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ORIGINAL ARTICLE

Genotoxic Evaluation of Subcytotoxic Concentration of Cathine-Rich *Catha edulis* in Human Peripheral Blood Lymphocytes

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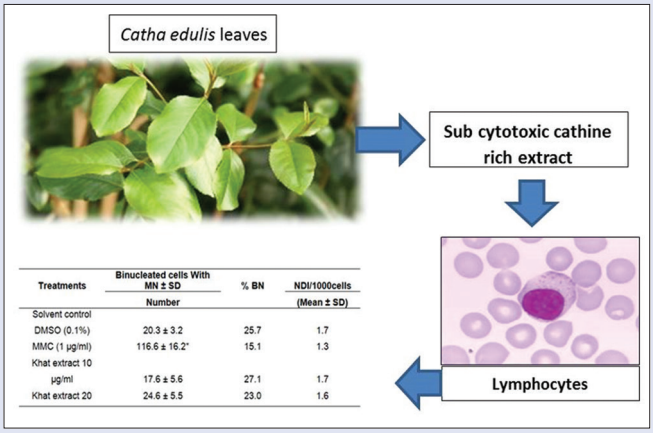
ABSTRACT

Background: *Catha edulis* is an evergreen plant belonging to the *Celastraceae* family, used by the people of Africa, Yemen, and some part of Saudi Arabia as a recreational plant. They chew it alone or in combination with alcohol for attaining euphoria. **Objective:** There are contradictory studies in the genotoxic potential of the leaves of this plant. So far, the accomplished studies were conducted with much high doses. Hence, the current study has been designed to check the genotoxicity of khat-leaves extract with subcytotoxic concentration in human peripheral blood lymphocytes. **Materials and Methods:** We have employed comet assay, micronuclei (MN) analysis, and Ames test to evaluate the objectives. **Results:** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay has revealed that there is a dose-dependent reduction in cell viability with an IC_{50} value of 76.5 µg/ml at 24-h treatment. The results of the comet assay have showed a nonsignificant difference between control and treatment up to 40 µg/ml extract. There was no dose-dependent increase in the frequency of binucleated cells with MN and reduction of cell proliferation in all the doses. The extract showed no mutagenic effect, in both TA98 and TA100 strains even with S9 activation. **Conclusion:** Under the experimental conditions employed in the present study, khat has been found to be nongenotoxic at subcytotoxic level.

Key words: *Catha edulis*, cathine, comet assay, genotoxic, subcytotoxic

SUMMARY

- *Catha edulis* is cytotoxic against peripheral blood lymphocytes
- *C. edulis* leaf extract at subcytotoxic concentration is not genotoxic.



Treatments	Binucleated cells With MN ± SD Number	% BN	NDI/1000cells (Mean ± SD)
Solvent control			
DMSO (0.1%)	20.3 ± 3.2	25.7	1.7
MMC (1 µg/ml)	116.6 ± 16.2*	15.1	1.3
Khat extract 10 µg/ml	17.6 ± 5.6	27.1	1.7
Khat extract 20	24.6 ± 5.5	23.0	1.6

Abbreviations used: DMSO: Dimethyl sulfoxide; GIT: gastrointestinal tract; HPBL: Human peripheral blood lymphocytes; KCL: Potassium chloride; LCQ: Low-content quantification; MMC: Mitomycin C; RPMI: Roswell park memorial institute; SWGDRUG: Scientific working group for seized drugs.

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INTRODUCTION

Catha edulis is the botanical name of a controversial stimulant plant which is well known by its local names such as khat and qat in Yemen, qaat and jaad in Somalia, and Chat, Miraa, and Quaadka in Ethiopia.[1] The plant belongs to *Celastraceae* family. It is a large, slow-growing evergreen shrub found mainly in Africa and Arabian Peninsula. The individuals using this plant simply chew the fresh leaves to make life a little more pleasant. This, also a part of the social activity in rural areas, starts soon after breakfast and continues throughout the day for pleasurable and stimulating effect. It is chewed alone and sometimes together with cigar and alcohol.[2] The prevalence of khat chewing is widespread even in USA and Europe due to the migration of local people from Yemen, Somalia, Eritrea, and Saudi Arabia.

There is a major association between consumption of khat by chewing with some health hazards. Chronic khat abuse can affect exactly every human organ systems and makes long-term adverse effects as suggested by various studies. Central nervous system effects of khat chewing in humans include euphoria and mild excitement, which later progressively substituted by slight dysphoria, anxiety, insomnia, and anorexia.[3] Khat contains a phytochemical called cathinone, which is structurally closely similar to a well-known stimulant amphetamine. It is believed that cathinone is the main reason for the reported central nervous system effects.[4] Apart from stimulation, khat also has been reported to produce hyperactivity and logorrhea. In some case reports, khat consumption has been found to induce, schizophreniform psychosis

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and paranoid psychosis secondary to other psychiatric disorders.[5] Besides, there is growing concern about the effects of khat chewing on the cardiovascular system. It has been found to induce acute myocardial infarction, coronary heart failure, and ischemic conditions.[6] One of the studies suggests the elevation of systolic blood pressure in khat consumers due to stimulant effect in β1 adrenoceptor in the heart.[7] Khat has shown stomatitis, esophagitis, and gastritis in the GIT. Apart from this, anorexia and constipation are frequent with heavy users of khat.[8]

Significant attention has been given in the literature regarding khat's effect in cancer incidence and its clastogenic potential.[8] There are many contradictory studies in this regards. While some studies indicate its potential to induce cancer, others have showed its anticancer effects.[9] The induction of oral cancer is one among them.[10] Apart from oral, head-and-neck squamous cancer has also been reported.[11] However, the reported evidences until now are weak, which is mainly based on anecdotal case reports and uncontrolled studies. In many *in vitro* cell-based assays, the cytotoxic effects of khat have been established. Similar effects have been found *in vivo* rabbits too.[12] Recently, we have studied the mechanism of such cell death and found that the mode of cell death is apoptosis.[13]

Many studies have been reported the genotoxic potential of khat. Al-Zubairi, Ismail, Pei, and Rahmat found that there is no deoxyribonucleic acid (DNA) damage detected in both the khat-treated groups in a rat model but found chromosomal aberrations with high dose of 2000 mg/kg body.[14] In another study, Abderrahman and Modallal found that there is genotoxicity in mice bone marrow cells and that the effect was significant in higher concentrations of khat than in lower concentrations.[15] Tariq, Qureshi, Ageel, and Al-Meshal conducted an evaluation of genotoxic potential of khat in Swiss albino mice at 500 mg/kg of dose, and the study stated that the genotoxic effects may be totally or partially due to the presence of flavonoid components of khat.[16] Kassie, Darroudi, Kundi, Schulte-Hermann, and Knasmüller have found that khat consumption (160 g/day) did not lead to a detectable elevation of micronucleated bladder mucosa cells, and they found that the effects may be due to accompanied alcohol and tobacco consumption.[17] The previous studies all carried out in higher doses, which may probably increase the total percentage of genotoxic phytochemical/phytochemicals. It is crucial to differentiate the genotoxicity alone and toxicity associated with cytotoxicity and DNA damage, which appears only in noncytotoxic concentrations. Hence, to prevent false-positive results because of DNA damage stemming from necrosis or apoptosis, in the current study, we have investigated the genotoxicity in human peripheral blood lymphocytes (HPBL) using a subcytotoxic concentration of khat extract which is rich in cathine (the main phytochemical leads to adverse effects in khat).

METERIALS AND METHODS

Plant collection and extraction

Catha edulis leaves were kindly 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. Briefly, 50 g of leafy material was macerated in a plant mill into very small pieces. Two hundred milliliters of methanol were 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 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/tandem mass spectrometry (LC-MS/MS) for the presence of cathine.

Analysis of khat by liquid chromatography/tandem mass spectrometry

Khat extract was diluted in methanol and water (5:95) before analysis. Aliquots (10 µl) were injected into the LC-MS/MS system consisting of an LCQ Fleet Single Quadrupole Ion Trap Mass spectrometer (Thermo Scientific, USA). The analytes were separated on a Hypersil GOLD column (150 mm × 3 mm i.d.: 5 µm, 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 runtime 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.

Qualitative analysis was performed in the scanning mode, monitoring the following transitions: m/z 150 → 150 and m/z 150 → 132 for cathinone, and m/z 152 → 152 and m/z 152 → 134 for cathine (results were published earlier).

Determination of cytotoxicity

The study was carried out using blood samples from four healthy, nonsmoking male donors, aged 25–38 years after getting approval from ethical committee of Jazan University (Ethical approval: 1436-SCBRE-17). Tubes containing anticoagulant sodium heparin as anticoagulant were used to collect 10 ml of blood by venipuncture. The collected whole blood was centrifuged at 1000 rpm and 0.5 ml of buffy coat-rich plasma was used for cultures. This was used for the determination of cytotoxicity of extract by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay according to Gerlier and Thomasset with slight modification.[18]

Comet assay

The alkaline comet assay was performed according to Collins.[19] Lymphocyte culture was prepared by adding 0.5 ml of buffy coat-rich plasma to 4.5 ml of media RPMI. The media was supplemented with 20% foetal bovine serum, penicillin, and streptomycin. Stimulation of cells was performed by addition of 2% phytohemagglutinin. The cell culture was performed for 24 h then treated with different concentration of khat extract for next 24 h. The subcytotoxic concentration of extract was selected from MTT assay. The cell analysis was performed immediately using commercial kit CometAssay® Kit, Trevigen, according to the manufacturer instruction. To determine the extent and distribution of DNA damage, single-cell gel electrophoresis assay was conducted by examining at least scoring 100 comets from each sample. The analysis has been done according to the fluorescence intensity in the tail and classified to different group as described earlier.[19]

Micronuclei analysis

Micronuclei (MN) analysis was carried out using the standard technique.[20] Lymphocyte culture was prepared by adding 0.5 ml of the buffy coat-rich plasma to 4.5 ml of media RPMI. The media was supplemented with 20% fetal bovine serum, penicillin, and streptomycin. Stimulation of cells was performed by addition of 2% phytohemagglutinin. The cell culture was performed for 24 h then treated with 10, 20, and 40 µg/ml concentration of khat extract. Cytochalasin B was added to the culture at 44 h at a concentration of 6 µg/ml to arrest the cytokinesis. At 48 h, the binucleated lymphocyte cells have been harvested, washed in hypotonic solution (0.075 M KCL) at room temperature, and centrifuged. The cells

were then fixed with fixative (methanol/acetic acid (3:1, v/v)). Air-dried slides were then used for scoring. Scoring has been done according to the method described earlier.[20] In each treatment, the numbers of mononucleated, binucleated, and polynucleated cell per 500 cells per donor were counted for cell cycle kinetic analysis, the Nuclear Division Index (NDI) was calculated.

Ames test

Ames test were conducted following guidelines from the Organization for Economic Cooperation and Development with *Salmonella typhimurium* tester strains TA98 (frameshift) and TA100 (base-pair substitution), using a preincubation methodology both with (+S9) and without (−S9) metabolism. The khat extracts (10, 20, and 40 μg/ml/plates) were dispersed in 100 L sterilizer water by vortexing for 10 min. After that, it has been sonicated for 5 min before the incubation with tester strains. Two hundred milliliters of total strains were used for 4 h at 37°C with 80 rpm shaking. The samples with strains were then diluted with 2 ml motel top agar and transferred to minimum salt agar. 2-nitrofluorene (3 g/plate) and TA98 and nitrofurantoin (5 g/plate) were used as positive control for TA98 and TA100, respectively. All assays were conducted in triplicates for statistical significance.

Statistical analysis

The results obtained in the cytokinesis-block micronucleus (CBMN) and comet assays were subjected to analysis of variance followed by Student's *t*-test (CBMN) and Tukey's test (comet assay). GraphPad Prism® software (Fay Avenue, CA 92037, USA) was used to perform the statistical analyses. In both tests, the results were considered statistically significant at $P < 0.05$.

RESULTS

Cell growth inhibition assay

The inhibition of khat extract on HPBL cells viability was investigated through MTT assay. The cells were treated with different concentrations of khat for 24 h. Figure 1 shows that khat inhibited the cell viability of cells in a dose-dependent manner. The IC_{50} values for was 76.5 μg/ml at 24 h treatment. Up to 40 μg/ml, there was no reduction in cell viability more than 20%. Hence, dose below that level was considered as subcytotoxicity level, which was used for the further investigation.

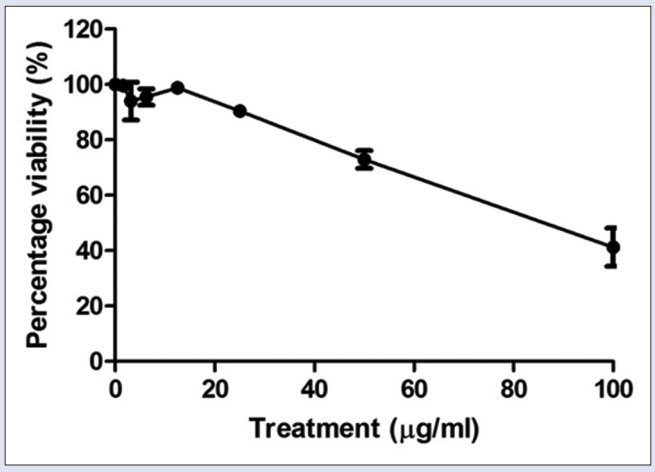

Figure 1: Cytotoxicity analysis of khat extract using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

Comet assay

The results of the comet assay (genotoxicity) are shown in Table 1. When the control was compared with khat treatment, a nonsignificant difference was noted. Even though at 40 μg/ml increased the class 2 comet from 1 to 1.6, the values were not significantly different.

Micronuclei analysis

Results of the MN analysis frequencies and the NDI for 48 h treatment has been obtained [Table 2]. As per the table, there is no significant genotoxicity. No dose-dependent increase in the frequency of binucleated cells with MN observed, even at one of the concentrations tested. By observing the results of NDI (cytotoxic effects of the khat extract on lymphocyte cultures), no reduction in cell proliferation has also been observed till 40 μg/ml concentration.

Ames test

Table 3 shows the number of revertant *Salmonella typhimurium* colonies, and TA98 and TA100 strains per plate, after treatment with the khat extract, in both the presence (+S9) and absence (−S9) of metabolic activation. The mutagenicity test showed that all tested concentrations

Table 1: Mean frequency and standard deviation of damaged cells, average distribution between the classes of damage, and average scoring for the assessment of genotoxicity of khat extract in human peripheral blood lymphocytes cells

Treatment	Comet class				Score
	0	1	2	3	
Control	71.0±2.0	28.0±1.0	1.0±1.7	0.0±0.0	27.0±2.1
DMSO (0.1%)	77.3±2.3	21.6±2.3	1.0±0.0	0.0±0.0	23.6±3.3
10 μg/ml	73.3±4.0	25.6±4.9	1.0±1.0	0.0±0.0	27.1±1.6
20 μg/ml	76.6±2.4	22.0±3.6	1.3±0.5	0.0±0.0	24.6±3.6
40 μg/ml	73.0±3.7	23.6±2.3	1.6±1.5	0.0±0.0	26.8±4.0

DMSO: Dimethyl sulfoxide

Table 2: The micronucleus frequency and Nuclear Division Index in human peripheral blood lymphocytes treated with khat extract and respective controls

Treatments	BN with MN±SD (*n*)	Percentage BN	NDI/1000 cells (mean±SD)
Solvent control	20.3±3.2	25.7	1.7
DMSO (0.1%)			
MMC (1 μg/ml)	116.6±16.2*	15.1	1.3
Khat extract (μg/ml)			
10	17.6±5.6	27.1	1.7
20	24.6±5.5	23.0	1.6
40	26.0±3.6	23.9	1.7

*Significantly different from control $P<0.05$. BN: Binucleated cells; NDI: Nuclear Division Index; MN: Micronuclei; SD: Standard deviation; DMSO: Dimethyl sulfoxide; MMC: Mitomycin C

Table 3: Mutagenic assessment of khat extract in *Salmonella typhimurium* TA98 and TA100 strains with (+S9) and without (−S9) metabolic activation

Dose (μg/ml)	Number of colonies/plate (mean±SD)			
	TA98		TA100	
	−S9	+S9	−S9	+S9
0	36.3±2.5	34.6±3.0	157.0±12.1	170.0±16.0
10	33.0±4.3	31.0±2.6	154.3±11.5	171.3±12.3
20	34.6±7.5	32.0±3.4	167.6±3.0	178.0±16.6
40	34.5±1.4	31.0±5.5	171.3±4.5	191.6±12.8
Positive control	908.3±22.6	-	757.0±24.4	-

SD: Standard deviation

of the extract, before and after metabolism by S9 fraction, produced no mutagenic effects in both the strains. In contrast, the used positive control showed induction of significant mutagenic effects in both strains.

DISCUSSION

Khat dependence is associated with high morbidity and societal and economical costs.[21,22] The khat ingestion in low doses results in several central nervous system effects, while at high doses and chronic use of khat can cause more serious adverse neurological, psychiatric, cardiovascular, dental, gastrointestinal, and genitourinary effects. Fresh leaves of khat contain the alkaloids of the phenylpropylamine type, of which the cathinone and cathine are the most important psychoactive constituents.

Oral administration of khat extract has been found to be associated with dominant lethal mutations together with lethal in mice.[23] Moreover, teratogenic effects also have been found in rats earlier. A study conducted in Africa on khat chewers has been found to be significant levels of micronucleated buccal mucosa cells, and it was 8-fold increases than control. However, the khat consumption did not lead to a detectable elevation of micronucleated bladder mucosa cells.[17] De Hondt, Fahmy, and Abdelbaset had treated rats with methanolic khat extract subcutaneously and studied the acute effect.[24] They observed that the administration of khat in acute level has the capacity to induce chromosomal aberrations. Moreover, Hassan, Gunaid, El-Khally, and Murray-Lyon have conducted a study of khat on bacteria to check the mutagenicity an revealed that an end-to-end association and breaks of chromatids and centric fusion and centromeric attenuation of chromosomes.[25] In addition, khat extract was reported to increase the frequency of micronucleated polychromatic erythrocytes, induced bone marrow depression, and reduce the mitotic index of the somatic cells.[26] Although investigations into the khat effects have spanned various physiological and metabolic effects, to date, there is no investigation on the effects of khat on DNA migration through the comet assay on HPBL.

The results of the present study showed that khat extract induced cytotoxicity on human PBL at higher dose. Meanwhile, the subcytotoxic dose failed to show that khat has the potential to promote genotoxicity. We choose subcytotoxic concentration in this study to make sure the DNA damage is not occurring due to cell death by apoptosis or necrosis. The comet assay (single-cell gel electrophoresis) is a simple method for measuring DNA strand breaks in eukaryotic cells. The current study reveals that there are no significant changes in the DNA damage even at 40 μg/ml dose concentration. Previously, Al-Zubairi, Ismail, Pei, and Rahmat used comet assay in rats to detect the DNA damage.[14] Even at higher dose (1000 and 2000 mg/kg body weight, for 90 days), they could not find any detectable range of DNA damage.

In the present research, we studied the occurrence of MN in human PBL cells treated with khat extract. MN assay is a general test to obtain information on genotoxic effects and on possible carcinogenic risks in humans. MN is extranuclear chromatin bodies in the cytoplasm formed when chromosome fragments or whole chromosomes are not incorporated into daughter nuclei during mitosis.[27] In support of the comet assay, our results revealed that exposure of human PBL to khat extract could not increase the frequency of MN dose-dependently and significantly. Moreover, the results obtained in the NDI showed that the there is no statistical significant cell death. NDI is commonly used as a measure of cytotoxicity.

Mutagenicity is like genotoxicity except that genotoxic effects are not necessarily always associated with mutations. All mutagens are genotoxic, however, not all genotoxic substances are mutagenic.[28] Since the MN and comet assay confirms that the subcytotoxic dose of khat is not genotoxic, we again confirm its mutagenic potential my Ames test. The chemical components of the extract, after undergoing metabolism, might form mutagenic products capable of inducing insertion and/or deletion of DNA base pairs (frameshift mutations). Hence, we conducted the study with and without S9 fraction. Our study showed that the used concentration of khat extract is no longer capable in producing mutagenic effect in TA98 and TA100 strains, thus revealing that low dose of khat is not mutagenic in nature.

In regard to the genotoxic potential, many studies have been carried out with khat extract. However, one thing which differentiates our study is the selection of dose. Kassie, Darroudi, Kundi, Schulte-Hermann, and Knasmüller have been observed that the *Catha edulis* at 20–85 g/day of khat has the potential to induce genotoxicity is significant when it is consumed together with alcohol, but not alone.[17] Al-Zubairi, Ismail, Pei, and Rahmat used 1000 and 2000 mg/kg body weight in rats but could not find any DNA damage in comet assay, instead they found chromosomal aberrations at higher doses.[14] Taken all these studies together and in comparison with our findings, it can be concluded that at lower doses khat may not have genotoxic potential. The capacity of khat to induce clastogenicity may be depended on higher doses use, long duration of use, and concomitant use of other substances of abuse such as cigar and alcohol. Under the experimental conditions employed in the present study, khat is not a genotoxic plant.

CONCLUSION

We suggest that under the experimental conditions employed in this study, khat has been found to be nongenotoxic at subcytotoxic level.

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Conflicts of interest

There are no conflicts of interest.

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