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Evaluation of the Cytotoxic and Genotoxic Potential of Khat (*Catha edulis* Forsk) Extracts on Human T Lymphoblastoid Cell Line

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This paper reports on an investigation of the cytotoxic and genotoxic potential of khat extract using a human T lymphoblastoid cell line (CEM). Exponentially growing CEM cells were cultured for 12 h in the presence of khat extract (0-2000 µg ml⁻¹). Statistically significant, dose-dependent increases in; CEM cell death at dose (> 400 µg ml⁻¹), in DNA damage at dose (>200 µg ml⁻¹) and in micronuclei frequency, at dose (>200 µg ml⁻¹) were observed. The genetic damage effects of khat extract on human cell line observed in this study could serve as a major contribution towards the understanding and creating of awareness of an increased risk of cancer amongst long-term khat consumers.

Key words: Khat (*Catha edulis*), cytotoxicity, genotoxicity, human T- lymphoblastoid cell cells

INTRODUCTION

In countries such as South and North Yemen, Djibouti, Kenya, Madagascar and Tanzania, fresh leaves of the plant khat (*Catha edulis*) are habitually chewed. Typically, a wad of leaves is formed in the mouth and kept there for several hours at a time^[1]. Although it is the leaves of khat, which are chewed, only the juice is swallowed. The result of this chewing is a range of amphetamine-like CNS-stimulant effects, due in large part to the presence of cathinone and cathine in the leaves.

Although several literatures exist on the chemistry and pharmacology of khat and its constituents^[2,3] relatively little is known about the effects of these compounds in biological systems. Khat and cathinone have been reported to produce significant mitodepression and clastogenic effects in root cells of *Allium cepa*^[4,5] and to induce congenital, teratogenic and mutagenic effects in experimental animals^[6,9]. Two epidemiological studies have been performed on human populations. The results of one of the studies revealed an increased incidence of oral cancer amongst long-term khat users, residing in the Asir area in Saudi Arabia^[10]. The other found that khat chewing and water pipe smoking was associated with a high frequency of tumours of the gastro-oesophageal junction or cardia.

The aim of this study was to investigate the cytotoxic and genotoxic effect of khat extract on human T-lymphoblastoid cell lines (CEM).

MATERIALS AND METHODS

Preparation of khat extracts: An aqueous extract of fresh khat was prepared by first washing leaves with sterile distilled water, then 200 ml of distilled water was added to 70 g of the leaves and boiled for 4 h under reflux conditions. After boiling, the extract was filtered through Muslin cheese cloth and reduced in volume. The supernatant was dried under vacuum (Savant Speedvac; Savant Instrumentation Corp, New York). The dried extracts were stored at -20°C until required.

Khat extract doses were expressed according to starting fresh plant material weight. Each ml of plant extract contained 2 g of fresh khat. The dried frozen extracts were reconstituted in distilled water just prior to use.

Cell line and culture conditions: Human lymphoblastoid (CEM) cell line^[11] were used in this study to investigate the cytotoxic and genotoxic potential of khat extract in human cells. The cells were cultured and maintained in RPMI 1640 medium supplemented with L-glutamine

(Gibco), 10% foetal calf serum (FCS) and 1% penicillin-streptomycin (Gibco). For routine maintenance, the cells were maintained at 1.0×10^5 to 5.0×10^5 cells ml^{-1} in plastic T-75 flasks (Corning), in a 5% CO_2 air, humidified atmosphere at 37°C. Exponentially growing cells were used in all experiments.

Pre-treatment of CEM cells with khat extracts: Exponentially growing CEM cells were incubated at 37°C in standard tissue culture flasks with a range of concentrations (0-2000 $\mu\text{g ml}^{-1}$) of khat extracts for 12 h in serum-free medium. All experiments were performed in triplicates.

Estimation of cell viability: The cytotoxic potential of khat extract on CEM cells was assessed following treatment using the trypan blue dye exclusion test. Viable cells, with intact cell membranes, did not take up the dye. Non-viable cells appeared blue^[12].

DNA damage analysis: The ability of khat extract to induce DNA damage in the CEM cells was investigated using the alkaline comet assay, according to the method of Singh *et al.*^[13]. The alkaline comet assay facilitates the detection of DNA single strand breaks and alkali-labile lesions. CEM cells treated with 150 μM hydrogen peroxide for five minutes at 4°C (to induce oxidative DNA damage) were used as positive controls in the alkaline comet assay. Khat treated CEM cells 2×10^4 were embedded in 1% low melting point agarose, overlaid on an existing 1% agarose gel on a frosted microscope slide. The cells on the slides were then lysed in a high salt alkaline buffer (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, 1% (v/v) Triton X-100, pH 10) for at least 1 h and then placed in electrophoresis buffer (0.3 M NaOH, 1 mM EDTA) for 40 min. to allow alkaline unwinding of the DNA, before being placed in a pre-chilled tank for 20 min at 25V, 0.30 amps. Following electrophoresis the slides were washed three times (5 min each wash) in a neutralising buffer (0.4 M Tris pH 7.5). The washed gels were then stained with 50 $\mu\text{g ml}^{-1}$ ethidium bromide and digitally analysed using UV microscopy and Comet 3.0 analysis software (Kinetic imaging, UK), counting 50 cells per slide. Each cell containing DNA damage had the appearance of a comet, when stained with ethidium bromide and viewed under fluorescent light, with a brightly fluorescent head and a tail whose intensity was related to the number of strand breaks (reported as %DNA in comet tail).

Determination of micronuclei frequency: The clastogenic potential of khat extract was assessed using the micronucleus assay. Following treatment of cells with

khat extract, the cells were washed twice with warm RPMI 1640 medium before being centrifuged and resuspended in 1 ml of growth medium at 37°C and incubated in the presence of cytochalasin-B (3.0 µg ml⁻¹; Sigma, Poole, England) for 12 h. The resultant cell suspension was then processed for micronuclei detection using the assay as described in Fenech and Morley^[14]. To visualise the micronuclei, slides were stained with 10% Giemsa, mounted with DPX and examined with a light microscope (x100). The micronuclei frequency was expressed as total number of micronuclei/400 CEM cells.

RESULTS

CEM cell viability following khat treatment: Khat extract concentrations (100-2000 µg ml⁻¹) caused a dose-dependent decrease in cell viability in CEM cells (Fig. 1). The reduction in cell viability was statistically significant from 400 µg ml⁻¹ (p<0.05). Maximum DNA damage was observed at 800 µg ml⁻¹ khat concentration. Treatment of CEM cells with 2000 µg ml⁻¹ of khat extract resulted in 100% CEM cell death (Table 1). Khat concentrations less than 1000 µg ml⁻¹ showed cell viability below 50% (Table 1).

Micronuclei frequency in CEM cells following treatment with Khat: The frequency of micronuclei following treatment of CEM cells with a range of doses of khat extract (0-500 µg ml⁻¹) showed statistically significant (Student t-test) dose-dependent increase in micronuclei frequency, from 200 µg ml⁻¹ with maximum effect at 300 µg ml⁻¹ (Fig. 2). At 1000 µg ml⁻¹ of Khat extracts concentrations, there was a significant reduction in the number of micronuclei frequency with maximum total reduction at 2000 µg ml⁻¹ (Table 2)

Levels of DNA damage in CEM cells following treatment with khat extract: The DNA damage levels in khat treated CEM cells showed a statistically significant dose-dependent increase in DNA damage levels from 200 µg ml⁻¹, with maximum damage at 800 µg ml⁻¹ (p<0.05). At 1000 and 2000 µg ml⁻¹ khat concentrations, maximum percentage tail DNA damage of 36.88 and 42.10% were recorded, respectively (Table 3). Low khat concentrations below 600 µg ml⁻¹ showed low tail DNA damage.

DISCUSSION

The results of this investigation have demonstrated that khat extract, at 200 µg ml⁻¹ doses induced cytotoxic, genotoxic and clastogenic effects in a human cell line *in vitro*. Two epidemiological studies on different human

Table 1: Effect of khat extract on human T-lymphoblastoid cell viability

Khat concentration (µg ml ⁻¹)	Cell viability (%)	P values
0	100.0±0.00	0.00
200	100.0±0.00	0.00
400	92.59±1.01	0.02
600	67.12±6.41	0.04
800	55.2±5.05	0.01
1000	48.08±2.09	0.003
2000	0.0±0.00	0.00

Table 2: Effect of Khat extract concentration on human lymphoblastoid (CEM) cell micronuclei damage

Khat concentration (µg ml ⁻¹)	Number of micronuclei/400 CEM cells	P values
0	8.67	0.00
200	12.60±1.10	0.00
400	16.92±2.60	0.02
600	20.33±2.30	0.04
800	18.33±2.03	0.01
1000	10.00±0.58	0.003
2000	0.0±0.00	0.00

Table 3: Effect of Khat extract on human lymphoblastoid (CEM) cell DNA damage

Khat concentration (µg ml ⁻¹)	Tail DNA %	P values
0	6.58±0.72	0.00
200	18.02±1.88	0.00
400	17.98±1.25	0.02
600	27.26±1.56	0.04
800	32.75±3.55	0.01
1000	36.88±4.12	0.003
2000	42.10±3.60	0.00

populations have been performed however, the results of which suggest that khat use may well be associated with an increased risk of cancer of the upper gastrointestinal tract. In the first study a high frequency of khat chewing and water pipe smoking was reported within a group of 3064 men and women with a high frequency of tumour of gastro-oesophageal junction or cardia^[15]. The second study, on the other hand, reported an increased incidence of oral cancer among 25 long term khat users who were non-smokers residing in the Asir area of Saudi Arabia^[10].

There is evidence that hydrolyzable tannins, which are present in high amounts in khat leaves^[16] may contribute to the cytotoxic, genotoxic and clastogenic effects. Tannins are defined as water-soluble polymeric phenolics that precipitate proteins. They have a large number of free phenolic hydroxyl groups that form strong hydrogen bonds with proteins and carbohydrates. It has been suggested that the cytotoxic effects of khat extract may be caused by the inhibition of *de novo* RNA synthesis^[17] which could be due to the formation of complexes with proteins^[18].

Reports from animal studies have increasingly implicated khat as a carcinogen^[10,15]. The tannin contents of khat have the ability to thicken the mucosa of the oropharynx and oesophagus in human, followed in some cases by malignancy^[19]. Tannins have also been shown to induce nucleosome-size fragmentation in HL-60 cells^[20,21].

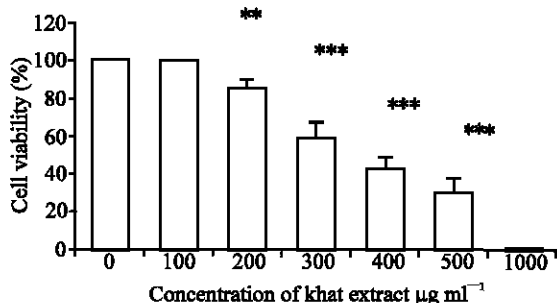


Fig. 1: Survival of CEM cells as a function of Khat extract concentrations. Each concentration is in 3 replicates from two experiments. **=(P<0.01), ***=p<0.0001 level of significance

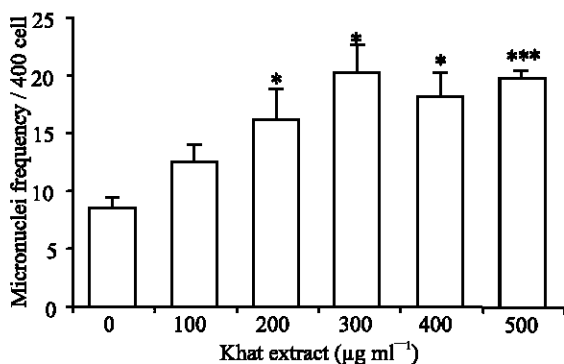


Fig. 2: Micronuclei frequency in CEM cells following treatment with Khat extract. *=Indicates micronuclei frequency significantly higher than control at 5% level of significance (Student t-test). ***=Indicates micronuclei frequency significantly higher than control at 1% level of significance (Student t-test)

Incidences of certain cancers, such as esophageal cancer has been reported to be related to consumption to tannin rich foods, such as betel nuts and herbal teas, suggesting that tannins might be carcinogenic^[22-26].

Other reports have indicated that the carcinogenic activity of tannins might be related to components associated with tannins rather than the tannins themselves^[27]. In an overview of esophageal carcinoma, it was considered that tannin could be an environmental risk factor among others, leading to this kind of cancer^[22,28].

In addition to tannin, there are other components in khat leaves, which may have been present in the aqueous extract, used in this present investigation. For example, terpenoids which have been shown to exhibit cytotoxic activity in human tumour cell lines^[29].

The results of present study indicated that an aqueous extract of fresh khat leaves contains cytotoxic and genotoxic compounds, which may well, contribute to the carcinogenic effects of long-term khat leaf chewing in human.

REFERENCES

1. Kalix, P., 1984. The pharmacology khat. Gen. Pharmacol., 15: 179-187.
2. Kalix, P. and O. Braeden, 1985. Pharmacological aspects of the chewing of khat leaves. Pharmacol. Rev., 37: 149-64.
3. Al-Meshal, I.A., M. Tariq, N.S. Parmer and A.M. Ageel, 1985. The anti-inflammatory activity of the flavonoid fraction of khat (*Catha edulis* Forsk). Agents and Action, 17: 3-4.
4. Kabarity, A. and G. Malallah, 1980. Mitodepressive effect of khat extract on the meristematic region of *Allium cepa* root tips. Cytologia., 45: 733-738.
5. Al-Meshal, I., 1987. Mitodepressive effect of (-)-cathinone, from *Catha edulis* (Khat), on the meristematic region of *Allium cepa* root tips. Toxicology, 25: 451-454.
6. Kamel, A.H., E.E.S. Hussain and E.M. Hammouda, 1967. Khat and congenital abnormalities. Current. Sci., 36: 62-63.
7. Hammouda, E. and E.H. Halbach, 1972. Medical aspects of the chewing of khat leaves. Bull. Narc., 32: 0-21.
8. Hondt, H.A., A.M. Fahmy and S.A. Abdelbaset, 1984. Chromosomal and biochemical studies on the effect of khat extract on laboratory rats. Environ. Mutagen., 6: 851-860.
9. Tariq, M., I.A. Al-Mesheal, N.S. Parmars, A.M. Ageel and S. Qureshi, 1986. Evaluation of genotoxic potential of khat (*Catha edulis*) in Swiss Albino mice, Mutagenesis, pp: 34-40.
10. Soufi, H.E. and M.T. Kameswaran, 1991. Khat and oral cancer. J. Laryngol Otol., 105: 643-5.
11. Foley, G.E. and H. Lazarus, 1967. The response *in vitro* of continuous culture of human lymphoblasts (CCRE-CEM) to chemotherapeutic agents. Biochem. Pharmacol., 16: 659-74.
12. Barnett, Y.A., 1995. Oxidant-induced Stress response in Lymphocytes Cells. Biochem. Mol. Biol. Int., 37: 2.
13. Singh, N.P., M.T. McCoy, R.R. Tice and E.L.A. Schnein, 1988. Simple technique for quantification of low levels of DNA damage in individual cells. Expt. Cell Res., 175: 185-191.
14. Fenech, M. and A. Morely, 1985. Measurement of micronuclei in lymphocyte. Mutation Res., 147: 29-36.

15. Gunaïd, A. and A. Sumairi, *et al.*, 1995. Oesophageal and gastric carcinoma in the Republic of Yemen. *Brit. J. Cancer*, 71: 409-410.
16. Islam, M.W., A.O. Al-Shabanah, M.M. Al-Harbi and A.N.M. Al-Gharably, 1994. Evaluation of Teratogenic Potential of Khat (*Catha edulis* Forsk.) in Rats. *Drug Chem. Toxicol.*, 17: 51-68.
17. Al-Ahdal, M.N., T.J. McGarry and M.A. Hannan, 1988. Cytotoxicity of khat (*catha edulis*) extract on cultured mammalian cells: effect on macromolecule biosynthesis. *Mutation Res.*, 204: 317-322.
18. Jess, D.G. Rees, 1995. Nutritional toxicology of tannins and related polyphenols In forage legumes. *Anim. Sci.*, 73: 1516-1528.
19. Drake, P., 1988. Khat-chewing in the Near East. *Lancet*, pp: 532-533.
20. Sakagam, H., N. Kuribayashi, M. Inada, T. Sakagam, M. Takeda, K. Fukuchi, K. Gomi, H. Ohata, K. Momose and Y. Kawazoe, 1995. Induction of DNA fragmentation by tannin-and lignin-related substances. *Anti-cancer Res.*, 15: 5B 2121-8.
21. Casalini, C., M. Lodovici, C. Briani, G. Paganelli, S. Remy, V. Cheynier and P. Dolara, 1999. Effect of complex polyphenols and tannins from red wine (WCPT) on chemically induced oxidative DNA damage in the rat. *Eur. J. Nutr.*, 38: 190-195.
22. Warner, T.E. and E.A. Azen, 1988. Tannins, salivary proline-rich proteins and Oesophageal Cancer. *Med. Hypotheses*, 26: 99-102.
23. O'Gara, R. and C. Lee, 1971. Carcinogenicity of extracts off selected plants from Curacao after oral and subcutaneous administration to rodents. *J. Natl. Cancer Inst.*, 46: 131.
24. O'Gara, R. and C. Lee, 1968. Biologic Screening of selected plant material for carcinogens. *Cancer Res.*, 28: 2272.
25. Morton, J.F., 1979. Plant tannins and oesophageal cancer, In: Deichmann, W(Ed.). *Toxicology and Occupational medicine (Proce. 10th Inter-Amer. Conf. on toxicology and Occupational Medicine)*, Elsevier, North Holland, pp: 129-137.
26. Morton, J.F., 1992. Widespread tannin intake via stimulants and masticatories, especially guarana, kola nut, betel vine and accessories. *Basic Life Sci.*, 59: 739-65.
27. Chung, K.T., T.Y. Wong, C.I. Wei, Y.W. Huang and Y. Lin, 1998. Tannins. *Human Health: A Review. Crit. Rev. Food Sci. Nutr.*, 38: 6, 421-64.
28. Sharma, D., 1999. Carcinoma of oesophagus-aetiological factors and epidemiology: and overview. *J. Indian Med. Assoc.*, 97: 360-4.
29. Zheng, G.Q., 1999. Cytotoxic terpenoids and flavonoids from *Artemisia annua*. *J. Nat. Prod.*, 62: 1518-21.