cancer cells

In the present study, caspase-3, -8 and -9 inhibitors were used to check the specific pathways involved in the induction of apoptosis of SW480 and SW48 cells followed by KRL treatment. It was noted that KRL treatment showed 59-67% growth inhibitions at 1.0 mg/mL when compared to that of control cells, whereas cells pre-treated with caspase-3, 9 inhibitors were insensitive to KRL treatment (Figure 6). Consequently, the cytotoxic activities of KRL against SW480 and SW48 cells were decreased remarkably down to 15-19% in cells pre-treated with caspase-3 and -9 inhibitors (Figure 6). However, SW480 and SW48 cells pre-treated with caspase-8 inhibitor were remain sensitive to KRL treatment and had shown 55-65% cells growth inhibition (Figure 6A-6B). The expression of caspases -3, -8, -9 in SW480 and SW48 followed by treated with KRL in cells pretreated with respective inhibitors in Figure 6C-6D.

4. Discussion

Cancer is one of the leading causes of death after cardiovascular diseases. Several drugs are using for the treatment of different types of cancer. Most of them affect cell replication, therefore tumour growth. Mode of action of the most of drugs are nonselective and therefore causing undesirable and potentially toxic effects by affecting on the different macromolecules (such as DNA) and also important metabolic pathways for both malignant and normal cells. [17, 18]. Thus, the researchers are finding more selective and less toxic molecules. Lectins are such type of molecules those exert antitumor activities and cytotoxicity towards different cancer cells *in vivo* and *in vitro* through the induction of apoptosis and autophagy [19-24]. Severe damage and remarkable decreased of the cell viability and cell proliferation was observed when malignant cells are exposed to different concentrations of lectin [25-28]. These anti-cancer properties of lectins were dose and time dependent and had shown increasing activities with the increased lectin concentration and increased exposure time to the cells [25-28]. The mechanisms of action of cell death of different cancer cell lines by the most of lectins were not elucidated completely. Recently, we have purified KRL, characterized KRL and the anticancer properties were evaluated against EAC cells, a cancer cell line derived from mouse. The lectin inhibited EAC cells with the induction of apoptosis [9]. To check the antiproliferative activities of KRL against human cancer cell lines, we have selected colorectal cancer cell lines (SW480 and SW48) as it ranked third in occurrence among all types of cancer with the mortality rate of about 50% [29].

The lectin inhibited SW480 cell proliferation approximately 42, 54 and 67% at the doses of 0.25, 0.5 and 1.0 mg/mL, respectively. On the other hand ~35, 49 and 59% cell growth inhibition was observed at the doses of 0.25, 0.5 and 1.0 mg/mL, respectively for SW48 cells. In our earlier studies, we found that pea lectin inhibited 62% of SW48 and 63%

of SW480 cell growth at the concentration of 1 mg/ml. The cell growth inhibition was also a dose dependent manner. From the comparison of the above studies, KRL is more effective against SW480 cell lines. Effects on the proliferation of SW480 by different lectin were reported by other groups [25, 30]. Concanavalin A (Con A), jacalin (AIL) and (Ulex europaeus agglutinin) (UEA) helped the proliferation of SW480 while wheat germ agglutinin (WGA) and tepary bean lectin inhibited the proliferation at all concentrations [30,31]. Cell growth inhibition was also studied by colony formation assay. Colony formation by SW48 and SW480 cell lines were significantly inhibited by KRL. The inhibitory effects were higher than that of the pea lectin [16].

Lectins bind with cell surface carbohydrates when it comes into contact of the cell or can be internalized into the cells which can trigger a wide variety of signals those includes induction of cell cycle arrest or apoptosis, suppression of telomerase activity, and inhibition of angiogenesis and ribosomal inactivation [32-40]. Proliferation of T lymphocytes can be stimulated, tumor necrosis factor alpha (TNF)- α activity can be raised and anti-inflammatory interleukin (IL)-10 activity can be also inhibited by lectin as a result immune system can be activated [32-40]. Different chemotherapeutic agents are used for the treatment of cancer those caused apoptosis in cells. Apoptosis is a program cell death characteristics of which include chromatin condensation, chromosomal DNA fragmentation, cell shrinkage, blebbing, nuclear fragmentation and global mRNA decay. In the present study induction of apoptosis followed by KRL treatment was observed by Hoechst 33342 dye. Induction of apoptosis was further confirmed by FITC labelled Annexin V where apoptotic cells populations in SW480 and SW48 cell lines were significantly increased after treatment KRL.

A key protein in apoptosis process is known as caspase-3 that causes DNA fragmentation as well as DNA damage and can be activated in the apoptotic cell both by extrinsic mitochondrial pathway where caspase-8 activates caspase-3 or intrinsic

mitochondrial pathways where caspase-9 activates caspase-3 though the activation of other proteins. SW48 and SW480 cell proliferation was almost similar to that of the control when treated with KRL in the presence of Caspase-3 and caspase-9. While any remarkable change was not observed for caspase-8. Expression level of Caspase-3 and caspase-9 were further studied by western blotting analysis. It was noted that remarkable increased of expression level of these proteins were observed after treatment of SW448 and SW480 cell lines with KRL. These result suggested the involvement mitochondrial intrinsic pathway in the apoptosis process. In our earlier experiment we also found that pea lectin induced apoptosis in the intrinsic mitochondrial pathway both in SW48 and SW480 cell lines. A 113 KDa nuclear protein known as poly(ADP-ribose) polymerase (PARP) plays a key role in repairing single strand and double strand breaks of DNA [41] whose expression level is high and helps for the development of colon cancer [42,43]. PARP1 expression level was significantly decreased after treatment of cells with KRL. Expression level of another anti-apoptotic protein in the cell lines was also decreased after treatment. Beside the anti-apoptotic proteins, expression level of apoptotic proteins such as p53, Bax was checked. It was noted that Bax expression significantly upregulated in KRL treated cells in comparison to that of control cells. Also, it was noted that when p53 gene was mutated then abnormal cell proliferation occurs which is in turns, causes cancer. The level of p53 is low in normal cell but the expression of p53 may increase during DNA damage that may cause cell cycle arrest as well as cause apoptosis. After treatment with KRL, expression level of P53 was increased remarkable. Although in the cell lines p53 gene was mutated, it contains DNA binding capability, thus p21 expression was increased significantly and binds directly to the cyclin-dependent kinases and inhibits their kinase activity thereby causing cell cycle arrest [44]. Cell cycle arrest was further studied by flow cytometry and G₀/G₁ phase was increased remarkable

in SW480 while percent of G₂/M cell population increased for SW48 cell line. This result indicates that KRL inhibited cell growth by arresting at different phases of the cell cycle.

5. Conclusion

In conclusion, KRL inhibited the proliferation of SW48 and SW480 colorectal cancer cells by the induction of apoptosis as confirmed by fluorometric assay, flow cytometric study, caspase inhibitors and different protein expressions. Mitochondrial intrinsic pathway apoptosis was activated followed by KRL treatment.

Conflict of interests: Authors declare no conflict of interest.

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Figure legends

Fig.1. Effect of KRL on cell proliferation of SW480 and SW48 human colon cancer cells. Treatment of SW-480 (**A**) and SW-48 (**B**) cells with KRL at different doses (0.25, 0.5 and 1.0 mg/mL) induce significant reduction of proliferation in comparison to control cells on different days of initial treatment. Level of significance * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared with that of control cells.

Fig.2. Effect of KRL on the colony formation of SW480 and SW48 colon cancer cells. **A)** Treatment of SW480 and SW48 cells with KRL decreased the colony formation capacity significantly. **B)** Bar graphs presented the number of colonies generated from KRL treated and control cells. **C)** Surviving fraction of cells obtained from KRL treated and control cells. Results are shown as mean $\pm$ SD. Level of significance ** $p < 0.01$ and *** $p < 0.001$ when compared with that of control cells.

Fig.3. Induction of apoptosis in colon cancer cell lines. (**A**) annexin-V and propidium-iodide positive cells after treatment with KRL and control cells. (**B**) Percent of apoptotic cells after treatment with KRL. (**C**) & (**D**) representing cell morphological changes of SW480 and SW48 cells after treatment with KRL followed by staining with Hoechst 33342 fluorescence dye and under phase contrast microscopy, respectively. Images are taken from three individual experiments and scale bar is 20 μ m.

Fig.4. Effects of KRL on cell cycle distribution in SW480 and SW48 cells. The effects of KRL on the cell cycle of SW480 and SW48 cells were evaluated by flow cytometry. (A) Representing histogram of control SW480 and SW48 cells and after treatment with KRL. (B) & (C) Bar graph showing the percentages of each cell cycle phase's distributions obtained from three independent experiments for SW480 and SW48 cells, respectively.

Fig.5. Expression of apoptotic and growth related proteins PARP1, p21, p53, BCL2, BAX, caspase-3 and -9 after treatment with KRL. GAPDH used as loading control. Results are shown as mean $\pm$ SD. Level of significance ** $p < 0.01$ and *** $p < 0.001$ when compared with that of control cells.

Fig.6. Effects of Caspase-3, -8 and -9 inhibitors on the cytotoxic activities of KRL on the SW480 (A) and SW48 (B) cell lines. The expression of caspases -3, -8, -9 in SW480 (C) and SW48 (D) followed by treated with KRL in cells pretreated with respective inhibitors.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Highlight

1. KRL showed strong anti-proliferative activity against SW48 & SW480 cells.
2. Apoptosis was confirmed by Annexin V, Hoechst-33342 and caspase inhibitors.
3. G₀/G₁ & G₂/M cell cycle arrested for SW480 & SW48 cell lines, respectively.
4. Expression levels of Caspase-3, -9, Bax, & p53 proteins were increased significantly.
5. Expression levels of PARP1 decreased significantly followed by KRL treatment.