Khat promotes human breast cancer MDA-MB-231 cell apoptosis via mitochondria and MAPK-associated pathways

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Abstract. Khat (*Catha edulis* Forsk) is a flowering evergreen plant in Eastern Africa and Southwestern Arabia. Consumption of Khat has been associated with the development of oral cancer, but its mechanism of action on the molecular level remains unclear. The present study demonstrated the cytotoxic effect of khat extracts on the human breast cancer cell line MDA-MB-231. Trypan blue exclusion assays, flow cytometry, fluorescent and electron microscopy, as well as western blotting were used to analyze the effects of Khat on the cell viability of breast cancer cells, expression of apoptotic-associated proteins and the levels of reactive oxygen species (ROS). The results of the present study demonstrated that treatment with 400 μg/ml khat was able to induce cell death in breast cancers, with an increase in the protein expression of apoptosis regulator Bax and a decrease in the expression of B-cell lymphoma 2, along with a decrease in ROS levels in a time-dependent manner. Furthermore, the expression of activated c-Jun N-terminal and extracellular regulated protein kinases was increased in khat-treated cells compared with untreated cells. Mitochondria participated in cell apoptosis through the release of apoptogenic proteins to the cytosol and the generation of excess reactive oxygen species. The results of the present study suggest that khat induces MDA-MB-231 cell apoptosis via MAPK activation and mitochondrial-mediated death.

Introduction

Khat is an herb that is used by millions of people worldwide for its psychostimulatory effects, primarily in Africa and the Middle East. The alkaloid in khat is the major ingredient that can be efficiently extracted and absorbed orally (1). Khat contains numerous pharmacologically active compounds (2). Cathinone is a major alkaloid compound in fresh khat leaves, which is relatively unstable and rapidly metabolized into cathine (norpseudoephedrine) and norephedrine (1). Furthermore, other alkaloids, including phenylpentenylamines and cathedulines may exert pharmacological effects (3,4). The majority of their pharmacological effects have been suggested to be mediated by releasing biogenic amines through preferential binding with the norepinephrine receptor, partially through binding with dopamine and 5-hydroxytryptamine receptors (5).

Khat chewing has been reported to impact the sexual behavior of males and females. Studies have shown that khat possesses aphrodisiac activities (6-8) and may be used to cure premature ejaculation (9). In addition, khat has been reported to be a psychostimulant (10). Medicinal uses of Khat for the treatment of depression, hunger, fatigue, obesity and gastric ulcers have also been previously reported (11). Khat can affect the cardiovascular, digestive, endocrine, hepatobiliary, respiratory and genitourinary systems (12). Previous studies have indicated that khat is associated with oral cancer development (13) and hepatic cell apoptosis (14). To the best of our knowledge, no studies have investigated the effects of khat consumption on breast cancer.

Programmed cell death may occur via apoptosis, necrosis or excessive autophagy, of which, mitochondria serve an essential role in its regulation (15,16). The B-cell lymphoma 2 (Bcl-2) family is involved in mitochondria-mediated cell death by affecting the stability of the outer mitochondrial membrane (17). Anti-apoptotic Bcl-2 and Bax have been demonstrated to be associated with spontaneous apoptosis in acute myeloid leukemia cells *in vitro* (18). A previous study has suggested the use of the Bax to Bcl-2 ratio in patient cells to predict clinical response and outcome (19). Mitochondria participate in cell death mechanisms through the release of apoptogenic proteins into the cytosol and generation of excess reactive oxidative species (ROS). The mitochondrial respiratory chain is a major source of cellular ROS and therefore, represents a target for the effects of ROS production (20).

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Khat-induced hepatocyte apoptosis is primarily regulated through the sustained activation of the c-Jun NH2-terminal kinase (JNK) signaling pathway and partially through the extracellular signal-regulated kinase (ERK) signaling cascade (14). JNK and ERK are part of the mitogen-activated protein kinase (MAPK) signaling pathways. The aim of the present study was to investigate the effects of khat on the viability and apoptosis of breast cancer cell MDA-MB-231.

Materials and methods

Khat extraction. Khat plants were purchased from a local Yemeni market, Sana'a, Yemen. Fresh khat leaves with soft stems were collected in the summer, weighed, washed three times with distilled water and dried for three days in a clean, and dry room away from sunlight. Subsequently, the leaves were weighed, packed in a tight foil packet and stored at 4˚C. Dried khat leaves (100 g) were chopped into sections (5 mm) and dissolved in 100 ml 95% ethanol, centrifuged at 5,031 x g for 5 min at room temperature. The supernatant was filtered using filter paper. Ethanol (100 ml) was added to the remaining leaves, and the procedure was repeated. The extracted ethanol khat suspension was concentrated using a rotary evaporator (LabTech, Inc., Hopkinton, MA, USA) at 30˚C at a speed of 0.44 x g until 70% of the ethanol solvent had evaporated. The viscous solution was diluted with 100 ml distilled water and stirred at 201 x g for 60 min at room temperature. The filtered liquid was stored at -70˚C for 24 h and subsequently dried using a lyophilization apparatus (Lyophilization Technology, Inc., Warminster, PA, USA). Generally, 100 g dried leaves yielded 8 g khat extract powder. The major alkaloids in khat leaves are cathine, cathinone and norephedrine. The mean concentrations of cathinone, cathine and norephedrine in fresh khat leaves are 0.95, 1.98, and 0.54 mg/g, respectively (21-23). The lyophilized khat extracts were dissolved in Hank's balanced salt solution (HyClone; GE Healthcare Life Sciences, Logan, UT, USA without Ca^{2+} or Mg^{2+} to achieve a final concentration of 200 μg/ml and sterilized using a filter with a pore size of 0.2 μm.

Cell culture and treatment. MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (HyClone; GE Healthcare Life Sciences) with 10% fetal bovine serum (Zhejiang Tianhang Biotechnology Co., Ltd., Hangzhou, Zhejiang, China) and maintained in a humidified incubator at 37˚C with 5% CO_2. When 60-70% confluence was achieved, the cells were treated with 400 μg/ml khat and left to culture for 4, 8, 16, or 24 h at 37˚C. The cells in the control group were not treated with khat.

Analysis of cell viability. The cells were seeded into 6-well plates at 60-70% confluence, overnight at 37˚C. The cells were subsequently exposed to various concentrations of Khat (20, 200, 300 and 400 μg/ml) for 4, 8, 16 and 24 h. Cells were digested using 0.25% pancreatic enzyme liquid (without EDTA) and the resultant suspension was collected and centrifuged at 168 x g for 5 min at room temperature. Then the cells were stained with 0.2% trypan blue or 5 min at room temperature prior to evaluation under an inverted light microscope. The cells with blue-labeled nuclei were considered dead.

Annexin V/propidium iodide (PI) staining assay. Apoptosis was assessed using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Jiangsu KEYGEN BIOTECH Co., Ltd., Nanjing, Jiangsu, China) according to the manufacturer's protocol. Following drug treatment, cells in the 6-well plates were digested using 0.25% pancreatic enzyme liquid (without EDTA), suspension was collected and centrifuged at 168 x g for 5 min at room temperature, then washed with phosphate-buffered saline (PBS) twice and resuspended in 500 μl of staining buffer with 5 μl FITC-conjugated Annexin V and 5 μl PI staining solution. The cells were incubated on ice for 30 min and analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) within 1 h. ModFit V3.2 software (BD Biosciences) was used to analyze the cell apoptosis rate. Rates of basal cell apoptosis and necrosis were determined in the untreated control group. The rates of cell apoptosis were determined following ≥3 independent experiments and each experiment was performed in triplicate.

Staining of apoptotic cells with Hoechst 33258. The cells were treated with khat and fixed in 4% formaldehyde containing Hoechst 33258 (10 μg/ml). Following incubation with 400 μg/ml khat for different times (0, 4, 8, 16 and 24 h) at 37˚C, nuclear morphology was examined at x200 magnification with an incident light fluorescent microscope to assess apoptotic features. The apoptotic cells were determined as the fraction of intensely stained, condensed and fragmented nuclei.

Transmission electron microscopy. Cells underwent a 16-h incubation with 400 μg/ml khat and were observed by transmission electron microscopy. The cells were fixed in 0.1 M sodium cacodylate buffer (pH 7.4) with 2% glutaraldehyde. The cells were rinsed with sodium cacodylate buffer and post-fixed in 1% osmium tetroxide. The cells were dehydrated with graded ethanol (50, 70 and 90% for 10 min each) and embedded in epoxy resin. The sections were double-stained with 3% uranyl acetate and lead citrate. The cells were examined under a JEOL 1230 transmission electron microscope (JEOL, Ltd., Tokyo, Japan), and micrographs were produced using an Agfa Arcus II scanner (Agfa Corporation, Shanghai, China) and Adobe Photoshop software (version 7.0.1; Adobe Systems Europe, Ltd., Maidenhead, UK).

Western blot analysis. The cells were harvested and lysed on ice for 30 min in radioimmunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM NaF, 1 mM Na3VO4, 5 mM sodium pyrophosphate and a protease inhibitor tablet [Jiangsu Keygen Biotech Co., Ltd.]). The cell lysates were centrifuged at 39,514 x g for 10 min at 4˚C, and the supernatants were used for western blotting. The total protein concentration was determined using the BCA Protein assay reagent (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The lysates were denatured at 100˚C following the addition of SDS loading buffer. The proteins were separated on 4-20% SDS-PAGE gels and subsequently transferred to nitrocellulose blotting membranes. Following blocking of the membranes with 5% non-fat dry milk in 1X TBST for >1 h at room temperature, the membranes were incubated with 1:1,000 diluted primary

antibodies [p-JNK (cat. no. sc-135642), JNK (cat. no. sc-1648), p-ERK (cat. no. sc-81492), ERK (cat. no. sc-271270), Bcl-2 (cat. no. sc-7382), Bax (cat. no. sc-7480) and Caspase-9 (cat. no. sc-73548); all Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA] diluted in 5% non-fat dry milk in 1X TBST for >12 h at 4˚C. Membrane-bound primary antibodies were detected with 1:3,000 diluted secondary antibodies (affinity purified goat anti-rabbit IgG (cat. no. GB23303), goat anti-mouse IgG (cat. no. GB23301), Servicebio Biotechnology, Ltd., Wuhan, Huber, China) conjugated to horseradish peroxidase for 1 h at room temperature. The blots were visualized using the ECL chemiluminescence reagent kit (Servicebio Biotechnology, Ltd., Wuhan, Hubei, China), and analyzed using the ChemiDoc XRS gel imaging device (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Measurement of ROS production. The production of ROS was measured using flow cytometry with dichlorofluorescein-diacetate (DCFH-DA) as previously described (24). The cells were treated with khat (400 μg/ml) for 4, 8, 16 and 24 h, dissociated with trypsin-EDTA, washed twice with cold PBS, and suspended in PBS ($1x10^6$ cells/ml). The cell suspension (500 μl) was transferred into a tube and incubated with DCFH-DA at a final concentration of 5 μM for 30 min at 37˚C. ROS production was assessed by determining the DCF fluorescence intensity from $1x10^4$ cells by flow cytometry.

Statistical analysis. Data are presented as the mean ± standard deviation and analyzed using the SPSS software (version 17.0; SPSS Inc., Chicago, IL, USA). One-way analysis of variance (followed by Student-Newman-Keuls post-hoc test) was used to analyze the significance of differences among groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of Khat treatment on viability of MDA-MB-231 cells. The inhibitory effect of Khat was assessed by detecting the viability of MDA-MB-231 cells. As shown in Fig. 1, following Khat treatment at concentrations of 20, 200, 300 and 400 μg/ml, the viability of MDA-MB-231 cells was inhibited in a dose- and time-dependent manner, with the greatest effect seen at the concentration of 400 μg/ml following a treatment duration of 8 h. Therefore, a concentration of 400 μg/ml Khat was selected for use in subsequent experiments.

Effect of khat treatment on apoptosis of MDA-MB-231 cells. To investigate the effect of 400 μg/ml khat on apoptosis of MDA-MB-231 cells, the cells were double-stained with Annexin V and PI, prior to detection using flow cytometry. Following serum-free culture for 24 h, MDA-MB-231 cells were exposed to 400 μg/ml khat for 4, 8, 16 and 24 h prior to the apoptosis assay. As shown in Fig. 2, the apoptotic rates of the control, and cells exposed to khat for 4, 8, 16 and 24 h were 7.76±2.86, 9.7±1.59, 10.58±2.03, 13.2±4.14, and 25.3±5.09%, respectively. At each of the time point indicated, the treated cells were stained with Hoechst 33258. The nuclei of the cells in the control group were regular and round-shaped (Fig. 3A-C). By contrast, the nuclei of the majority of khat-treated (400 μg/ml; 16 h) cells were condensed and fragmented, which

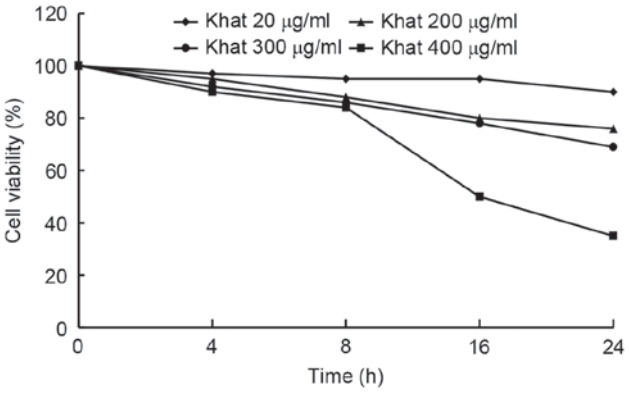

Figure 1. Effect of Khat treatment on the viability of MDA-MB-231 cells. MDA-MB-231 cells were treated with various concentrations of khat for a range of times (0, 4, 8, 16 and 24 h), Cell viability was measured using a trypan blue exclusion assay.

are characteristic of apoptotic cells (Fig. 3D and E). When observed under the electron microscope, the khat-treated cells exhibited chromatin condensation and nuclei shrinkage, which further confirms apoptosis (Fig. 3F).

Effect of Khat treatment on the expression of apoptosis-associated proteins and the activation of MAPKs. The expression of caspase-9 was determined in MDA-MB-231 cells prior to and following khat treatment using western blot analysis. The expression of caspase-9 was not detected following Khat treatment (Fig. 4). Following incubation with 400 μg/ml khat for different times (0, 4, 8, 16 and 24 h), the expression of pro-apoptotic protein Bax markedly increased compared with untreated cells (i.e., cells at 0 h). Conversely, the expression of anti-apoptotic protein Bcl-2 markedly decreased compared with expression levels at 0 h.

The involvement of MAPKs in khat-induced apoptosis of MDA-MB-231 cells was subsequently investigated by measuring the expression of the activated form of JNK and ERK. As shown in Fig. 4, the size of the bands for p-JNK seem to be less intense at 16 and 24 h than those at 0, 4 and 8 h. The band for p-ERK at 4 h is higher-intesity than that at 0 h. Similarly, the band at 24 h is higher intensity than that at 16 h, while the total JNK and ERK expression were not changed. Therefore, the expression of p-JNK and p-ERK were increased when cells were treated with khat compared with untreated cells (i.e., those at 0 h). It is likely that activation of ERK and JNK signal in MDA-MB-231 cells may mediate the cellular apoptotic response to khat treatment. To confirm the possible roles of JNK and ERK in khat-induced apoptosis, cell viability and apoptotic rates of MDA-MB-231 cells were determined in response to inhibitors of JNK (SP600125) and ERK (PD98059). As shown in Fig. 5, treatment with SP600125 and PD98059 significantly reversed khat-induced cell death compared with untreated cells. These results indicated that JNK and ERK are involved in khat-induced apoptosis in MDA-MB-231 cells.

Effect of khat treatment on ROS production. ROS has been considered as a potential regulator of apoptosis (25). Therefore, in the present study, the level of ROS was determined in

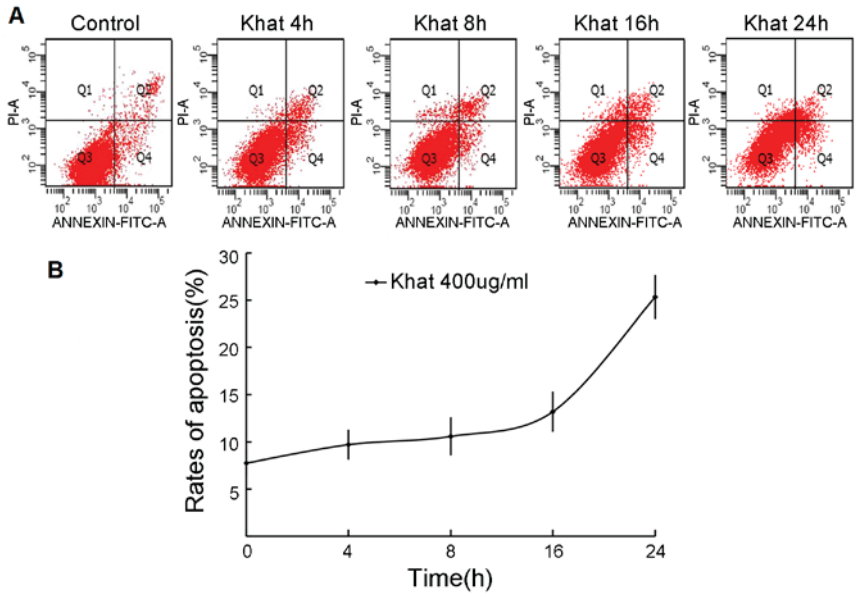

Figure 2. Analysis of apoptosis using Annexin V and PI staining. (A) MDA-MB-231 cells were double stained with Annexin V and PI, and analyzed using flow cytometry. Four populations were indicated as non-apoptosis dead cells (Q1), late apoptosis cells (Q2), viable cells (Q3) and early apoptosis cells (Q4). (B) The rate of apoptosis of cells treated with 400 μg/ml khat between 0 and 24 h. PI, propidium iodide.

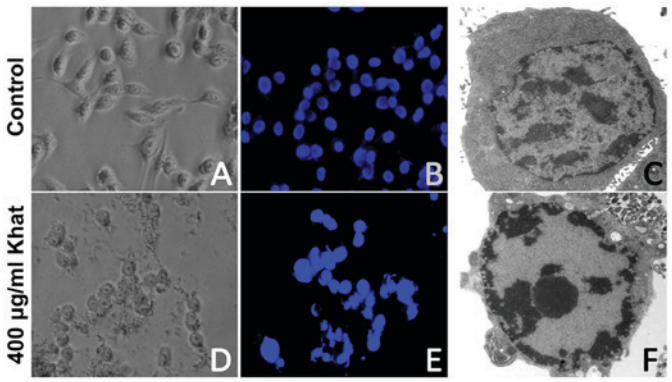

Figure 3. Morphological and ultrastructural features of untreated and khat-treated MDA-MB-231 cells for 16 h. The untreated control cells were observed under (A) an inverted contrast light microscope (magnification, x20), (B) a fluorescence microscope following staining with Hoechst 33258 (a DNA fluorochrome) (magnification, x20) and (C) an electron microscope (magnification, x200). Khat-treated cells were observed under (D) an inverted contrast light microscope (magnification, x20), (E) a fluorescence microscope (magnification, x20) and (F) an electron microscope (magnification, x200).

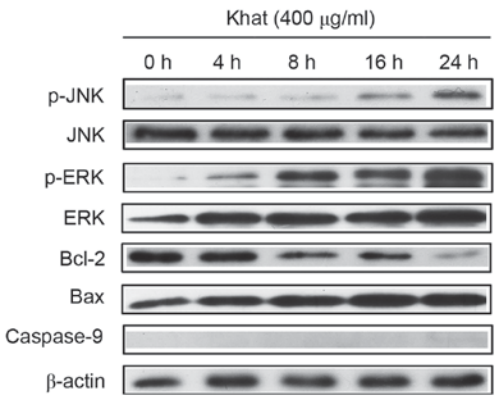

Figure 4. Expression of apoptosis-associated proteins and MAPKs (p-JNK, JNK, p-ERK, ERK). MDA-MB-231 cells were incubated with 400 μg/ml khat for 4, 8, 16 and 24 h; apoptosis- and MAPK-associated protein expression was detected by western blotting. MAPK, mitogen-activated protein kinase; p, phosphorylated; ERK, extracellular signal-regulated kinase; JNK, janus kinase; Bax, apoptosis regulator Bax; Bcl-2, apoptosis regulator Bcl-2.

khat-treated cells. MDA-MB-231 cells were treated with 400 μg/ml khat for 4, 8, 16 and 24 h and subjected to flow cytometry for detection of ROS. As shown in Fig. 6, ROS production in cells treated with 400 μg/ml khat for 4 h was higher than control cells (0 h cells). Then the ROS production decreased in 8-24 h groups. khat induced a time-dependent decrease in ROS production.

Discussion

In the present study, the cell viability assay indicated that khat treatment inhibited the viability of MDA-MB-231 cells in a time- and dose-dependent manner. Flow cytometric analysis demonstrated that 400 μg/ml khat induced apoptosis in MDA-MB-231 cells in a time-dependent manner, which was consistent with the cell viability assay.

Apoptosis is a major mechanism of cancer suppression and is characterized by morphological and ultrastructural

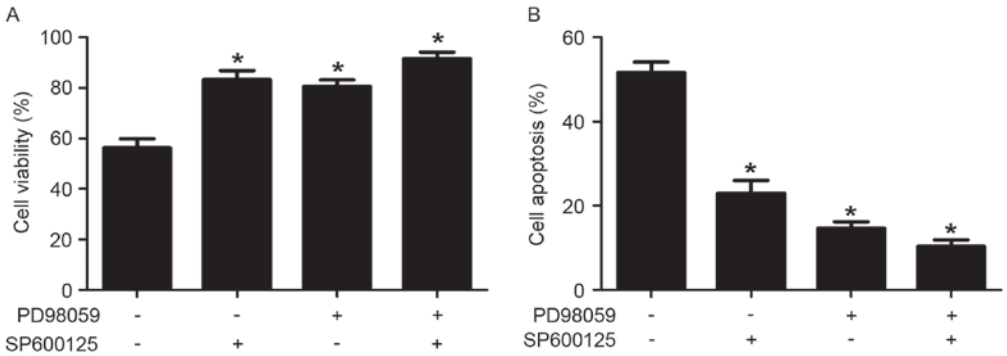

Figure 5. Inhibitors of extracellular signal-regulated kinase (PD98059) and c-Jun NH2-terminal kinase (SP600125) significantly decreased khat-induced apoptosis in MDA-MB-231 cells. (A) Cell viability was detected using a trypan blue exclusion assay (B) Cell apoptosis was analyzed using flow cytometry. *P<0.01 compared with cells not treated with PD98059 and SP600125.

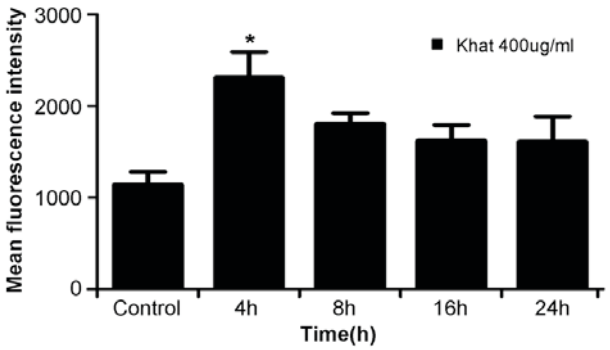

Figure 6. Effect of khat treatment on ROS production. Flow cytometry was used to assess ROS production in khat-treated (400 μg/ml) MDA-MB-231 cells. ROS production in cells treated with 400 μg/ml khat for 4 h was higher than control cells and then decreased in 8-24 h groups. *P<0.01 compared to control cells. ROS, reactive oxygen species.

changes associated with caspase-regulated biochemical processes (26,27). Khat contains a complex phytochemisty, with fractions, including flavonoids that are able to induce apoptosis and act against cancer cells (28). In the present study, Hoechst 33258 staining and transmission electron microscopy results indicated that khat induced apoptosis in MDA-MB-231 cells, and caused morphological changes, including microvilli loss, cell membrane bubbling, nuclear chromatin condensation, cytosolic compartment vacuolization and apoptotic body formation.

The MAPK signaling pathway includes the ERK, JNK and p38 pathways, which are involved in cell survival, proliferation and apoptosis. A previous study by the present authors demonstrated no significant difference in p38 expression in hepatic cells treated with khat and untreated cells (29). Therefore in the present study, the expression levels of ERK and JNK were examined in khat-treated breast cancer cells. The activation of ERK has been previously demonstrated to be involved in cell cycle progression and proliferation (30), whereas JNK is generally activated in response to stress and toxicants that induce cell apoptosis (31). Prior reports demonstrated that sustained JNK activation results in apoptosis (32,33). In the present study, western blotting demonstrated that the levels of p-JNK and p-ERK were increased following treatment with khat in a time-dependent manner, whilst treatment with JNK and ERK inhibitors significantly reversed the khat-induced cell death. These results suggest that khat-induced breast cancer cell apoptosis is primarily mediated by the activation of the JNK signaling pathway.

The Bcl-2 protein is known to be a suppressor of apoptosis (34), and Bax is a promoter of apoptosis (35). The results of the present study revealed that Bcl-2 expression was decreased and Bax was increased in a time-dependent manner in khat-treated MDA-MB-231 cells, with an increase in Bax/Bcl-2 ratio. A previous study reported that the Bax/Bcl-2 ratio may be a more important apoptosis indicator than either promoter alone (36). The mitochondrial-mediated apoptosis signaling pathway is regulated by the Bcl-2 family of anti-apoptotic proteins (Bcl-2, Bcl-xl and myeloid cell leukemia 1) and pro-apoptotic proteins (Bax, Bcl2-associated agonist of cell death and Bcl-2 homologous antagonist/killer) (17-20). Therefore the results in the present study suggest that khat induces apoptosis in MDA-MB-231 cells via the mitochondrial-mediated apoptosis pathway. Additionally, no caspase-9 protein expression was detected in MDA-MB-231 cells, suggesting khat-induced apoptosis is not modulated by the classical apoptosis pathway.

In humans, oxidative stress has been identified to be involved in various pathologies, including cancer, arteriosclerosis, type II diabetes, ischemia/reperfusion injury, chronic inflammatory processes and various neurodegenerative diseases (37). Excess ROS can induce the oxidation of macromolecules and has been identified to be involved in ageing, mtDNA mutations and cell death (38). The generation of ROS by mitochondria serves an essential role in the release of cytochrome c and other pro-apoptotic proteins, which can trigger caspase activation and apoptosis (20). In the present study, ROS levels were higher in khat-treated breast cancer cells compared with that of the control cells. This result further suggests that khat-induced apoptosis is modulated by the mitochondrial-mediated apoptosis pathway.

In conclusion, the results of the present study suggest that khat causes apoptosis in MDA-MB-231 cells via sustainable activation of JNK and the mitochondrial-mediated apoptosis pathway. To the best of our knowledge, this is the first study to demonstrate that khat may suppress the breast cancer by inducing the apoptosis of breast cancer cells.

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