

Catha edulis Extract Induces H9c2 Cell Apoptosis by Increasing Reactive Oxygen Species Generation and Activation of Mitochondrial Proteins

Syam Mohan, Siddig Ibrahim Abdelwahab¹, Yahya Hasan Hobani², Suvitha Syam², Adel S. Al-Zubairi³, Rashad Al-Sanousi¹, Magbool Essa Oraiby⁴

Medical Research Centre, Jazan University, ¹Substance Abuse Research Center, Jazan University, ²Faculty of Applied Medical Sciences, Jazan University, ⁴Confirmatory and Specialized Analyzers Unit, Poison Control Centre, Jazan, ³Faculty of Applied Medical Sciences, Albaha University, Al Baha, Kingdom of Saudi Arabia

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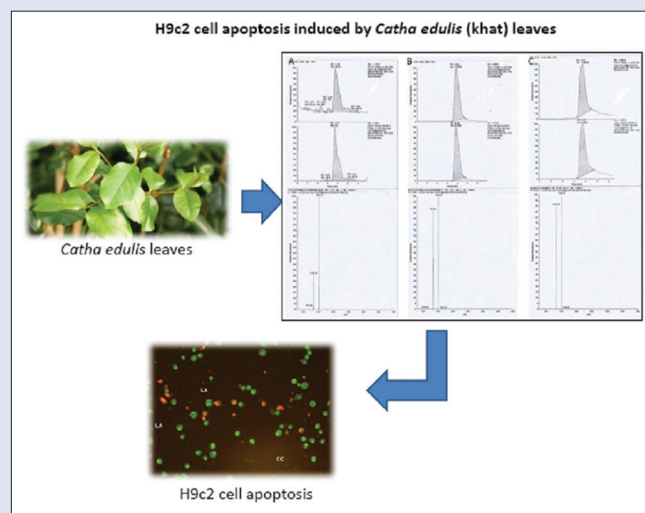
ABSTRACT

Background: *Catha edulis* (Khat) is an evergreen shrub or small tree, traditionally used by various peoples of the Arabian Peninsula and Africa as an integral component of the socioeconomic traditions. It is believed that the psychostimulant nature and toxic nature of khat is primarily due to the presence of cathinone and cathine respectively. Studies have shown that khat chewing is closely associated with cardiac complications, especially myocardial infarction. Hence in this study, we exposed cathine-rich khat extract in a cardiomyoblast H9c2 (2-1) cell line to check the cell death mechanism. **Materials and Methods:** Extraction of *Catha edulis* leaves was done and the presence of cathine was confirmed with LC-MS-MS. The anti-proliferative activity was assayed using MTT and apoptosis detection by acridine orange/propidium iodide assay. The expression of Bcl-2 and Bax protein and caspase-3/7 expression were analyzed. The level of reactive oxygen species generation was also evaluated. **Results:** The khat extract showed an IC₅₀ value of 86.5 µg/ml at 48 hours treatment. We have observed significant early apoptosis events by intervened acridine orange within the fragmented DNA with bright green fluorescence upon treatment. The Bcl-2 expression in the treatment with IC₅₀ concentration of khat extract for 24, 48 and 72 hours of incubation significantly decreased with increase in bax level. The increased activation of caspase-3/7 was significantly observed upon treatment together with significant increase of ROS was detected at 24 and 48 hours treatment. **Conclusion:** Collectively, our results provide insight into the mechanisms by which *Catha edulis* leaves mediate cell death in cardiomyocytes.

Key words: Apoptosis, *Catha edulis*, cathine, myocardial infarction, toxicity

SUMMARY

- *Catha edulis* (Khat) is an evergreen psychotropic shrub or small tree
- Extraction of khat leaves was done and the presence of cathine was confirmed with liquid chromatography-mass spectrometry-mass spectrometry
- The khat extract showed an IC₅₀ value of 86.5 µg/ml at 48 h treatment in H9c2 (2-1) cell line
- The observed cell death was associated with increased expression of Bcl2 and caspase-3
- Significant increase of reactive oxygen species was also detected in the cell with treatment.



Abbreviations used: CNS: central nervous system; AMI: acute myocardial infarction; TLC: thin layer chromatography; ESI: electrospray ionization; FBS: fetal bovine serum; DMSO: dimethylsulfoxide; AO: acridine orange; PI: propidium iodide; HRP: horseradish peroxidase; HBSS: hank's balanced salt solution; DCFH-DA: 2',7'-dichlorofluorescein diacetate; NAC, 10 mM: NAC: N-acetyl cysteine; ROS: reactive oxygen species.

Correspondence:

Dr. Syam Mohan,
Medical Research Centre,
Jazan University, P. O. Box 114,
Jazan, Kingdom of Saudi Arabia.
E-mail: syammohanm@yahoo.com
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INTRODUCTION

Catha edulis (Khat) is an evergreen shrub or small tree, traditionally used by various people of the Arabian Peninsula and Africa as an integral component of the socioeconomic traditions. In their social gatherings, it has been chewed over the course of several hours to facilitate interpersonal communication and for its euphoric effects.^[1] It is also used by general class of people to boost their performance and to get rid of fatigue.^[2] Moreover, people widely chew khat believing that it improve memory, alleviate headaches, common cold, make

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them alert, think clearly, improve depression, and to have aphrodisiac effects.^[3]

There was a long series of chemical studies focusing on the identification of khat's active principle and the characterization of its alkaloid content. Even though many compounds such as tannins, flavonoids, glycosides, and other compounds are present, the plant is famous due to the presence of two major phytochemicals such as cathinone and cathine due to its similarity in structure toward a known psychostimulant amphetamine.^[4] It is believed that the psychostimulant nature of khat is primarily due to the presence of cathinone, the β -keto analog of amphetamine. Due to its keto-amine base, the stability of this compound is always compromised and found only in fresh leaves.^[5] Upon drying, the enzymatic degradation happens and cathinone is converted to cathine (norpseudoephedrine). On contrary, Chappell and Lee^[6] showed that drying khat leaves does not actually destroy the cathinone contained within them. Apart from cathinone and cathine, khat also contains other phytochemicals such as merucathinone, pseudomerucathine, and merucathine.

Khat plant containing cathinone and cathine has been subjected to extensive pharmacological investigation using *in vitro* system and in animals. Cathinone appears to affect neurotransmission in a similar manner to amphetamine by triggering presynaptic dopamine release and also by inhibiting the reuptake of dopamine.^[7] Apart from the main effect, it also holds effects on the cardiovascular system including augmentation of blood pressure, vasoconstriction, and an increase in heart rate.^[8,9] Khat has been found to increase the body temperature and has analgesic properties and antimicrobial activity. Apart from these pharmacological properties, khat also exerts some toxicological or adverse effects such as producing central nervous system-related hallucinations, increased states of paranoia, aggression, and dependence.^[10,11] Fokunang, Ireland,^[12] had reported an increased chance of mutagenicity, carcinogenicity, *in vitro* and *in vivo* models. Yet another study shows that khat decreased the sperm count in

roosters and also negatively impacted sperm volume and fertilization rates. Al-Ghamdi^[13] has recently reported khat to be a causative agent of acute hepatotoxicity in a series of cases. Besides, there is an increased risk of myocardial infarction during chewing sessions,^[14] and heavy khat chewers have been estimated to have a 39-fold increase in myocardial infarction risk.^[15] Khat may increase the risk of cerebral infarctions and hemorrhoids. A case-control study done by Al-Motarreb *et al.*^[16] indicates that khat chewing is associated with acute myocardial infarction (AMI) and it is an independent dose-related risk factor for the development of myocardial infarction. Among these factors that lead to myocardial infarction, cardiac myocyte apoptosis is an important contributor to myocardial dysfunction and heart failure in patients with AMI.^[17]

Unfortunately, many studies have focused on the health effects of khat by purely investigating cathinone, while cathinone plays only a minor role in the toxicity of khat, but it is cathine that is responsible for the unwanted systemic effects.^[18] In light of this rationale, the current study has been designed with an aim to investigate the role of cathine-rich khat extract-induced apoptosis in cardiac myocyte.

MATERIALS AND METHODS

Plant collection and extraction

C. edulis leaves were provided for this research by the Ministry of Interior, Saudi Arabia. The fresh bundles were transported to the laboratory and kept in -80°C immediately. The extraction of the plant materials has been done according to the protocol of SWGDRUG organization with slight modification.^[19] Briefly, 50 g of leafy material was macerated in a plant mill into very small pieces. Two hundred milliliters of methanol was added and sonicated for 15 min. The mixture was filtered through a Whatman filter paper to separate the liquid from the solid plant material. The alcohol solution was evaporated to near dryness under a stream of air. The small volume is reconstituted in 0.02 N sulfuric acid, extracted

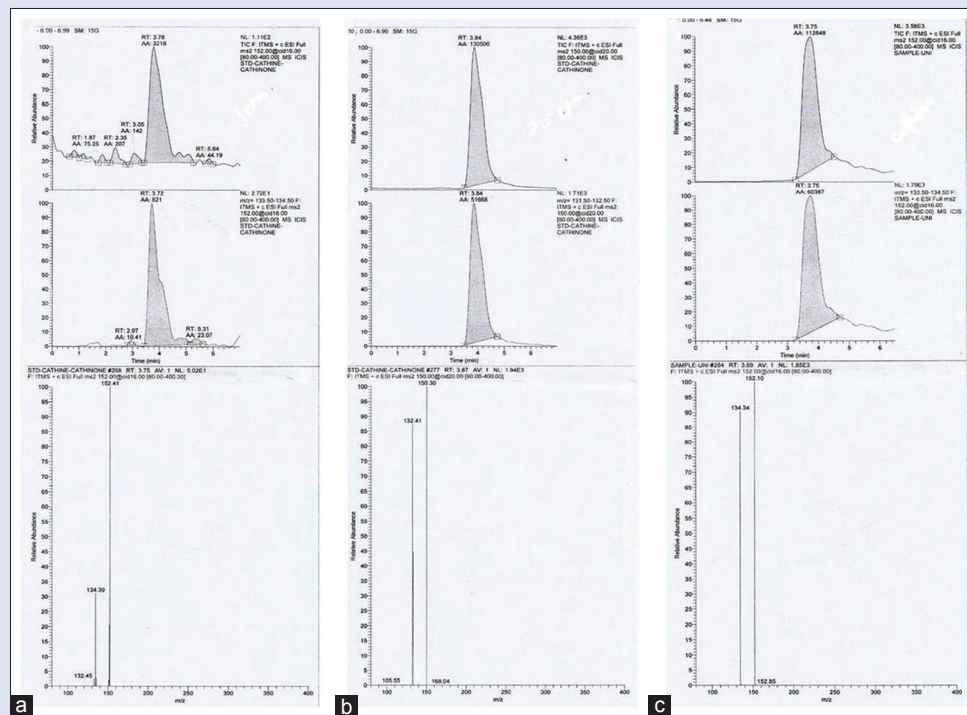


Figure 1: Liquid chromatography-mass spectrometry-mass spectrometry of the cathine and cathinone alkaloids. Mass spectrometry analysis of diluted (1:1000) STD cathine (a: Ion m/z 152) and STD cathinone (b: Ion m/z 150) showing specific ion scan spectra. (c) Detection of cathine in the khat extract

into chloroform, and separated. The aqueous acidic layer was made basic with saturated sodium bicarbonate and extracted into dichloromethane. This extract was then analyzed by thin-layer chromatography and liquid chromatography-mass spectrometry-mass spectrometry (LC-MS-MS) for the presence of cathine.

Analysis of khat by liquid chromatography-mass spectrometry-mass spectrometry

Khat extract was diluted in methanol and water (5:95) before analysis. Aliquots (10 μ l) were injected into the LC-MS/MS system consisting of a LCQ Fleet Single quadrupole Ion Trap MS (Thermo Scientific, USA). The analytes were separated on a Hypersil GOLD column (150 mm \times 3 mm i.d.: 5 μ m, Thermo Scientific, USA). The compounds were eluted by isocratic mobile phase made from 85% of 10 mmol ammonium formate buffer and 15% of 0.1% formic acid in acetonitrile. The run time was 7 min with a flow rate of 0.30 ml/min. The compounds were then positively charged in the LC-MS interface using electrospray ionization at the positive mode. To verify the presence of specific alkaloids in the khat extract, diluted samples of (methanol: Water; 5:95) cathinone hydrochloride and norpseudoephedrine (cathine) hydrochloride (Lipomed, Switzerland) were used as standard [Figure 1].

Qualitative analysis was performed in the scanning mode, monitoring the following transitions: m/z 150 \rightarrow 150 and m/z 150 \rightarrow 132 for cathinone and m/z 152 \rightarrow 152 and m/z 152 \rightarrow 134 for cathine.

Cell culture

H9c2 (2-1) (rat cardiomyoblast) cells were obtained from American Type Culture Collection (ATCC, VA, USA). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). The cell suspension (1×10^5 cells/ml) was plated out into 96 well-plates. Khat extract was dissolved in dimethylsulfoxide (DMSO) and the final concentration of DMSO was 0.1% (v/v). Different concentrations of the sample were prepared in serial dilutions. DMSO (0.1%) was used as the control.

The cytotoxicity profile of the extract was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) microculture tetrazolium viability assay. Thereafter, the various concentrations of samples were plated out in triplicates. Each plate included untreated cell controls and a blank cell-free control. After 48 h of incubation, MTT (5 mg/ml) was added to each well and the plates were incubated for further 4 h and the media was removed. DMSO was later added into each well to solubilize the formazan crystals. The absorbance was read at a wavelength of 595 nm using a microtiter plate reader. The cytotoxicity of khat extract on cells was expressed as IC₅₀ values (the sample concentration reducing the absorbance of treated cells by 50% with respect to untreated cells).

Detection of apoptosis

Khat-induced cell death in H9c2 (2-1) cells was quantified using acridine orange (AO) and propidium iodide (PI) double-staining according to standard procedures and examined under fluorescence microscope. Briefly, cells were plated at a concentration of 1×10^5 cells/ml and treated with khat extract for 24, 48, and 72 h consecutively. The cells were then spun down at 300 \times g for 10 min. Supernatant was discarded and the cells were washed twice using PBS after centrifuging at 300 \times g for 10 min to remove the remaining media. Ten microliters of fluorescent dyes containing AO (10 mg/ml) and PI (10 mg/ml) were added into the cellular pellet at equal volumes. Freshly stained cell suspension was dropped into a glass slide and covered by a cover slip. Slides were observed under ultraviolet-fluorescence microscope within 30 min before the fluorescent color starts to fade. The percentages of viable, early apoptotic, and late apoptotic cells were determined in >200 cells. Quantification of the different cell population has been done as described earlier.^[20]

Bcl-2 and Bax expression

The level of expression of Bcl-2 and Bax was determined by colorimetric procedures by using rat Bcl-2 and Bax ELISA kit (Neo Biolabs, MA, USA) according to the manufacturer's instruction. Briefly, Bcl-2 and Bax protein from the cell lysate specifically bound to the primary antibody and detected by horseradish peroxidase (HRP) conjugated secondary antibody. HRP-conjugated secondary antibody provided sensitive colorimetric detection, which was determined by measuring the absorbance at 450 nm and the reference wavelength of 630 nm using a microplate reader.

Caspase-3/7 activity assay

A caspase-3/7 activity was measured using luminescence-based assay, caspase-Glo™ 3/7 assay (Promega, Madison, WI, USA). Cells were cultured in 96 well-culture plates in 50 μ l of RPMI 1640 supplemented with 10% FBS and incubated for 24 h. Cells were then treated with different concentrations of khat. At the end of incubation, 100 μ l of assay reagent was added to be incubated for 1 h at room temperature. Luminescence was measured using a microplate reader [Figure 5].

Measurement of reactive oxygen species generation

The production of intracellular reactive oxygen species (ROS) was measured using 2',7'-dichlorofluorescein (DCF) diacetate. Briefly, 10 mM dichloro-dihydro-fluorescein diacetate (DCFH-DA) stock solution (in methanol) was diluted 500-fold in Hank's balanced salt solution (HBSS) without serum or other additives to yield a 20 μ M working solution. After 24 h of exposure to khat, the cells in the 96 well-black plate was washed twice with HBSS and then incubated in 100 μ l working solution of DCFH-DA at 37°C for 30 min. Fluorescence was then determined at 485-nm excitation and 520-nm emission using a fluorescence microplate reader.

Statistical analysis

All values were reported as mean \pm standard error of the mean, $P < 0.05$ was considered significant.

RESULTS

Cell growth inhibition assay

MTT assay, a standard colorimetric assay for measuring cellular growth, was used to check the H9c2 cardiomyoblast cell growth inhibition by khat extract. The cells were treated with different concentrations of khat for

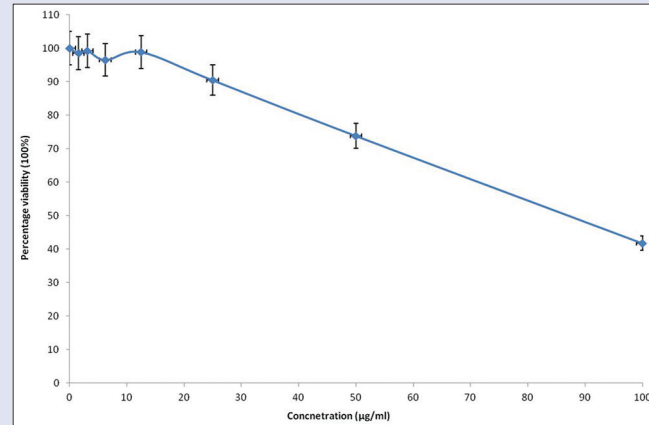


Figure 2: Dose-response curve of cell viability (%) against khat extract concentration at 48 h. The value is the mean of three independent experiments

48 h. As shown in Figure 2, khat inhibited the cell viability of cells in a dose-dependent manner. The IC_{50} values was 86.5 $\mu\text{g/ml}$ at 48 h treatment.

Khat-induced morphological effects resembling apoptotic cell death

The cells were treated with khat and then evaluated for the apoptosis features morphologically. Briefly, the cells were scored under fluorescence microscope to quantify viable, early apoptotic, late apoptotic, and secondary necrosis. We counted 200 cells arbitrarily and differentially, together with the untreated negative control. The results showed that khat generated morphological features that relate to apoptosis in a time-dependent manner [Figure 3a-d]. We have observed the early apoptosis by intervened AO within the fragmented DNA with bright green fluorescence. At the same time, control cells were observed with a green intact nuclear structure. At 24 h treatment with khat, moderate apoptosis was seen by nuclear chromatin condensation. Furthermore, in the late stages of apoptosis, changes such as presence of reddish-orange color due to the binding of AO to denatured DNA were observed after 48 h and 72 h of treatment. A statistically significant ($P < 0.05$) difference was observed in the cell population while doing differential scoring of treated cells [Figure 3e].

To determine whether intracellular ROS generation is required for khat-induced apoptosis, we inhibited ROS generation by pretreating

cells with the ROS scavenger, N-acetyl cysteine (NAC) for 1 h before exposure to khat extract. The results in Figure 3e show that the incidence of apoptosis has been significantly reduced upon NAC pretreatment.

Khat-induced apoptosis associated with Bax/Bcl-2 regulation

Both Bax and Bcl-2 were estimated using Elisa kit. In this assay, the Bcl-2 expression after treatment with IC_{50} concentration of khat extract for 24, 48, and 72 h of incubation decreased significantly ($P < 0.05$) as compared to the control. Meanwhile, the pro-apoptotic protein bax was increased significantly for 48 and 72 h ($P < 0.05$) [Figure 4].

Khat-induced apoptosis associated with caspase-3 activation

In the present study, the signaling pathway involved in the induction of apoptosis in H9c2 cardiomyoblast cells was examined through the caspase-3/7 activity assay. When the cells were treated with IC_{50} concentration of khat extract for 24, 48, and 72 h, the caspase-3/7 activation was found. The increased activation of caspase-3/7 was significantly increased upon treatment ($P < 0.05$).

Khat-induced cell death includes increased reactive oxygen species formation

Since the generation of ROS is associated with apoptosis, the intercellular ROS levels in khat-treated H9c2 cardiomyoblast cells were investigated through oxidation-sensitive DCF fluorescence intensity. The nonfluorescent H_2DCFDA easily permeabilized through cell membrane and got oxidized into highly fluorescence DCF in the presence of ROS. The increase in fluorescence intensity is directly proportional to the increased ROS formation in the cell. A time-dependent increase in DCF fluorescence was detected in treated cells [Figure 6]. A significant increase of ROS was detected at 24 and 48 h treatment.

DISCUSSION

The use of plants as a source of drug of abuse is as old as recorded history. Hallucinogenic compounds found in some plants made it widely used by people. The desired effects of such plants and its adverse effects are highly variable and unreliable, producing different effects in different

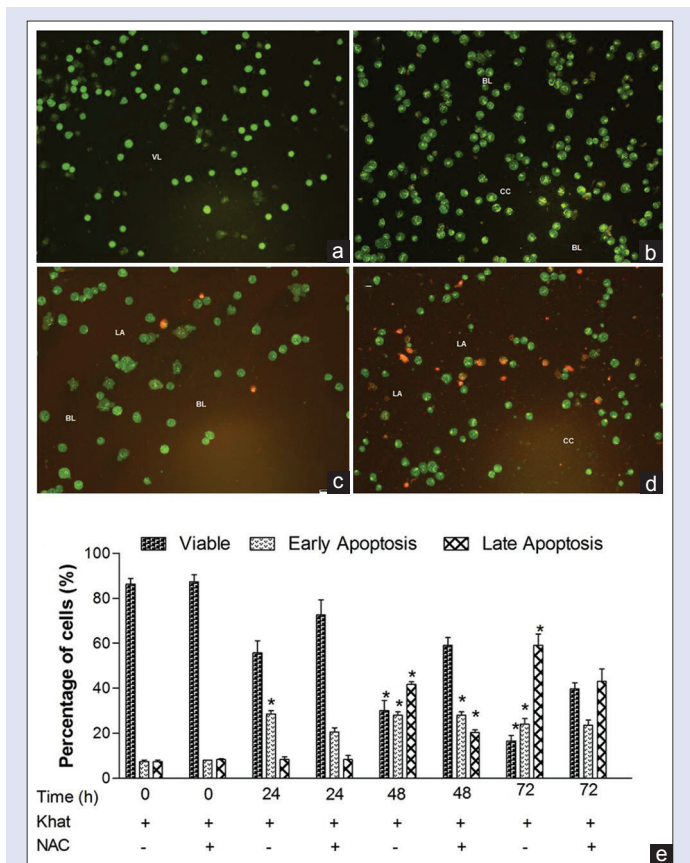


Figure 3: Fluorescent micrograph of acridine orange and propidium iodide double-stained cells. Cells were treated at IC_{50} of khat extract with and without acetyl cysteine N-acetyl cysteine at time-dependent manner. (a) Untreated cells showed normal structure without prominent apoptosis. (b) Early apoptosis features were seen after 24 h representing intercalated acridine orange (bright green). (c) Blebbing and nuclear margination were noticed in 48 h treatment. (d) Late apoptosis was seen in 72 h incubated cells. (e) There was a significant ($P < 0.05$) difference in the cell populations among the different criteria

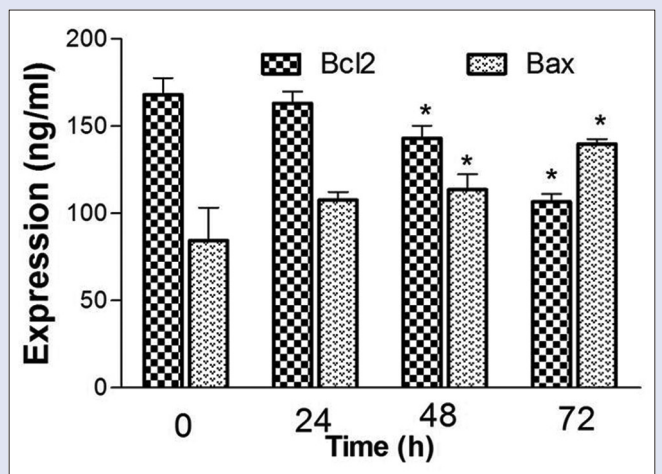


Figure 4: The expression of Bcl-2 and Bax protein in H9c2 (2-1) cells treated with khat extract, evaluated by rat Bcl-2, Bax ELISA kit. Results are expressed as the mean \pm standard deviation. Statistical significance is expressed as * $P < 0.05$

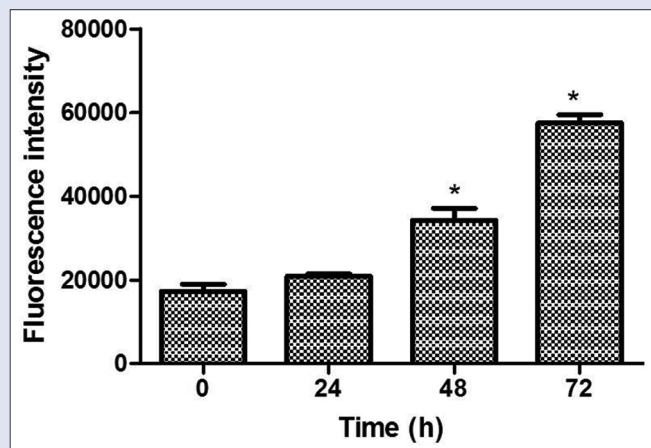


Figure 5: Relative luminescence expression of caspase 3/7 in H9c2 (2-1) cells treated with khat extract. Triplicates of each treatment group were used in each independent experiments. Statistical significance is expressed as $*P < 0.05$

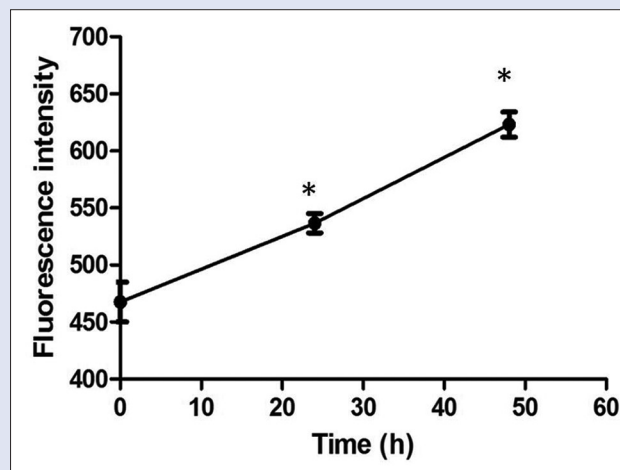


Figure 6: Effects of khat on H9c2 (2-1) cells reactive to oxygen species generation. Dichlorofluorescein-fluorescence intensity after 24 and 48 h. Values are mean \pm standard deviation from three independent experiments. Triplicates of each treatment group were used in each independent experiment. The statistical significance is expressed as $*P < 0.05$

people at different times. This is mainly due to the significant variations in the amount and composition of active compounds.^[21] One such plant is *C. edulis*, which is more than a psychotropic plant. It is part of a life style and plays a dominant role in celebrations, marriages, and political meetings. The leaves of this plant are the rich source of cathine and cathinone, both are considered as addiction forming moiety due to its structural similarity to amphetamine.^[1] Normally, fresh leaves contain a higher proportion of the desirable cathinone. Where the content of cathine is relatively higher, it causes more unwanted systemic effects.^[4] Studies have shown that khat chewing is closely associated with cardiac complications, especially myocardial infarction. Hence, in this study, we report that cathine-rich extract in cathine alkaloids induces cell death in H9c2 (2-1) cell lines.

The factors that lead to the development and progression of heart failure are still not fully understood. Studies show that cardiac myocyte apoptosis is an important contributor to myocardial dysfunction and heart failure in patients with AMI.^[17] In canine models of pacing-induced heart failure and heart failure due to chronic ischemic injury, loss of cardiomyocytes due to apoptosis was detectable by TUNEL staining, whereas in control myocardium, only rare cardiomyocytes stained positive.^[22,23] Apoptosis is a mode of cell death, which is a strictly controlled process and characterized by chromatin condensation, DNA fragmentation, and membrane blebbing.^[24,25] It was very clear in this study with the AO/propidium iodide double-staining which differentiated viable cells from early and late apoptotic cells. The observed chromatin condensation and marginization are seen earlier than the break-up of the genomic DNA in apoptosis cells.^[26]

It is known that the apoptosis of mitochondrial pathway is regulated by members of the Bcl-2 family proteins, and the Bcl-2 family can be divided into either antiapoptotic (e.g. Bcl-2, Bcl-XL, and Mcl-1) or pro-apoptotic (e.g. Bax, Bad, Bak, Bcl-Xs, and NOXA) members.^[27] The antiapoptotic protein Bcl-2 is considered an important cellular component and prevents cell apoptosis. Pro-apoptotic Bax promotes apoptosis. It is the balance between the pro-apoptotic and anti-apoptotic Bcl-2 family members that determines whether the cell survives or undergoes apoptosis. This ratio is crucial in cell apoptosis in response to drugs.^[28] Previous studies have indicated that apoptosis which upon treatment with khat is associated with cell death in various cells.^[29-32] Moreover, the cell death was associated with mitochondrial protein expression.^[33] The results of the present study also demonstrated that the levels of Bcl 2 were attenuated and that of levels of Bax were increased

in response to khat-induced apoptosis in H9c2 cells. These changes in the levels of Bcl-2 family members may have a direct effect on mitochondrial membrane pore formation and consequent activation of caspase-3.

Mitochondria release intracellular cytochrome c, which interacts with dATP and Apaf-1 in the apoptosome complex and subsequently activates the procaspase-9 into active caspase-9 and toward the executioner caspase-3.^[34] Caspase-3/7 is one of the effector caspases that is involved in the final execution of dying cells in both intrinsic (mitochondrial) and extrinsic (death receptor) pathways. To initiate this process, the mitochondria must be involved. Earlier, we have found the involvement of two important mitochondrial proteins Bcl-2/Bax. To understand the mechanism of action induced by the khat extract, caspase-3/7 activity and Bcl-2/Bax protein expression have been evaluated. The results showed that the khat induced time-dependent increases in caspase-3/7 activity in H9c2 cells. Our results were consistent with the findings reported by Dimba *et al.*^[29] Further study is required to fully elucidate the details of other caspases in the khat-induced apoptosis.

A number of studies have reported that increased levels of ROS production is a contributing factor to apoptosis in cardiac myocytes.^[35,36] In the current study, ROS production was identified at increased levels following khat-induced apoptosis in H9c2 cells, consistent with previous studies.^[31,37] Increasing ROS production has been demonstrated to account for apoptosis in response to khat in numerous cell lines, including oral keratinocytes, fibroblasts, and hepatic cells.^[31,32] In addition, the results of the present study indicate that increased levels of ROS production may be one of the mechanisms by which khat induces apoptosis in H9c2 cells. In support to this, the pretreatment of cells with NAC significantly reduced the incidence of apoptosis in Figure 3e.

Taken together, we conclude that khat is a potent agent to suppress the growth of H9c2 cells *in vitro*. The growth inhibition was in large part mediated via apoptosis-associated mitochondrial dysfunction and regulation of Bcl-2 signaling pathways. Furthermore, the cell death was found to be closely associated with ROS generation and resulting in the activation of caspase-3. These results are likely to provide insight into the mechanisms by which *C. edulis* leaves mediate cell death in cardiomyocytes, leading to cardiomyopathy. The positive outcomes of our research could be a strong basis for the development of cardiomyopathy associated with khat consumption, which warrants further investigations including in animal model.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Syam Mohan

ABOUT AUTHOR

Dr. Syam Mohan is an Associate Professor at the Medical Research Center, Jazan University, Saudi Arabia. He is currently serves as Director, Central Laboratory and Head of Bio-Medical Research Unit. His research interest is in the area of drug discovery and pharmacological and toxicological evaluation of herbal plants.