

Khat (*Catha edulis*) generates reactive oxygen species and promotes hepatic cell apoptosis via MAPK activation

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Abstract. A number of studies have suggested an association between khat (*Catha edulis*) chewing and acute liver lesions or chronic liver disease. However, little is known about the effects of khat on hepatic cells. In the current study, we investigated the mechanism behind khat-induced apoptosis in the L02 human hepatic cell line. We used cell growth inhibition assay, flow cytometry and Hoechst 33258 staining to measure hepatocyte apoptosis induced by khat. Western blot analysis was used to detect the expression levels of caspase-8 and -9, as well as those of Bax and Bcl-2. We also measured reactive oxygen species production. The results indicated that khat induced significant hepatocyte apoptosis in L02 cells. We found that khat activated caspase-8 and -9, upregulated Bax protein expression and downregulated Bcl-2 expression levels, which resulted in the coordination of apoptotic signals. Khat-induced hepatocyte apoptosis is primarily regulated through the sustained activation of the c-Jun NH₂-terminal kinase (JNK) pathway and only partially via the extracellular signal-regulated kinase (ERK) cascade. Furthermore, the khat-induced reactive oxygen species (ROS) production and the activation of the ROS scavenger, N-acetyl-L-cysteine (NAC), attenuated the khat-induced activation of JNK and ERK. Our results demonstrate that khat triggers the generation of intracellular ROS and sequentially induces the sustainable activation of JNK, which in turn results in a decrease in cell viability and an increase in cell apoptosis.

Introduction

The chewing of khat leaves (*Catha edulis*) is a widespread recreational custom in Eastern Africa and the Arabian Peninsula. The plant contains the alkaloids, cathine and cathinone, which have amphetamine-like properties and produce a variety of

pleasurable effects. A significant number of people chew khat leaves to experience the euphoric and stimulating effects (1).

Apart from its neurological effects, khat can also act systemically and its use has been associated with tachycardia, hypertension, gastrointestinal disturbances and relaxation of the bladder wall (2).

There is increasing evidence indicating that khat-related hepatotoxicity also occurs in animals (3,4). The use of khat has been associated with severe liver injury, such as acute hepatitis (5,6) and chronic liver disease (7). However, studies on the toxicological potential of khat are insufficient (8), particularly regarding the mechanism(s) by which khat induces the reported liver lesions.

Apoptosis is a regulated form of cell death that is distinguishable from necrosis by its distinct morphological features, which include cytoplasmic shrinkage, plasma membrane blebbing and nuclear chromatin condensation (9), as well as biochemical features such as the externalization of phosphatidylserine, DNA fragmentation and the activation of specific caspases (10,11). Many plant-derived substances induce apoptosis in mammalian cells (12,13). Khat has been found to induce caspase-dependent apoptotic cell death in various leukemic cells (14), as well as in normal human oral keratinocytes and fibroblasts (15). Reactive oxygen species (ROS) are unstable molecules, ions or radicals that are generated through normal cellular metabolic processes. They include free radical species, such as superoxide anion and hydroxyl radical, as well as non-radical species, such as hydrogen peroxide. These molecules are involved in a number of normal cellular processes, such as gene expression (16), and proliferation and differentiation (17). Exogenous and endogenous stress may generate excessive amounts of ROS that cause damage to DNA, proteins and lipids, which induce cell cycle arrest, premature senescence (18) and the activation of pathways leading to cell death (19).

Khat has been found to reduce free radical metabolizing enzymes (20) and to induce apoptotic cell death via the regulation of oxidative stress in primary normal oral keratinocytes and fibroblasts (15).

Given that hepatocyte toxicity is an important toxic effect of khat, we used L02 human hepatic cells as an *in vitro* model in the current study to evaluate the cytotoxic effects of khat. Furthermore, we also examined the activation of ROS and mitogen-activated protein kinase (MAPK) signaling in L02 cells exposed to khat.

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Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM) was obtained from HyClone Laboratories (Logan, UT, USA). Fetal bovine serum (FBS) was provided by Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). Antibodies against caspase-8 (13423-1-AP) and caspase-9 (10380-1-AP) were supplied from the Proteintech Group, Inc. (Chicago, IL, USA). Antibodies specific for Fas (sc-1023), Bcl-2 (sc-7382), Bax (sc-7480), extracellular signal-regulated kinase (ERK; sc-271270), phospho-ERK (p-ERK; sc-81492), p38 (sc-7149), phospho-p38 (p-p38; sc-101759), c-Jun NH2-terminal kinase (JNK; sc-1648), phospho-JNK (p-JNK; sc-135642) and β -actin (Sc-1616r) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Khat extraction. *Catha edulis* Forsk (khat) purchased from Sana'a, Yemen was used in this study. Fresh khat leaves along with soft stems were collected during the summer period, weighed, washed with distilled water three times and allowed to dry for three days in a clean, dry room protected from sunlight. After drying, the plant was weighed, packed in a closed foil packet and stored at 4°C until use. Khat was extracted from the leaves as described previously (14,21,22). Briefly, dried khat leaves (100 g) were swiftly chopped into small (5 mm) pieces and dissolved in 100 ml of 95% ethanol, centrifuged at 5,000 rpm for 5 min and the supernatant then filtered with Whatman filter paper. Ethanol (100 ml) was added to the remaining leaves and the procedure was repeated. The ethanol khat extract was concentrated using a rotary evaporator (Labtech, Inc., Hopkinton, MA, USA) at 30°C with a rotation speed of 70 rpm until 70% of the ethanol solvent had evaporated. The resulting viscous solution was diluted with 100 ml of distilled water and then stirred at 1,000 rpm with for 1 h at room temperature. The filtrate was kept frozen at -70°C for 24 h and then dried by lyophilization (Lyophilization Technology, Inc., Ivyland, PA, USA). Typically, 100 g of dried leaves yielded 8 g of khat extract powder. As previously described, high performance liquid chromatography analysis confirmed that the alkaloids in the khat extract consisted of 80% cathine and 20% norephedrine; no cathinone was detected (14,21,22). Lyophilized khat extract was dissolved in Hank's buffered salt solution (HBSS) without Ca^{2+} , Mg^{2+} to a final concentration of 200 $\mu\text{g}/\text{ml}$ and filter (0.2 μm) sterilized before being added to the cells.

Cell culture and treatment. L02 cells were cultured in DMEM containing 10% FBS and maintained at 37°C in a 5% CO_2 humidified incubator. The cells were grown to 60-70% confluence before the experiments were conducted. L02 cells were exposed to various concentrations of khat (10, 50 and 100 $\mu\text{g}/\text{ml}$) and incubated at 37°C, 5% CO_2 for up to 24 h. The cells not treated with khat served as the control group.

Cell growth inhibition assay. The number of viable cells was determined using the trypan blue exclusion assay as previously described by Ahmed *et al* (23). Briefly, the cells were seeded in 6-well plates to 60-70% confluence, incubated overnight,

and then exposed to the indicated concentrations of khat for 4, 8, 16 and 24 h. Floating and adhering cells were collected and stained with 0.2% trypan blue for 5 min at room temperature before they were examined under a fluorescence microscope (Olympus, Tokyo, Japan). Following the internalization of the dye into the cells, the cells whose nuclei were stained blue were considered dead. The results are expressed as a percentage of the control.

Staining of apoptotic cells with Hoechst 33258. Following treatment with khat, the cells were washed with 0.1 mol/l PBS (pH 7.2) and resuspended in the same buffer. The cells contained in 100 μl of cell suspension (1×10^6 cells/ml) were incubated with 1 ml Hoechst 33258 (1 mg/ml in distilled water) for 10 min. Apoptotic cells were evaluated under a fluorescence microscope.

Annexin V/PI staining assay. Apoptosis was assessed by measuring the membrane redistribution of phosphatidylserine using an Annexin V-FITC apoptosis detection kit according to the manufacturer's instructions. After drug treatment, the cells were collected, washed twice with PBS and resuspended in 500 μl of staining solution containing FITC-conjugated Annexin V antibody (5 μl) and PI (5 μl of 250 mg/ml stock). After incubating the cells in ice for 30 min, the cells were analyzed by flow cytometry (FACSCalibur; Becton-Dickinson, San Jose, CA, USA). Basal apoptosis and necrosis were also determined in the untreated cells. The percentage of cells undergoing apoptosis was determined by three independent experiments.

Transmission electron microscopy. The cells were fixed in 0.1 M Na-cacodylate buffer, pH 7.4 containing 2% glutaraldehyde. The samples were rinsed with buffer and post-fixed in 1% osmium tetroxide. The specimens were dehydrated using graded ethanol and embedded in epoxy resin. Ultra-thin sections were double-stained with uranyl acetate and lead citrate. Specimens were examined under an electron microscope (Jeol 1230; Jeol Ltd., Tokyo, Japan), and the micrographs were processed using an Agfa Arcus II scanner and Adobe Photoshop 7.0.1 software.

Western blot analysis. The cells were harvested and lysed on ice for 30 min in modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM NaF, 1 mM Na_3VO_4 , 5 mM sodium pyrophosphate and a protease inhibitor tablet). The cell lysates were centrifuged at 14,000 \times g for 15 min, and the supernatant was recovered. The total protein concentration was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). The lysates were denatured by boiling in SDS sample buffer. The proteins were separated on SDS/PAGE 4-20% SDS-polyacrylamide gels and then transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using a semi-dry transfer cell (Bio-Rad). After blocking the membranes, the membranes were probed with the appropriate primary antibodies. Membrane-bound primary antibodies were detected using secondary antibodies that were conjugated to horseradish peroxidase. Western blots were visualized using enhanced

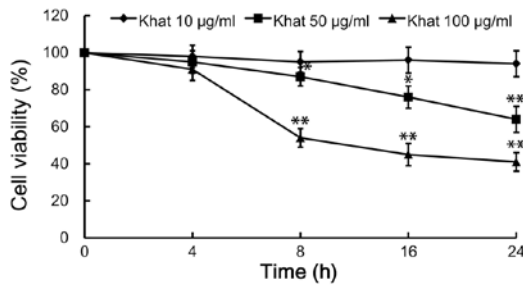


Figure 1. Effect of khat on the viability of L02 cells. L02 cells were incubated with various concentrations of khat for different periods of time, followed by the analysis of cell viability using the trypan blue exclusion assay. The khat-treated group was compared to the control group (* $P < 0.05$, ** $P < 0.01$; $n = 3$).

chemiluminescence detection reagents (Sigma) according to the manufacturer's instructions. The quantification of protein bands was performed via scanning using the Bio-Rad Gel Doc™ XR and ChemiDoc™ XRS systems and analyzed using Quantity One 1-D analysis software version 4.6.3.

Measurement of ROS production. The generation of ROS was measured by flow cytometric analysis using dichlorofluorescein-diacetate (DCFH-DA) as a substrate (24). Briefly, following treatment with khat at a concentration of 100 µg/ml for 4, 8, 16 and 24 h, the cells were harvested, washed twice with cold PBS, and suspended in PBS (1×10^6 cells/ml). The cell suspension (500 µl) was placed in a tube, loaded with DCFH-DA to a final concentration of 5 µM, and incubated for 30 min at 37°C. ROS production was assessed based on the DCF fluorescence intensity from 10,000 cells that was obtained by flow cytometry.

Statistical analysis. Data are expressed as the means \pm SD and analyzed using SPSS 10.0 statistical software (SPSS Inc., Chicago, IL, USA). The one-way ANOVA procedure followed by LSD post hoc tests was used to determine the significance of differences among groups ($P < 0.01$ and $P < 0.05$).

Results

Effect of khat on the viability of L02 cells. The inhibitory effect of khat was evaluated by measuring the viability of L02 cells. At the indicated concentrations of 10, 50 and 100 µg/ml, treatment with khat inhibited the growth of the L02 cells in a time-dependent manner, compared to the control group ($P < 0.05$, $P < 0.01$). In the current study, we also found that khat significantly decreased the viability of the L02 cells at the concentration of 100 µg/ml following treatment for 8 h (Fig. 1). Therefore, the concentration of 100 µg/ml of khat was selected for the remaining experiments.

Effect of khat on apoptosis of L02 cells. To investigate whether the reduction in cell viability was due to apoptosis, cytometric analysis and morphological observation of the cells were performed. We double-stained the cells with Annexin V and PI and analyzed the results using flow cytometry. Following serum starvation for 24 h, the L02 cells were exposed to 100 µg/ml of khat for 4, 8, 16 and 24 h, yielding apoptotic rates of 4.7 ± 1.4 , 21.7 ± 3.1 , 33.4 ± 4.4 and $39.5 \pm 4.7\%$, respectively (Fig. 2). For a further assessment of apoptosis, we analyzed chromatin condensation and apoptotic bodies. the L02 cells were treated with the indicated concentrations of khat for 8 h. The vehicle-treated cells exhibited regular and round-shaped nuclei. Bu contrast, the majority of cells treated with

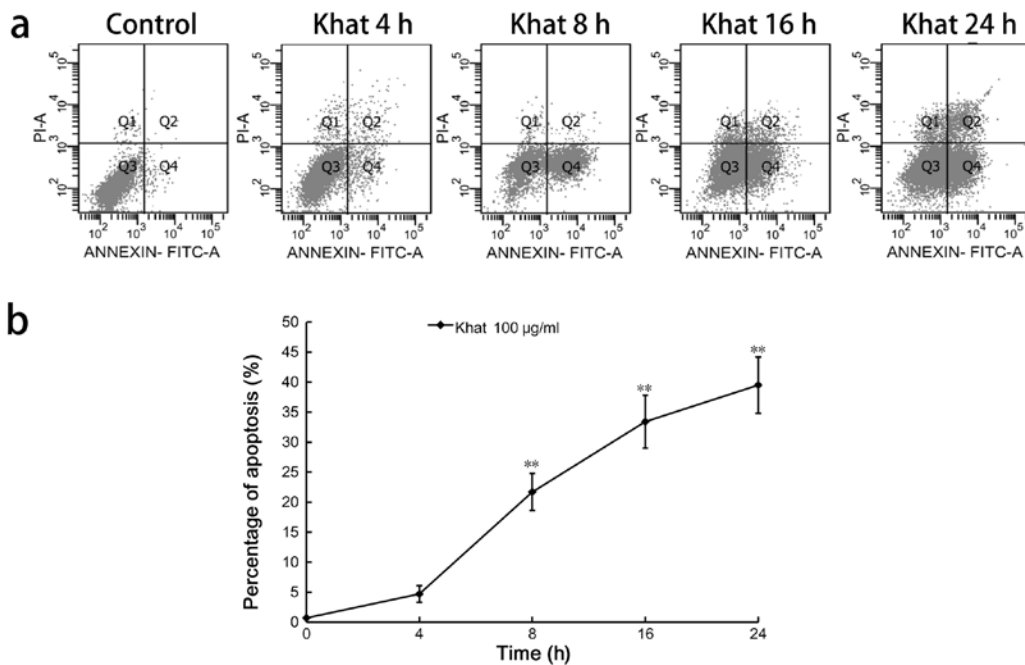


Figure 2. Evaluation of apoptotic cells using the Annexin V staining assay. (a) Apoptosis was quantified using Annexin V-PI staining. L02 cells were stained with Annexin V and PI and then analyzed by flow cytometry. Four subpopulations and their quantities are indicated as follows: non-apoptotic dead cells (Q1), late apoptotic cells (Q2), viable cells (Q3) and early apoptotic cells (Q4). The numbers in Q4 are the percentage of early apoptotic cells. (b) The percentage of early apoptotic cells following treatment with 100 µg/ml of khat was measured using flow cytometry. At least three independent experiments were performed for each condition, and at least 300 cells were counted for each measurement.

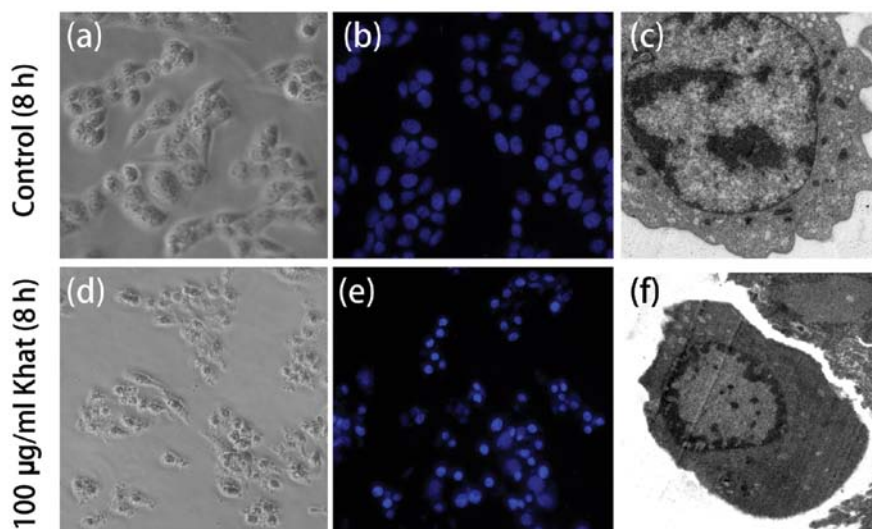


Figure 3. Morphological and ultrastructural features of khat-treated L02 cells. L02 cells were grown in 12-well plates up to half-maximal confluency and then treated for 8 h with 100 $\mu\text{g/ml}$ of khat. (a and d) The cells were observed under an inverted phase contrast light microscope at a 200-fold magnification or (b and e) stained with Hoechst 33342 and observed under a fluorescence microscope at x200 magnification. (c and f) The cells were observed under a transmission electron microscope as described in Materials and methods at x2,000 magnification.

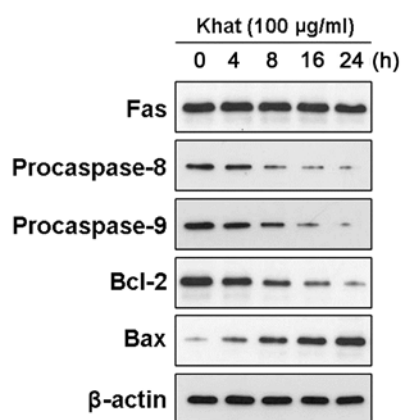


Figure 4. The involvement of apoptosis-related protein expression in khat-induced apoptosis. L02 cells were treated with khat at a concentration of 100 $\mu\text{g/ml}$ for different periods of time, and the cell lysates were analyzed by western blot analysis using the appropriate antibodies. The blots shown are from the same typical experiment, which involved three representative experiments.

khat (100 $\mu\text{g/ml}$) were stained with Hoechst 33258, which is bound to chromatin. The treated cells exhibited primary characteristics of apoptotic cells, namely the condensation and fragmentation of nuclei. When observed under an electron microscope, the khat-treated cells showed chromatin condensation and nuclear shrinkage consistent with apoptosis (Fig. 3).

Effect of khat on the expression of apoptosis-related proteins.

We assessed the activated caspase-8 and -9 levels in the L02 cells before and after treatment with khat by western blot analysis. Khat exposure significantly activated caspase-8 and -9 in a time-dependent manner (Fig. 4). After incubation with khat at the concentration of 100 $\mu\text{g/ml}$ for different periods of time (0, 4, 8, 16 and 24 h), the intensity of the bands corresponding to procaspase-8 and -9 decreased. However,

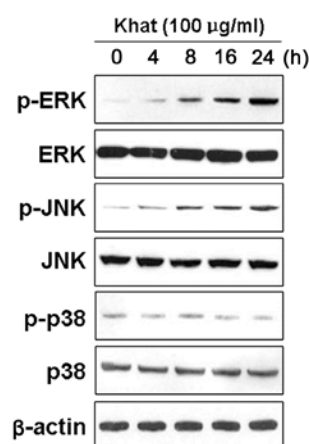


Figure 5. Timecourse of khat-induced accumulation of phosphorylated ERK, JNK and p38 MAPK. L02 cells were incubated with 100 $\mu\text{g/ml}$ khat for the indicated periods of time. The cell lysates were subjected to western blot analysis and probed using the appropriate antibodies. The data shown are from a typical experiment involving three representative experiments.

under the same experimental conditions, the expression level of Fas remained stable following treatment with khat, which suggested that caspase activation not stimulated by Fas but by other death signaling pathways.

In addition, Bax protein expression levels in L02 cells treated with khat demonstrated a marked increase compared to the control protein expression levels. Conversely, the L02 cells treated with khat showed a marked decrease in Bcl-2 protein levels.

Effect of khat on the activation of MAPKs.

In order to clarify the involvement of MAPKs in the khat-induced cell death of L02 cells, the levels of phosphorylated MAPKs (p38, ERK and JNK) were investigated by western blot analysis (Fig. 5). The phosphorylation of JNK and ERK was significantly

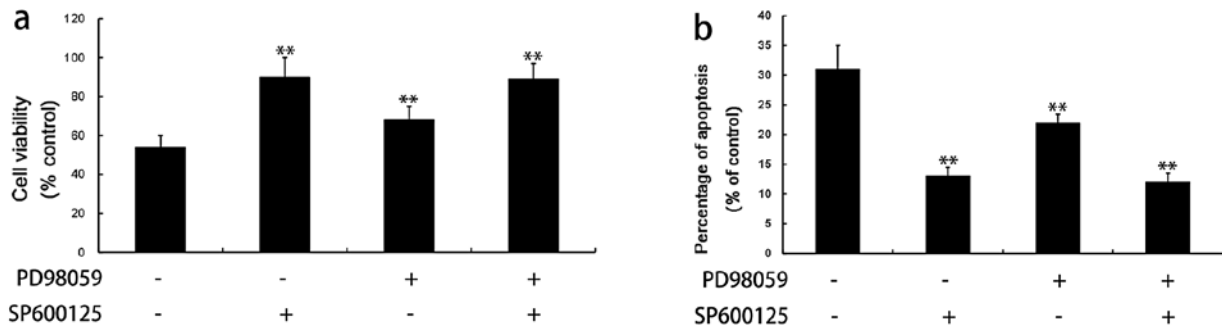


Figure 6. The inhibition of ERK1/2 and JNK kinases attenuates khat-induced cytotoxicity and apoptosis. L02 cells were pre-treated with 50 μ M ERK1/2 inhibitor (PD98059) or 10 μ M JNK inhibitor (SP600125) for 1 h, or the vehicle control followed by treatment with 100 μ g/ml of khat for 8 h. Following incubation, cell viability was measured using the (a) trypan blue exclusion assay, and (b) apoptosis was analyzed by flow cytometry. **P<0.01 compared to the khat-treated group.

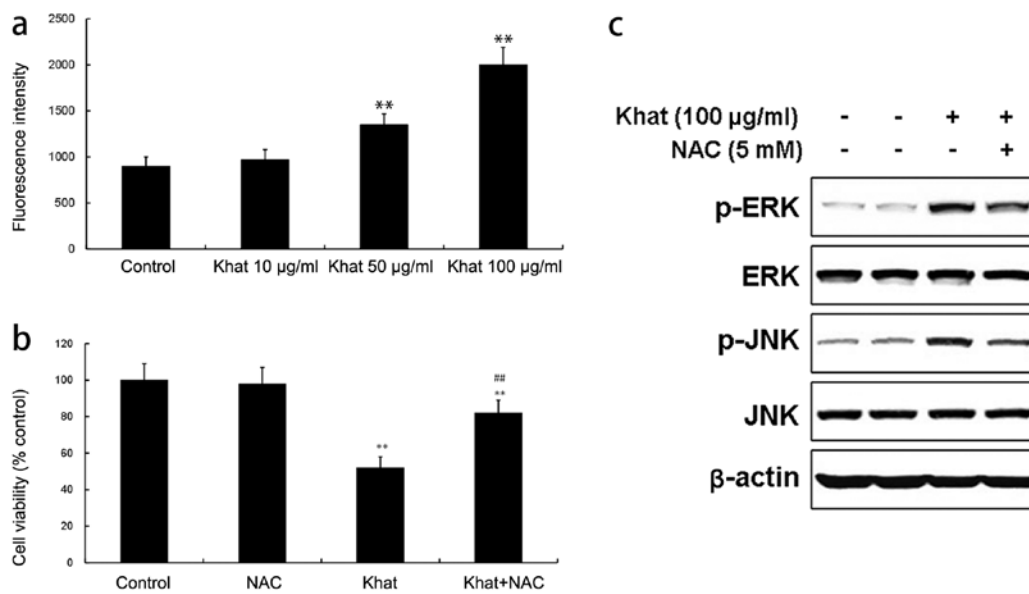


Figure 7. Effect of khat on intracellular ROS production. (a) L02 cells were exposed to various concentrations of khat for 8 h and then analyzed for ROS production. (b) L02 cells were pre-treated with 5 mM NAC for 2 h or the vehicle control followed by treatment with 100 μ g/ml of khat for 8 h. Following incubation, cell viability was measured using the trypan blue exclusion assay. (c) The effects of the inhibition of ROS on the ERK and JNK signaling pathways are shown. L02 cells were pre-treated for 2 h with 5 mM of NAC and then stimulated for 8 h with 100 μ g/ml of khat. The phosphorylation of ERK1/2 and JNK were measured by western blot analysis.

increased following treatment with khat at the concentration of 100 μ g/ml. However, the phosphorylation levels of p38 were not altered under the same conditions. The total levels of each MAPK were not altered during the incubation period. Therefore, the ERK and JNK signaling pathways may be involved in the response of L02 cells to khat.

To further evaluate the possible roles of MAPKs in khat-induced apoptosis, we examined cell viability and apoptotic rates in the presence or absence of specific inhibitors of JNK (SP600125) and ERK1/2 (PD98059). SP600125 and PD98059 prominently reversed the khat (100 μ g/ml)-induced cell death observed in the trypan blue exclusion assay and flow cytometric analysis (Fig. 6), which was concomitant with the block of Bcl-2 reduction as well as caspase-8 and -9 activation (data not shown). These results suggest that both JNK and ERK are involved in the apoptotic progression caused by khat in L02 cells.

Effect of khat on ROS in L02 cells. ROS have been implicated as potential modulators of apoptosis. In the L02 cells, treatment with khat caused a dose-dependent accumulation of intracellular ROS (Fig. 7a).

To further determine the role of khat-induced ROS generation in the activation of JNK and ERK, the L02 cells were pre-treated with 5 mM N-acetyl-L-cysteine (NAC), a specific inhibitor of ROS, for 2 h and then incubated with 100 μ g/ml of khat for 8 h.

NAC prominently reversed khat (100 μ g/ml)-induced cell death, which was observed using the trypan blue exclusion assay (Fig. 7b). NAC markedly decreased the activity of ERK and JNK induced by khat (Fig. 7c). However, NAC had no effect on total ERK1/2 and JNK expression. These results suggest that khat-induced ROS accumulation may contribute to the activation of ERK and JNK in L02 cells.

Discussion

In the current study, we investigated the role of khat in the apoptosis of L02 cells and further clarified the underlying molecular mechanisms. To the best of our knowledge, the current study is the first report the role of ROS generation and MAPK signaling pathways in khat-induced hepatocytic apoptosis.

The results of the cell viability assay indicated that treatment with khat inhibited the growth of L02 cells in a time-dependent manner compared to the control group (Fig. 1). In agreement with these data, flowcytometric analysis revealed that 100 $\mu\text{g/ml}$ of khat induced apoptosis in L02 cells in a time-dependent manner (Fig. 2). The khat-treated L02 cells underwent morphological changes, such as plasma membrane blebbing, cell shrinkage and condensation of nuclear chromatin (Fig. 3).

The induction of cell death by khat was synchronous, and occurred in the majority of cells in a concentration-dependent manner (Fig. 2). These results suggest that these effects may be elicited through a specific mechanism(s). Our results indicate that caspase-8 and -9 may be involved in the cascade of cellular events leading to khat-induced cell death. However, under the same experimental conditions, the expression level of Fas remained stable following treatment with khat, which suggests that the activation of caspases is not stimulated by Fas, but by other death signaling pathways.

The Bcl-2 protein is a well-known suppressor of apoptosis that homodimerizes with itself or heterodimerizes with the homologous protein, Bax, which is a promoter of apoptosis (25,26). These two proteins are critical mediators of apoptosis, and their expression ratio is regulated by an apoptotic inducer. In the current study, we found that khat upregulated Bax protein expression and downregulated Bcl-2 expression levels, which increased the Bax/Bcl-2 ratio in relation to apoptosis coordination. These data suggest that khat causes hepatic apoptosis by modulating the Bax/Bcl-2 ratio.

A previous study indicated that the MAPK signaling pathway is involved in the cytotoxicity induced by bifenthrin (27). Therefore, it is important to investigate the roles of MAPK signaling molecules in khat-induced hepatocyte toxicity. The MAPK family includes the ERK, JNK and p38 kinase, which are involved in cell survival, proliferation and apoptosis in response to various growth or stress stimuli.

The activation of ERK has been implicated in cell proliferation and cell cycle progression (28), whereas JNK and p38 are more commonly activated in response to stress and toxicants that induce cell apoptosis (29). Importantly, a number of studies support the concept that sustained JNK activation leads to apoptosis (30,31). This hypothesis was also supported by the current study. Our results demonstrate that treatment with khat causes a sustained JNK activation and that the specific inhibitor of JNK, SP600125, significantly blocks the khat-induced apoptosis of L02 cells.

Although ERK1/2 was also activated by khat, the inhibition of ERK activity only partially reversed khat-induced hepatocyte toxicity (Fig. 6). These results demonstrate that khat-induced hepatocyte apoptosis is predominantly mediated by the sustained activation of the JNK pathway and is only partially mediated by the ERK cascade.

Oxidative stress has been implicated as a mechanism of hepatocytic toxicity from numerous toxicants (32,33). In the current study, khat induced ROS production. This result suggests that ROS production may be attributed to khat-induced hepatocytic apoptosis. Previous studies have demonstrated the mechanistic involvement of alterations in signal transduction cascades in response to ROS generation (32,33). In the current study, we also determined that the antioxidant, NAC, attenuated the khat-induced activation of JNK and ERK. These results indicate that the outcome of the challenge with khat depends on the oxidative stress-induced activation of a series of signaling cascades that promote hepatocyte apoptosis.

In conclusion, the data from the present study suggest that the intracellular response in L02 cells following exposure to khat triggers the generation of intracellular ROS and sequentially induces the sustainable activation of JNK, which in turn results in a decrease in cell viability and an increase in cell apoptosis. To our knowledge, this is the first study that estimates the possible cytotoxic effects of khat on hepatocytes at the molecular level.

Acknowledgements

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