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Original Article

Evaluation of *in vitro* and *in vivo* anticancer activities of potassium koetjapate: a solubility improved formulation of koetjapic acid against human colon cancer

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Abstract

Background and purpose: The previous work on koetjapic acid (KA) isolated from *Sandoricum koetjape* showed its efficacy towards colorectal cancer however KA has poor water solubility which poses the biggest hindrance to its efficacy. In the present paper, an attempt was made to study the anti-colon cancer efficacy of KA's potassium salt *i.e.* potassium koetjapate (KKA) applying *in vitro* and *in vivo* methods.
Experimental approach: KKA was produced by a semi-synthetic method. A human apoptosis proteome profiler array was applied to determine the protein targets responsible for the stimulation of apoptosis. Three doses of KKA were studied in athymic nude mice models to examine the *in vivo* anti-tumorigenic ability of KKA.
Findings/Results: The results of this study demonstrated that KKA regulates the activities of various proteins. It downregulates the expression of several antiapoptotic proteins and negative regulators of apoptosis including HSP60, HSP90, Bcl-2, and IGF-1 in HCT 116 cells with consequent upregulation of TRAILR-1 and TRAILR-2, p27, CD40, caspase 3, and caspase 8 proteins. Additionally, KKA showed an *in vitro* antimetastatic effect against HCT 116 cells. These results are feasibly related to the down-regulation of Notch, Wnt, hypoxia, and MAPK/JNK and MAPK/ERK signalling pathways in HCT 116 cells besides the up-regulation of a transcription factor for cell cycle (pRb-E2F) pathways. In addition, KKA revealed potent inhibition of tumor growth.
Conclusion and implications: In sum, the findings indicate that KKA can be a promising candidate as a chemotherapeutic agent against colorectal cancer.

Keywords: Apoptosis; Colon cancer; Hypoxia; MAPK signalling pathways; Potassium koetjapate; TRAILR-1&2.

INTRODUCTION

Sandoricum koetjape Merr. is a medicinal plant belonging to the plant family Meliaceae, which is indigenous to Malaysia and the Southeast Asian region. It has a varied range of traditional medicinal applications for the curing of inflammatory conditions and digestive disorders (1).

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Different extracts and many isolated chemicals of *S. koetjape* have demonstrated potential pharmacological activities. The seeds of *S. koetjape* contain limonoids such as sandoricin and 6-hydroxy sandoricin that have shown antifeedant activity (2). While some trijugin type limonoids including sandrapins A-E were isolated from the leaves (3,4). Moreover, Pancharoen and colleagues successfully isolated two new andirobin types limonoids called sandropins A and B (5). Some comprehensive studies have also been done on the stem bark. Several triterpenoids were isolated from the stem bark including, katonic acid, indicic acid, koetjapic acid (KA), 3-oxo-12-oleanen-29-oic acid, alloaromadendrene caryophyllene oxide, spathulenol (6), 20-epikoetjapic acid, 3-epikatonic acid, and sandorinic acid A-C (7). Previously, it was documented that KA is the main anticancer compound of *S. koetjape* against colon cancer cell line (8). However, it was reported that KA has poor solubility in water and other cell culture-permitted solvents, posing a hurdle to further research and its clinical function. In our prior study, an effort was made to improve the aqueous solubility of KA by chemically modifying it to potassium koetjapae (KKA) using the salt formation method (9). The characterization of KKA was determined by solubility studies, Fourier transform infrared spectroscopy (FT-IR), differential scanning calorimetry (DSC) along proton nuclear magnetic resonance (1HNMR) analysis. Furthermore, KA and its potassium salt were analyzed by high-performance liquid chromatography (HPLC) technique (9). Next, the preliminary safety and *in vitro* anticancer properties of KKA were confirmed. The toxicological test showed that KKA was safe (not lethal) in rats with LD_{50} of 2000 mg/kg body weight and pronounced more anticancer efficacy than KA (9). Our recently published study demonstrated that bio-absorption of KKA from the oral route was considerably efficient with longer retention in the body than that of the intravenous route. Furthermore, the improved antiangiogenic activity of KKA was recorded which could probably be due to its increased solubility and bioavailability (10). In the present study, we examined the antitumor efficacy of KKA using *in vitro* and *in vivo* human colon tumor models and evaluated the molecular mechanisms involved in its antitumor activity.

MATERIAL AND METHODS

The materials, reagents, and instruments that used in this study were as follows: apoptosis antibody array kits (Raybiotech, Parkway, Norcross, GA, USA), betulinic acid (Fisher, USA), bovine serum albumin and fetal bovine serum (Chromadex, USA), human colorectal carcinoma (HCT 116, ATCC®), Cignal Finder™ 10-pathway reporter array system (Qiagen, Hilden, Germany), dual luciferase reporter system (Promega, Madison, USA), Matrigel (BD Bioscience. USA), NCR nu/nu nude mice (Taconic, USA), MTT (3-(4,5-dimethylthiazol-2-yl)2,5 diphenyl tetrazolium bromide (Sigma-Aldrich, USA), multi-well plates (Costar Corning, USA), RPMI-1640 medium (Gibco/ Invitrogen, Grand Island, NY, USA), incubator (Binder Fisher Scientific, Germany), inverted fluorescent microscop (Olympus, Japan), inverted light microscope (Matrix Optic (M) Sdn. Bhd, Japan), microplate reader (Thermolab Systems, Finland), biosafety cabinet (Esco, Singapore).

Migration assay

The anti-migratory activity of KKA on HCT-116 cells was measured by scratch assay (11,12). Briefly, HCT 116 cells were plated till the formation of a confluent monolayer. Then, the wound was created with micropipette tips. Next, the plates were treated with KKA. The images of wounds were taken after 12 and 18 h. Next, the width of the wound was evaluated under an inverted light microscope. Ten fields per well were taken pictures and about 30 readings per field were measured. The findings were described as the mean percentage of migration inhibition linked to control ± SD. The percentage of migration inhibition was evaluated using the equation below:

$$\text{Migration inhibition (\%)} = \left(1 - \frac{Ds}{Dc}\right) \times 100$$

where Ds stands for the distance traveled by cells treated with KKA and Dc for the distance traveled by cells treated with the vehicle (distilled water).

Cell invasion assay

The anti-invasive potential of KKA on HCT-116 cells was performed using a modified Boyden chamber method. Briefly, Matrigel (150 µL) containing 20% fetal bovine serum (FBS) was loaded into each well of a 96-well plate and then polymerized for 45 min at 37 ℃ in a cell culture incubator. Subsequently, 100 µL of cell suspension (5×10^3) was added to a 96-well plate. Considering the previous study of MTT assay and IC_{50} calculation, different concentrations (2.5 and 5 µM) of KKA samples were added to the cells. Distilled water and 5-fluorouracil (5-FU; 5 µM) were used as and positive controls, respectively. Later, old media in each well was eliminated followed by rinsing with phosphate-buffered saline (PBS) to wipe out the non-invading cells. The upper layer of Matrigel gel was cleaned with sterile cotton. Next, five chosen fields per well were photographed. Consequently, the number of cells invading the Matrigel matrix was counted. Finally, the percent inhibition of cell invasion was evaluated as a result of the number of cells invaded in the control group and KKA treatment groups. The results are shown as mean ± SD of percent inhibition of different group's cell invasions.

Transmission electron microscope

To demonstrate apoptotic types of HCT cells under treatment with KKA, a transmission electron microscope (TEM) was used. Briefly, HCT cells were cultured using RPMI-1640 culture medium and then incubated for 24 h. Subsequently, the old medium was replaced by a new RPMI1640 culture medium containing the KKA at concentrations of half of the IC_{50} and IC_{50}, in addition to one flask that contained distilled water as a negative control. The cells then were fixed, solidified in a two percent agar solution (Fisher, USA), cut into small slides, and kept in 50% ethanol. Next, the slides were placed in resin, followed by 24 h infiltration in Suprr's mixture, which had been changed repeatedly every day for 5 days. Subsequently, the specimens were embedded in resin. The semi-thin sections were marked with toluidine blue (Sigma-Aldrich, USA). Finally, the cells were photographed by a TEM (EFTEM, Carl-Zeiss, Germany) at 1600× magnification.

Human apoptosis proteome profiler array

To examine the effect of KKA on the expression pattern of key proapoptotic and antiapoptotic proteins in colon cancer cells, the human apoptosis proteome profiler array was applied following the manufacturer's guidelines. Briefly, 20×10^4 HCT 116 cells were seeded in a 6-well plate and treated with 7 µg/mL of KKA for 24 h. Afterwards, cells were lysed and protein concentration in each sample was calculated by the Bradford protein test. Then, the antibody array was prevented with 1 × blocking buffer at room temperature by occasional gentle shaking. Next, 500 µg/mL of protein sample from each treatment group was diluted in 1 × blocking buffer and then incubated overnight with an antibody array with continuous gentle shaking at 4 ℃. Afterward, protein samples were aspirated from the wells followed by washing the array slide using wash buffers. Subsequently, 70 µL of biotin-conjugated antibodies were included in the wells and the array slide was incubated at room temperature by occasionally gentle shaking. Next, the cocktail of antibodies was removed from the array wells and followed by washing the wells using wash buffers. Subsequently, 70 µL of labeled streptavidin was pipetted into each well and the array slide was incubated in the dark for 2 h with gentle shaking. Next, the streptavidin solution from the wells was removed and the slide was rinsed with wash buffers I and II, respectively. The slide was scanned at the Genomax® facility by an Agilent microarray scanner. Then, the achieved data from scanning was transferred into the analysis software followed by calculating the relative fold change in the expression layout of each protein. Image Studio Lite software (LI-COR, Biotechnology) was used to Identify and label each spot in the array. Finally, a Heat map was obtained by Genespring GX software 13.

In vivo antitumor studies

The *in vivo* antitumor ability of KKA was evaluated in the ectopic colon tumor form of human colorectal carcinoma cells (HCT 116) using athymic NCR nu/nu nude mice (Taconic Farms Inc USA). The investigational procedure was certified by the Animal Ethics Committee of Universiti Sains Malaysia [Reference

No. USM / Animal Ethics Approval / 2012 / (81) (462)]. Briefly, mice with a standard weight of about 25 g and aged 6-8 weeks were injected subcutaneously in the right flank along with 5×10^6 HCT 116 cells containing 200 µL of RPMI-1640 media. Later, when the mean tumor size reached almost 100 mm^3, the animals were divided into five groups (6 mice each). The negative control group was administered 100 µL of distilled water orally. The positive control group was under treatment with 10 mg/kg of capecitabine (Xeloda®; Roche Laboratories, Nutley, NJ, USA) dissolved in distilled water. The other three groups were orally administrated under treatment with 50, 100, and 200 mg/kg of KKA, respectively. The tumor size and body weight were measured before starting the treatment and then at 6-day intervals till the experiment was finished (when tumor volume in any of the treatment groups reached 1000 mm^3) (14,15). The tumor dimensions were calculated by a digital caliper. Then, the tumor volume in each group was determined (16,17). At the end of the experiment, the animals were euthanized by carbon dioxide along with cervical dislocation. Then, subcutaneous tumors were excised and were preserved in 4% paraformaldehyde. The tumor cross-sections were collected and stained with hematoxylin and eosin (H&E) (10).

Statistical analysis

The statistical differences between the treatments and the control were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test using GraphPad Prism (San Diego, CA, USA). The results are expressed as the mean ± SEM. *P*-values < 0.05 were considered statistically significant.

RESULTS

KKA prevents colon cancer cell migration

The *in vitro* scratch test revealed that KKA has significant anti-migratory activity against HCT 116 cells (Fig. 1). Treatment with two concentrations of 2.5 and 5 µM of KKA along with 5 µM of 5-FU caused a significant decrease of HCT 116 movement across the created wound. The average percentages of inhibition after 6 h treatment were 13.9, 20.41, and 23.1% at concentrations of 2.5 and 5 µM of KKA along with 5 µM of 5-FU, respectively.

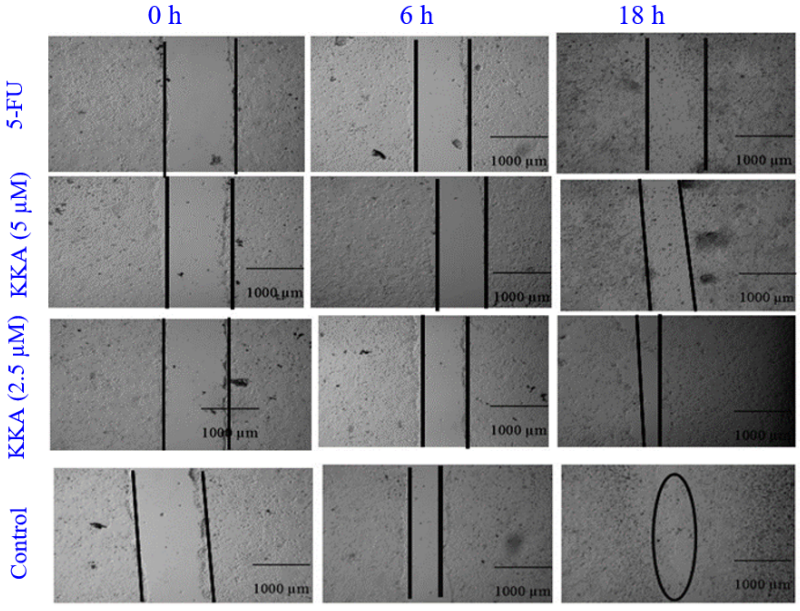

Fig. 1. Pictomicrographic representation of antimigratory result of KKA on HCT cells. HCT cells were treated with different concentrations of KKA, distilled water as the negative control, and 5-FU (5 µM) as the positive control. Significant prevention at 6 and 18 h contrary to the negative control which reveals complete wound closure (at 18 h). Photographs were taken at 4× magnification. KKA, Potassium koetjapate; 5-Fu, 5-fluorouracil.

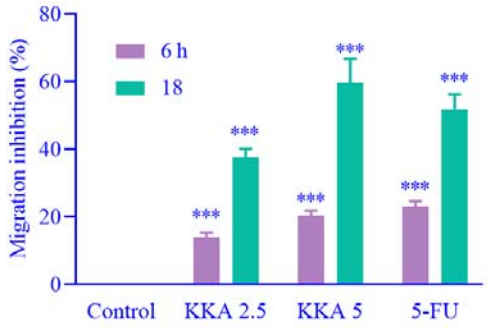

Fig. 2. Percentages of migration inhibition of HCT cells after 6 and 18 h under treatment with KKA at 2.5 and 5 µM. Cells treated with distilled water and 5-FU (5 µM) were used as the negative and positive controls, respectively. Data are expressed as means ± SD, n = 3. ***P < 0.001 Indicates significant differences in comparison with the control group. KKA, Potassium koetjapate; 5-Fu, 5-fluorouracil.

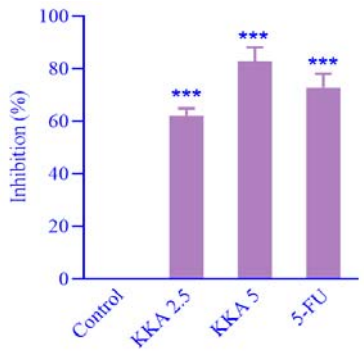

Fig. 3. The anti-invasive efficacy of KKA (2.5 and 5 µM) on HCT 116 cells. Cells treated with distilled water and 5-FU (5 µM) were used as the negative and positive controls, respectively. Data are expressed as means ± SD, n = 3.***P < 0.001 Indicates significant differences in comparison with the control group. KKA, Potassium koetjapate; 5-Fu, 5-fluorouracil.

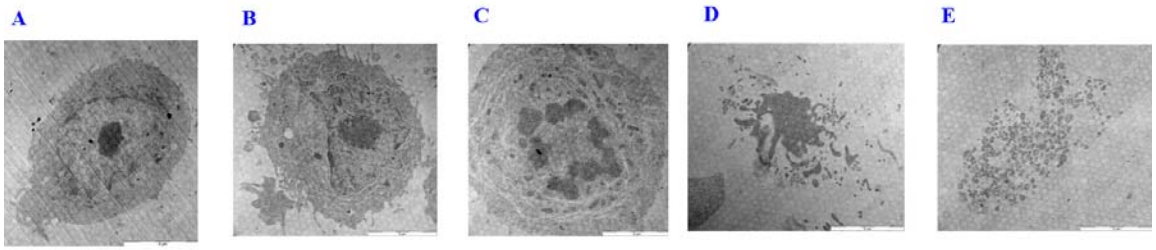

Fig. 4. The effect of KKA on apoptotic properties of colon cancer cells. (A) The cells that received distilled water show typical cell morphology with undamaged cell membranes. The cells treated with KKA at 3.5 and 7 µM revealed apoptotic morphological changes such as (B) chromatin condensation and blebbing of the cell membrane, (C) fragmentation, and (D and E) formation of apoptotic bodies. Photographs were taken at 1600× magnification along with a scale bar equal to 5 µm. KKA, Potassium koetjapate

The untreated cells showed complete wound closure within 18 h, while KKA presented percentages of migration inhibition of 37.43, 59.04, and 51.85 at concentrations of 2.5 and 5 µM of KKA along with 5 µM of 5-FU, respectively which are statistically significant when compared with negative control group. HCT 116 cells treated with 5 µM of KKA as well as 5 µM of 5-FU showed significantly higher inhibition of cell migration whereas other treated groups had no statistical variation in comparison with each other (Fig. 2). Wound closure of the negative control group reached 100% after 18 h.

KKA hinders colon cancer cell invasion

Figure 3 shows the anti-invasive activity of KKA on HCT 116 cells in a modified Boyden chamber assay. The cells invading the Matrigel base were detected in cells under treatment with KKA at 2.5 and 5 µM as well as 5 µM of 5-FU then evaluated comparably with the number of cells invaded in the negative control (distilled water) group followed by calculating percent inhibition. The percent inhibition of cell invasion treated with KKA (2.5 and 5 µg/mL) as well as 5 µM of 5-FU significantly increased compared to the control group (Fig. 3).

Observation of ultra-structural apoptotic arrangement in HCT cells treated with KKA

Figure 4A-E indicates several characteristic changes in HCT cells undergoing apoptosis after treatment with 3.5 and 7 µM of the KKA (Fig. 4B-E) while the cells treated with distilled water (Fig. 4A) show intact cell membranes with dense cellular content. Treatment with KKA at 3.5 and 7 µM indicated morphologic characteristics of apoptosis, like blebbing of the cell membrane, chromatin condensation, and fragmentation along with the formation of apoptotic bodies (Fig. 4B-E).

The effect of KKA on the expression of apoptotic proteins

Figure 5 shows the effects observed in the expression pattern of pro- and anti-apoptotic proteins in colon cancer cells treated with KKA (7 µM). KKA modulated the activity of about 15 proteins in a significant manner. It down-regulated the expression of several anti-apoptotic proteins and adverse regulators of apoptotic pathways involving B-cell lymphoma 2 (Bcl-2), heat shock protein (HSP) 60, HSP90, and insulin-like growth factor-1 (IGF-1) in HCT 116 cells. It also triggered the extrinsic apoptotic pathway by upregulating the expression of death receptors *i.e.* TRAILR-1 as well as TRAILR-2 along with upregulating the expression of IGF ligand-binding protein 6 (IGFBP-6), p27CD40, caspase 8 and caspase 3 proteins. The relative fold change values greater than 0 indicate up-regulation and the values lower than 0 demonstrate down-regulation of the protein targets. Table 1 illustrates the effect of KKA on apoptotic regulatory proteins in HCT 116 cells.

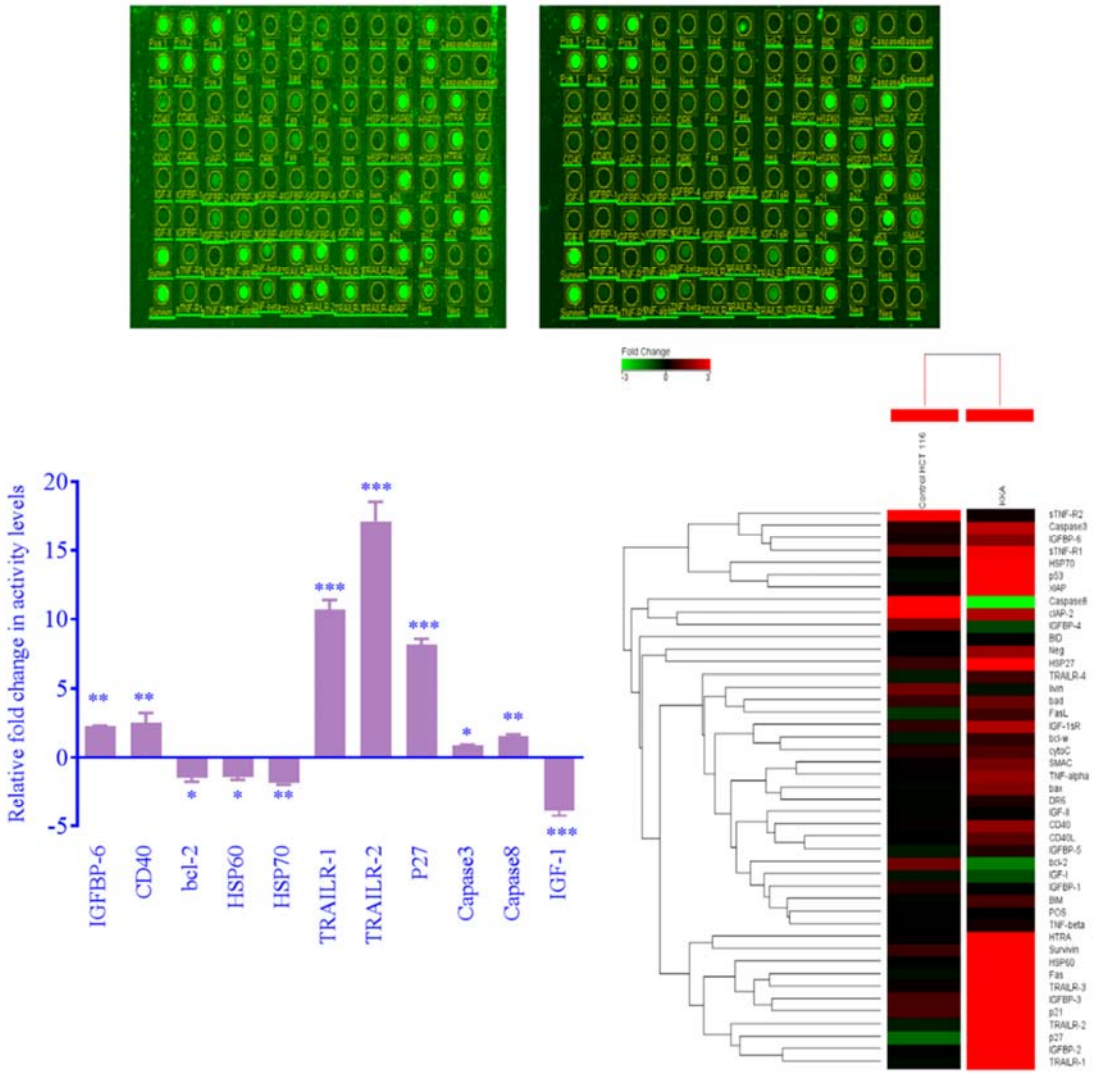

Fig. 5. Effect of KKA treatment on the expression of several proteins involved in the progression of apoptotic events. Heatmap characterizes signal strengths of each protein in the KKA treatment group and control group. Red bands in the cluster diagram show upregulation although green bands indicate downregulation of proteins. Values shown are mean ± SD of two experiments (n = 4 for every protein). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ indicate significant differences in comparison with the amount of zero. KKA, Potassium koetjapate; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Bcl-2, B-cell lymphoma-2; IGF1, insulin-like growth factor 1 receptor; IGFBP-6, insulin-like growth factor binding protein 6; CD40, cluster of differentiation 40; Hsp, heat shock protein.

Table 1. Effect of potassium koetjapate on the expression of proteins engaged in apoptotic events.

Protein	Regulation	Target report
Bcl-2	Down	Antiapoptotic
Bcl-w	Down	Antiapoptotic
Caspase-3	Up	Proapoptotic
caspase 8	Up	Proapoptotic
CD40	Up	Proapoptotic
Fas	Up	Proapoptotic
FasL	Up	Proapoptotic
HSP60	Down	Antiapoptotic
HSP70	Down	Antiapoptotic
IGFBP-2	Up	Proapoptotic
IGFBP-6	Up	Proapoptotic
IGF-1	Down	Antiapoptotic
p27	Up	Proapoptotic
TRAILR-1	Up	Proapoptotic
TRAILR-2	Up	Proapoptotic

The modulatory effects of KKA on carcinogenesis signalling pathways at the transcriptional phase

Figure 6 illustrates the efficiency of KKA on the expression levels of transcription factors for 10 major pro- and anti-tumorigenic signalling pathways in HCT-116 cells examined by the Cignal Reporter assay. The results show that KKA caused significant down-regulation of Notch, Wingless-int (Wnt), hypoxia, mitogen-activated protein kinase (MAPK) / extracellular signal-regulated enzyme kinase (ERK) and MAPK / Jun N-terminal kinase (JNK), and significant up-regulation of retinoblastoma protein (pRb)-E2F. Fold change values below 1 display down-regulation, whereas higher than 1 show up-regulation of specific transcription factors. (Fig. 6).

Antitumor activity of KKA

In-vivo anti-colon cancer effect of KKA was examined on HCT 116 sub-cutaneous tumor models in mice (NCR nu/nu nude). Before the start of the tumor xenograft study, the preliminary safety profile of KKA was assessed in an acute toxicity rat model following OECD guidelines. The outcome of the acute toxicity study illustrated that KKA was not lethal up to the dose of 2000 mg/kg. Keeping in view the LD_{50} value of KKA, three doses including 100 mg/kg ($1/20^{th}$ of LD_{50}), 50 mg/kg ($1/40^{th}$ of LD_{50}), and 25 mg/kg ($1/80^{th}$ of LD_{50}) were selected for *in vivo* xenograft studies.

At regular intervals, tumor size was measured, and the images of tumors were captured at the end of the experiment. Figure 7A-F illustrates the inhibition of tumor growth during 21 days in nude mice treated with different doses of 25, 50, and 100 mg/kg of KKA and 10 mg/kg of capecitabine. Analysis of tumor size indicated significant prevention of tumor growth in treated animals in comparison with the untreated group, furthermore, the inhibition of tumor growth was time-dependent (Fig. 8).

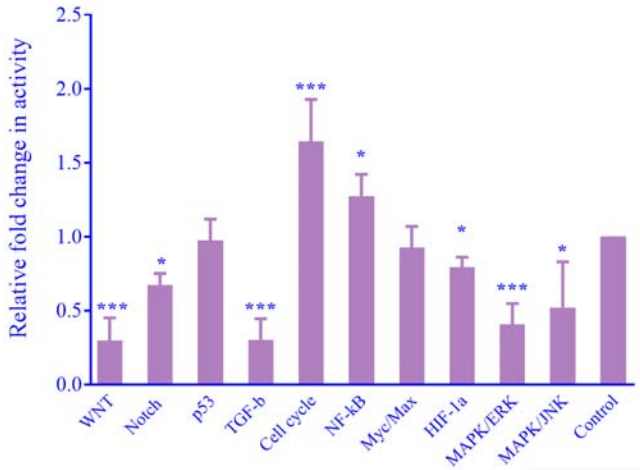

Fig. 6. Treatment with potassium koetjapate (7 µM) significantly altered the expression of multiple cells signaling pathways in colon cancer cells (HCT 116). The results show that KKA caused significant down-regulation of Notch, Wnt, hypoxia, MAPK / ERK, MAPK / JNK, and significant up-regulation of pRb-E2F. Fold change values below 1 display down-regulation, whereas higher than 1 show up-regulation of specific transcription factors. Data are expressed as means ± SD, n = 3. **P* < 0.05 and ****P* < 0.001 indicate significant differences in comparison with the control group. Wnt, Wingless-int; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated enzyme kinase; JNK, Jun N-terminal kinase; pRb, retinoblastoma protein.

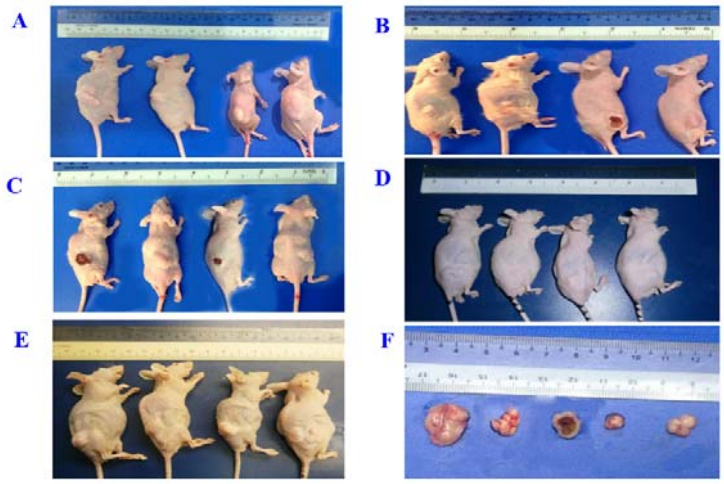

Fig. 7. Subcutaneous tumors established in NCR nu/nu nude mice. *In-vivo* antitumor efficacy of KKA was determined using an athymic mice xenograft model bearing HCT-116 tumors at day 21 post-inoculation. (A) Mice from the negative control (distilled water); (B) mice under treatment with KKA at 25mg/kg; (C) mice under treatment with KKA at 50 mg/kg; (D) mice under treatment with KKA at 100 mg/kg; (E) mice under treatment with capecitabine at10 mg/kg (positive control); (F) tumor morphology of the representative groups of animals (from left to right: (1) negative control; (2) 25 mg/kg of KKA; (3) 50 mg/kg of KKA; (4) 100 mg/kg of KKA; (5) 10 mg/kg of capecitabine. KKA, Potassium koetjapate.

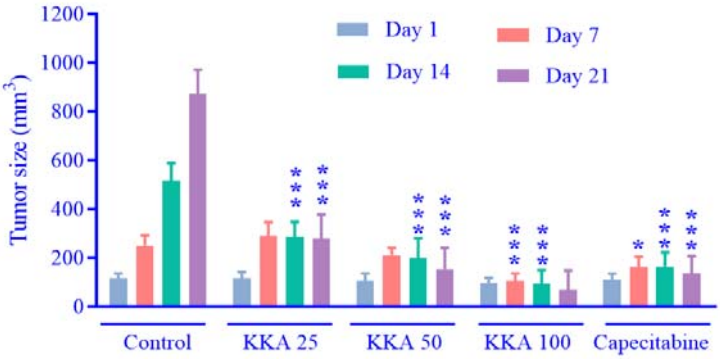

Fig. 8. Antitumor efficacy of potassium koetjapate in ectopic xenograft colon cancer model. Values are expressed as mean ± SD (n = 5 - 6 per group). *P < 0.05 and ***P < 0.001 indicate significant differences in comparison with the control group.

The groups treated with KKA at 25, 50, and 100 mg/kg and also 10 mg/kg of capecitabine demonstrated significant growth inhibitory effect after the 1st week. week of oral treatment. Furthermore, at the dose of 100 mg/kg, KKA shows significantly, better activity than the standard reference, capecitabine, whereas no difference was observed at the dose of 50 of KKA compared to the capecitabine-treated group during the last week of *in vivo* experiment. Tumor growth inhibition values proved that KKA can lead to potent inhibition of tumor growth as 68.15%, 82.35%, and 92.76% at 25, 50, and 100 mg/kg, respectively comparable to the tumor growth inhibition induced by capecitabine which was 84.37%.

Analysis of tumor size indicates a significant restraint of tumor growth in treated animals in comparison to the untreated mice (Fig. 8).

Moreover, by comparing the weight of animals in the treated groups on day 1 with that of day 21, the mean percentage of weight gain or loss at the end of the experiment was measured which shows no statistical differences between the study groups (Fig. 9). Animals treated with doses of 25, 50, and 100 mg/kg of KKA, 10 mg/kg of capecitabine, and distilled water showed 9.6, 10.1, 13.8, 15.89, and 12.5% reduction in body weight at the end of experiment.

In addition, Fig. 10 illustrates photomicrographic tumor slides stained with H&E. In addition, the tumor cross-sections were analyzed for the extent of the apoptotic/necrotic areas. Oral administration of different doses of KKA and capecitabine (10 mg/kg) for 21 days resulted in a decline in viable tumor cells with a consequent increase in the percentage of death colon cancer cells whereas tumor sections of the negative control-treated group displayed closely packed living tumor cells along with minor necrotic area.

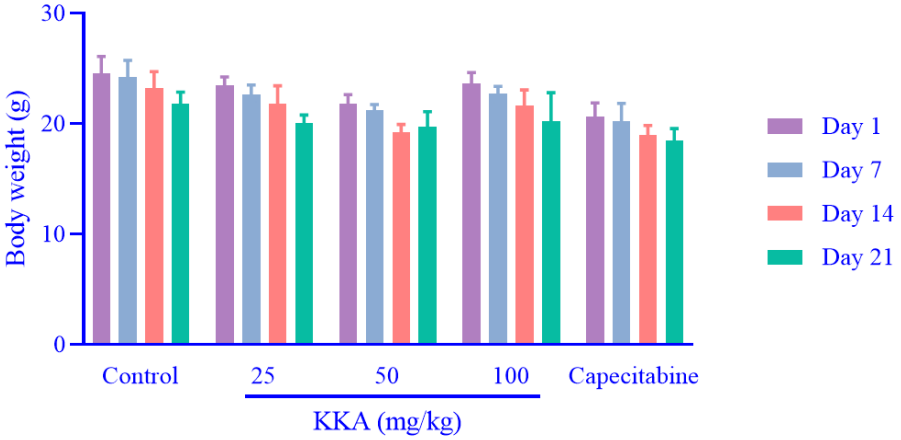

Fig. 9. Changes in body weight revealed in different groups under treatment. Values are expressed as mean ± SD, n =6. Reductions in body weight by less than 20% demonstrate the safe nature of treatments.

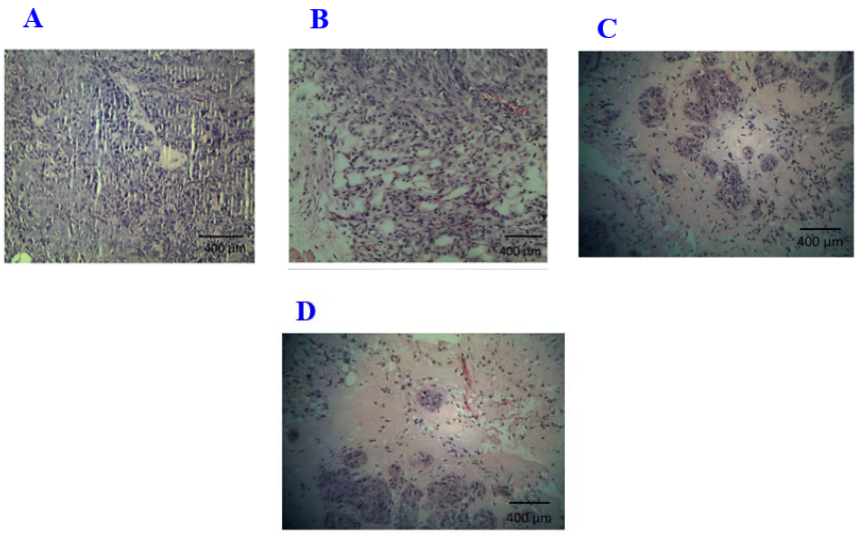

Fig. 10. Hematoxylin and eosin-stained cross sections from tumor tissues of (A) the negative control treated with distilled water; (B) the positive control group under treatment with 10 mg/kg of capecitabine; (C) the group treated with KKA at 50 mg/kg; (D) the group treated with KKA at 100 mg/kg. Necrotic/apoptotic areas are less in the negative control group than in the other groups. The pictures were taken at a magnification of 10×.

DISCUSSION

Colorectal carcinoma is considered the 3rd most prominent reason for cancer death worldwide whereas the chemo drug 5-FU applicability is limited because of its non-specificity, minimal bioavailability as well as toxicity (18). Recently, the secondary metabolites in natural products such as triterpenoids represent a promising role in the development of anticancer agents (19,20). For example, oleanolic acid is found to inhibit cancer growth in a mouse model and has similar radioprotective properties in hematopoietic tissues. Derivatives of oleanolic acid for example 2-cyano-3,12-dioxooleana-1,9(11) dien-28-oic acid, showed a range of significant effects including inhibition of proliferation in several human cancers (20). In our previous study, MTT cytotoxicity assay was used to obtain and compare the IC_{50} values of both compounds KA and KKA against different colorectal cancer and normal epithelial cell lines (9). Among the tested compounds, KKA demonstrated a strong cytotoxic effect against human colorectal carcinoma, HCT 116, and HT 29 cells, with IC50s of 7 and 12 µm, respectively. However, KKA displayed poor cytotoxicity against the normal fibroblast (NIH-3T3) cell lines (9). The selectivity index, which suggests the cytotoxic selectivity of the compound versus cancer cells and its safety in the non-cancerous cells was defined from the ratio of the IC50 acquired from the test on normal cells (NIH-3T3) compared to the IC50 for cancer cells (HCT 116). Interestingly, the selectivity index of KKA for HCT 116 cells was 11 which was more than two times greater than that of the native compound (KA). The cytotoxic effect of KKA was examined on the HCT 116 cells with a sequence of in-vitro experiments (9).

In the current research, the possible molecular procedures that are contributing to the proapoptotic activity of KKA were investigated *via* a human apoptosis protein profiler array. The findings demonstrated that KKA significantly down-regulated the expression of Bcl-2, Bcl-w, HSP60 as well as HSP70 proteins along with up-regulation of IGF-1, TRAILR-1, TRAILR-2, p27, CD40, Fas, Fas ligand (FasL), IGFBP-2, IGFBP-6, caspase 8, and caspase 3 proteins (Fig. 5). Deficiencies in apoptotic routes can boost cancer cell survival and give resistance to anti-neoplastic medicines. One pathway being concerned for cancer therapy is the anti-apoptotic Bcl-2 family proteins such as Bcl-2, Bcl-w as well as Bcl-XL which block the BH3-domain of pro-apoptotic proteins. The activities responsible for mitochondrial pathways of apoptosis are strongly controlled by the members of the Bcl-2 family proteins (21). Overexpression of anti-apoptotic Bcl-2 family members has been linked with chemotherapy resistance in multiple human cancers including colon cancer, leukemia, and glioblastoma. Therefore, inhibiting the anti-apoptotic Bcl-2 family proteins can improve apoptosis hence preventing drug resistance to chemotherapy (22). In the present research, the apoptosis antibody array test has demonstrated that KKA significantly down-regulated the expression of Bcl-w as well as Bcl-2 proteins.

Numerous HSPs have been proven to prevent the release of cytochrome c along with the activation of caspase-9 in colorectal cancer (CRC). Interestingly, it was found that down-regulation of HSP60 provoked by IGFBP7 may trigger IGFBP7's tumor-suppressive natural behavior in CRC (23). It was also reported that HSP70 plays a significant role in the suppression of the apoptosis pathway (24,25). The findings of the current study highlighted that KKA significantly up-regulated the expression of IGFBP-2 and IGFBP-6 while down-regulating the expression of IGF-1, HSP60 as well as HSP70 proteins.

It was also reported that Bcl-2 overexpression increased the survival of colon cancer cells by safeguarding the cells from a dose-dependent stimulation of apoptosis through TRAIL followed by preventing cleavage of Bid caspase-8 in addition to caspase-3. Moreover, it was revealed that TRAIL enhances apoptosis in colonic cancer cell lines through changing Bax relocation and cytochrome c release (26). TRAIL is a class II membrane-bound tumor necrosis factor (TNF) family ligand that is extremely homologous to cytotoxic FasL. It was confirmed that Fas (CD95) is a member of the TNF family which

is comprehensively expressed on various cell varieties including human malignant tumor cells. It was also found that FasL interaction triggers apoptosis in Fas-positive targets. It is also reported that using the compounds that block Fas or FasL inhibits apoptosis in colon carcinoma cells (27).

The IGF system contains ligands, receptors, and IGFBPs. Several studies have indicated that IGF-1 links to tumor development in adults. For example, a 6-year study reported that the maximum amounts of IGF-1 cause more than two times the risk of colorectal carcinoma (28). IGFBP-6 is a relatively selective inhibitor of IGF-2 behaviors hence preventing proliferation as well as survival of various cells. It is also believed that it has several IGF-independent acts such as elevation of apoptosis in a variety of cells and preventing angiogenesis (29,30). The expression of IGFBP6 suggested as a tumor suppressor, is diminished in large quantities of cancer cells (29,30). Interestingly, the results of the present study show that KKA significantly up-regulated the expression of IGFBP-2 and IGFBP-6 proteins while down-regulated the expression of IGF-1 likely in a consequent result. Significant stimulation of cyclin-dependent kinase inhibitors for example p27and p21 is associated with the stimulation of apoptosis and hindering of antiapoptotic members of the Bcl-2 family. The cell cycle regulator protein p27 has been connected with the induction of apoptosis in some cancer cells such as colon cancer (31). Apoptosis antibody array study has shown that whereas KKA significantly up-regulates p27, it downregulates the expression of Bclw and Bcl-2 antiapoptotic proteins.

These findings suggest that KKA-induced apoptotic mechanisms involve both mitochondrial and death receptor pathways of apoptosis. Therefore, the possible mechanisms that make KKA a potential apoptosis inducer are regulating the expression of various proteins engaged in different apoptosis routes which may link to each other and finally activate caspases-3/7, resulting in the demolition of HCT 116 cells. However, the results have shown noticeably that KKA triggers the death receptor pathway along with mitochondrial-mediated apoptosis in colon cancer cells. Additional studies are still essential to verify the exact processes of cell death induction by KKA.

Moreover, in the present study, carcinogenic pathways that may have contributed to the observed apoptotic cell death and antimetastatic properties of KKA in HCT 116 cells were investigated using a cell-based dual luciferase reporter array system (Fig. 6). The results of this study indicated that KKA (7 μM/mL) can significantly down-regulate Notch, hypoxia, Wnt, MAPK/ERK as well as MAPK/JNK signalling pathways in HCT 116 cells. In addition, significant up-regulation of transcription factors for the cell cycle (pRb-E2F) pathway was observed. The findings of this study are mostly comparable with the earlier report for KKA except that in the previous study, no significant modifications were found in the transcriptional regulation of Notch and pRb-E2F in addition to p53 and TGFβ signalling pathways (8). Notch signalling plays a critical role in the pathogenesis in addition to the development of human malignancies. The findings show that Notch and Wnt signals jointly modulate cell proliferation as well as tumorigenesis in the CRC (32). It was reported that Notch prevents apoptosis through suppression of p27 and Atonal BHLH transcription factor 1 suggesting that the inhibition of Notch signalling can be an imperative mechanism to improve CRC chemotherapy (33). In the present study, KKA significantly blocked the activity of Notch signalling pathways. The Wnt signalling pathway was abnormally up-regulated in CRC. Several mutations in multiple oncogenes and tumor suppressor genes such as Wnt pathway components (*e.g.* APC and AXIN2) are detected. Epigenetic suppressing of Wnt inhibitors through DNA hypermethylation has also been proposed as another common mechanism to trigger the Wnt pathway (34). The increased frequency of mutations in Wnt pathway agents detected in CRC illustrates the capability of Wnt signalling as a therapeutic aim (35). The present study found that KKA reduces the expression level of TCF/LEF (Wnt) suggesting the down-regulation of the Wnt pathway. Other studies also revealed down-regulation of Wnt signalling, reduction of cell

proliferation rates as well as induction of apoptosis (8).

Hypoxia-inducible factor 1 (HIF-1) triggers the transcription of genes that are involved in crucial aspects of cancer biology such as invasion and cell survival. HIF-1α overexpression is linked to raised patient mortality in some types of cancer including colon cancer. HIF-1 activity results in the upregulation of genes that are involved in apoptosis resistance, metastasis, metabolic adaptation, and angiogenesis. The findings show a connection amongst HIF-1α expression, angiogenesis, and tumor growth in colon cancerous cells. HIF-1α protein synthesis is modulated by stimulation of ERK/MAPK pathways. The findings of the present study indicated the capability of KKA to prevent the hypoxia pathway in the colon cancerous cells and make KKA a good anticancer agent candidate.

Furthermore, JNK and ERK pathways as the active members of the MAPK family were also examined in the current study. The findings revealed that KKA can cause a significant downregulation of MAPK-correlated genes. The findings also have shown that down-regulation of the expression of MAPK gene products results in the down-regulation of the HIF-1α signalling pathway and induction of apoptosis. Moreover, it was found that prevention of MAPK/ERK pathways in pancreatic cancer leads to the activation of forkhead box transcription factors which can result in apoptosis through caspase-3 activation (36). According to the LD_{50} of KKA, which was expected to be more than 2000 mg/kg of body weight, three doses of 25, 50, and 100 mg/kg of KKA were chosen for *in vivo* antitumor studies. Even though 5-FU is still the most commonly used medicine for the treatment of colorectal carcinoma, about 33% of patients suffer from adverse effects. Thus, to improve the cytotoxicity as well as therapeutic efficiency of this drug, recent research has concentrated on the bi-modification of 5-FU. Capecitabine or Xeloda® is a cytotoxic agent derived from 5-FU which has been designed to avoid the unsatisfactory toxicity of 5-FU (37,38). In the present study, the data obtained from an *in vivo* antitumor study against human colon cancer demonstrated a significant decrease in the growth of subcutaneous tumors in KKA- and capecitabine-treated animals. The effective tumor growth inhibition induced by KKA starts at the beginning of the 2nd week of treatment. Tumor growth inhibition values which were calculated at the end of the experiment have shown that KKA has promising efficacy in hindering the growth of colon cancer so that KKA at 100 mg/kg displayed strong *in vivo* growth inhibitory effects comparable with positive control (capecitabine). Significant dissimilarities were observed in tumor cross-sections obtained from KKA-treated animals in comparison with the negative control group. Tumor sections in the negative control group were composed of sheets of living tumor cells with eosinophilic cytoplasm whereas loose clusters of tumor healthy cells with variation in nuclear size and abundant cytoplasm were observed all over the tumor sections obtained from KKA-treated animals. KKA probably causes nucleus shrinkage in the HCT tumor cells followed by fragmentation of the nucleus (karyorrhexis) which is highlighted by widespread apoptotic/necrotic areas in tumor sections with abundant fragmented nuclei of dying cells. These changes are probably due to the secondary form of necrosis which is found to occur after apoptosis (39). Therefore, cell death induced by KKA finally results in tumor shrinkage as observed in the treated animals. In sum, these findings suggest that KKA can be a valuable compound to treat colon cancer.

CONCLUSION

From the outcome of the present research, it can be concluded that KKA could be a promising and effective candidate as a chemotherapeutic agent against colorectal cancer.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

S.F. Jafari wrote the manuscript. A.M. Abdul Majid, M. Keshavarzi, M.B. Khadeer Ahamed, A.S. Abdul Majid, and M. Naseri supervised and funded the study and revised the manuscript. S.F. Jafari, F.S. Al-Suede, M. Asif, Md Sultan Khan, and L.A.E. Hassan carried out the experiments and analyzed the data. The finalized article was read and approved by all authors.

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