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ORIGINAL ARTICLE

Chronic Khat (*Catha edulis*) Chewing and Genotoxicity: The Role of Antioxidant Defense System and Oxidative Damage of DNA

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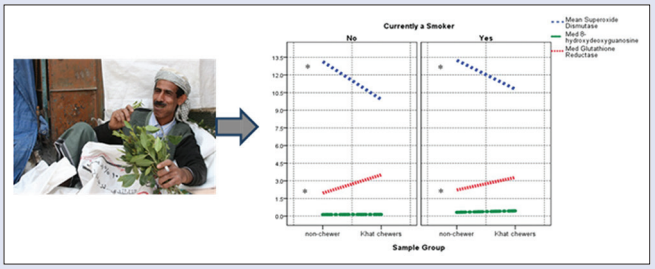
ABSTRACT

Background: Khat chewing is culturally endemic in the Southern areas of Saudi Arabia. Many health-related issues such as the incidence of carcinogenicity and comorbidities associated with the chewing of khat have been reported earlier. **Objectives:** Mainly, the objective of the study was to assess the risk of genotoxicity caused due to oxidative stress that may result from khat chewing among chronic chewers from Jazan Province of Saudi Arabia. **Materials and Methods:** Hundred and twenty-two adult males were recruited after signing informed consent. Ninety participants were chronic khat chewers (CKCs), while the control group consisted of 32 non-chewers of matched age and gender for the study group. A blood sample was collected from all participants and kept at 4°C until the time of assay. The activity of superoxide dismutase (SOD) and glutathione reductase (GR) were estimated in erythrocyte lysate as well as 8-hydroxydeoxyguanosine (8-OHdG) level in plasma. **Results:** There is a decreased SOD activity, which might be due to an increase in the endogenous production of reactive oxygen species resulted from the increase in lipid hydroperoxides. Furthermore, the significant elevation in GR activity was observed, reflecting the presence of highly toxic compounds. The 8-OHdG levels were higher in khat chewers group in comparison to the control, but this increase was statistically insignificant. **Conclusion:** CKCs will be at considerable risk of oxidative stress as a result of a significant reduction in antioxidant enzymes. This would be a good reason for them to quit chewing khat for health benefits.

Key words: Addiction, *Catha edulis*, chronic chewing, detoxification system, DNA oxidative damage, genotoxicity

SUMMARY

- *Catha edulis* (khat) leaves are widely chewed by adults in East Africa, Yemen, and the Southern area of Saudi Arabia for its mild stimulant effect
- Being pharmacology related to amphetamine, it was hypothesized that khat may be promoting the synthesis of reactive oxygen and nitrogen species and would also inhibit serum-free radical scavenging enzymes along with significant inhibition of serum acetylcholine esterase
- The significant elevation in glutathione reductase activity reflects the presence of highly toxic compounds. These compounds would be either exogenous as pesticides that are commonly sprayed in large amounts on khat trees or endogenous as a result of increased production of reactive oxygen species
- Although our study showed a lack of significant increase in the levels of 8-8-hydroxydeoxyguanosine in chronic khat chewers (CKCs), it may still explain, at least in part, the possible correlation between CKC and the oxidative DNA damage through the negative impact on the levels of the antioxidant enzymes.



Abbreviations used: 8-OHdG: 8-Hydroxydeoxyguanosine; CKCs: Chronic khat chewers; EDTA: Ethylenediaminetetraacetic acid; ERK: Extracellular signal-regulated kinase; GR: Glutathione reductase; GSH: Glutathione (reduced form); GSSG: Glutathione (oxidized form); GST: Glutathione S-transferase; HRP: Horseradish peroxidase; JNK: c-Jun NH2-terminal kinase; MAPK: Mitogen-activated protein kinase; MDA: Malondialdehyde; NAC: N-acetyl L-cysteine; PBS: Phosphate-buffered saline; ROS: Reactive oxygen species; SOD: Superoxide dismutase.

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INTRODUCTION

Khat (*Catha edulis* Forsk) leaves are widely chewed by adults in East Africa, Yemen, and the Southern area of Saudi Arabia for a mild stimulant effect.[1-3] Jazan Region in Saudi Arabia has the highest prevalence of khat use in the kingdom.[4] The most controversial and active phytochemical in khat leaves is cathinone, which is both structurally and pharmacologically related to amphetamine.[2-5] Cathinone is always present only in fresh leaves, as it decomposes quickly while drying. Hence, users usually prefer to chew the fresh leaves. Khat chewing is highly prevalent in those areas with a negative impact on the socio-economic and public health status.[6,7] Recently, khat chewing habit

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has been started to move with the immigrants from these countries to different areas of the world.[8,9]

Being pharmacologically related to amphetamine, it was hypothesized that khat may also be promoting the synthesis of reactive oxygen and nitrogen species in the same way. Khat may be also inhibiting free radical scavenging enzymes in the serum along with significant inhibition of serum acetylcholine esterase.[10] It has been shown that khat and/or its alkaloid use may significantly decrease the levels of free radical metabolizing/scavenging enzymes, leading to an increase of free radical concentration and toxicity caused by khat. The same study revealed that oral consumption of khat has been shown to enhance activities of glutathione S-transferase (GST) and catalase but demonstrated no effect on superoxide dismutase (SOD).[11]

A more recent study has also reported that the production of reactive oxygen species (ROS) induced by khat activates ROS scavenger and N-acetyl L-cysteine. Similarly, khat activates the c-Jun NH2-terminal kinase (JNK) and the extracellular signal-regulated kinase. The authors have claimed that khat triggers the generation of intracellular ROS, which induces sustainable activation of JNK, with a subsequent decrease in cell viability and an increase in cell apoptosis.[12]

Among several biomarkers of oxidative DNA damage, the formation of 8-hydroxydeoxyguanosine (8-OHdG) is an omnipresent, predominant marker. 8-OHdG, one of the oxidative DNA damage byproducts, is probably formed and enhanced by chemical carcinogens.[13-16]

Khat is usually chewed and kept inside the mouth for hours during the chewing sessions. Whether these free radicals and other ROS that are constantly being generated *in vivo* and cause oxidative damage to biomolecules would lead to the same effect as the process of khat chewing or not is yet to be known. Hence, we conducted this study with two main objectives. The first objective was to assess the level of DNA damage caused by ROS and the subsequent risk of genotoxicity as a result of chronic khat chewing (CKC) in Jazan. The second objective was to estimate the level of the antioxidant enzymes, SOD, and glutathione reductase (GR) in CKC.

MATERIALS AND METHODS

Subjects

A hundred and twenty-two adult Saudi male volunteers accepted to participate in this study. Ninety of them were CKCs (age; 28.6 ± 6.2). We defined CKC as chewing khat at least 3 days/week regularly for the past 5 years prior to the study. The nonchewer group comprised 32 participants who never chew khat (age; 26.1 ± 4.5). Both the groups were recruited from Jazan Region, at the far South-west of the Kingdom of Saudi Arabia. The snowball sampling technique was used to recruit chronic chewers. The control group comprised the relatives and friends of CKC who never chew khat. All participants have signed an informed consent form prior to be involved in the study. The informed consent was written in accordance with the updated version of the Declaration of Helsinki and the study was then approved by the Institutional Review Board of Jazan University (IRB# CDREC-12).[17]

Exclusion criteria

For both khat chewers and non-chewers, the exclusion criteria consisted of patients experiencing other possible known causes of genetic damage that are present in Jazan Region including hereditary (sickle cell disease and Thalassemia), chronic diseases (diabetes mellitus, hypertension), and occupational or environmental exposures (pesticides, heavy metals, and ionizing radiation).

Erythrocyte lysate preparation

A venous blood sample (5 ml) was collected from all participants using ethylenediaminetetraacetic acid-coated vacutainers. The samples were then kept at 4°C till used for the analysis.

Centrifugation of the collected blood samples was done at 2500 ×*g* for 5 min at 4°C, then red blood cell pellet was washed twice with ×1 phosphate-buffered saline (PBS). The erythrocyte pellet was then lysed in 4 pellet volumes of cold deionized H_2O by mixed well by consecutive pipetting followed by centrifugation at 8500 ×*g* for 10 min at 4°C. After that, the supernatant was transferred to a new clean tube and placed on ice for the determination of SOD and GR enzyme activities. The protein level was estimated by Lowry method.[18] The level of 8-OHdG was estimated in plasma.

Assay of 8-hydroxydeoxyguanosine

The measurement of 8-OHdG was done using a commercial kit (OxiSelect oxidative DNA damage competitive ELISA kit from Cell Biolabs, Inc., USA) with slight modification. Briefly, an 8-OHdG/BSA conjugate preabsorbed microplate was loaded with an unknown sample and 8-OHdG standard in duplicate. 8-OHdG conjugate (1 mg/ml) was diluted to make 1 μg/ml concentration stock using PBS to prepare the 8-OHdG-coated plates. Then, 100 μL of this stock was added to all the wells and incubated at 4°C overnight. The contents of the plates were removed the next morning and the plates were washed one time with dH_2O and the remaining liquid in the plates was completely emptied using a blotting paper. The empty wells were then added with 200 μL of assay diluents and were kept it at room temperature for blocking for 1 h. The plate was transferred to 4°C followed by the removal of assay diluent immediately before use. Fifty microliter of each sample or 8-OHdG standard was added to 8-OHdG conjugate-coated plate. The plate was then incubated at room temperature for 10 min using an orbital shaker followed by the addition of 50 μL of anti-8-OHdG monoclonal antibody to each well, then by three times, it was washed by 250 μL ×1 washing buffer. A 100 μL of horseradish peroxidase secondary antibody-enzyme conjugate was then added to all wells before the incubation of the plate at room temperature for 1 h using an orbital shaker. The plate was then washed three times with ×1 washing buffer, followed by the addition of 100 μL of substrate solution to each well including also blank wells. The plate was then incubated at room temperature using an orbital shaker till the color change (2–30 min) was observed, and then the reaction was stopped by adding 100 μL of stop solution onto each well including also blank wells. Absorbance was then read immediately (to avoid any color fading over time) using a spectrophotometer microplate reader at 450 nm as the primary wavelength. The 8-OHdG level in the unknown samples was calculated from the 8-OHdG standard curve.

Assay of superoxide dismutase

SOD enzyme activity was assayed by an ELISA kit purchased from CAYMAN Chemicals, USA. The assay kit utilized tetrazolium salt for the estimation of superoxide radicals resulted by xanthine oxidase and hypoxanthine enzymes. One unit of SOD is defined as the quantity of enzyme required to exhibit 50% dismutation of superoxide radicals. SOD assay measures all the three types of SOD (Cu/Zn, Mn, and Fe SOD). Each unknown and standard sample was performed in duplicate. The assay was performed by the addition of 200 μL diluted radical detector and 10 μL of sample to each well. Initiation of the reaction was done by adding 20 μL of diluted xanthine oxidase to all wells (of note, xanthine oxidase should be added as quickly as possible). The plate (96 wells) was carefully shaken for a few seconds to mix, covered, and then incubated on a shaker at room temperature for 30 min. The absorbance was then read at 440–460 nm using a plate reader.

Assay of glutathione reductase

GR activity was assayed using GR Assay Kit purchased from TREVIGEN, Inc., USA. The method of assay is spectrophotometric. The oxidation of Nicotinamide adenine dinucleotide phosphate (NADPH) to NADP+ was monitored as a decrease in absorbance at 340 nm. This decreased rate in A_{340} is directly proportional to the enzyme activity of GR in each sample. The unit definition for GR activity was expressed as the oxidation of NADPH or reduction of the oxidized form of glutathione (GSSG) since their molar ratio is 1:1.

All other used chemicals and reagents used were of highest-grade commercial products available.

Statistical analysis

Data analysis was done using SPSS Statistics ver. 17 (SPSS Inc. Chicago, IL, USA) for Windows. Variables were expressed as means ± standard deviation (SD); the difference between the groups was considered significant when $P < 0.05$.

RESULTS

There were total 122 participants in this study, whereas 90 were chewers and the rest were non-chewers. All the participants were in the age group of 17–45 years, with a mean of 27.9 ± 5.9. Among them, 28.6 ± 6.2 years were khat chewers and 26.1 ± 4.5 years were non-chewers. We have checked the height and calculated the body mass index (BMI) as well. The mean (±SD) height and weight of the participants among khat chewers were somewhat high and slightly low, respectively. Whereas, the mean values of BMI were found to be higher among chewers when compared to non-chewers. Nevertheless, there were no significant differences between khat chewers and non-chewers concerning their mean height, weight, and BMI ($P > 0.05$), as shown in Table 1.

The mean (±SD) of SOD was lower among khat chewers in comparison to non-chewers. The reduction in the concentration of SOD was statistically significant [$P = 0.002$; Table 1]. With respect to GR, there was a significant increase in its level in khat chewers group when compared to the corresponding control [$P = 0.001$; Table 1]. The data also showed a slight increase in the level of 8-OHdG in the khat chewers group in comparison to the corresponding control. However, this increase in 8-OHdG levels was only marginally significant [$P = 0.056$; Table 1].

Effect of smoking and other environmental exposures on superoxide dismutase, glutathione reductase, and 8-hydroxydeoxyguanosine

The data showed that the concentrations of the three mentioned enzymes in the Khat chewers and non-chewers groups are not related to the smoking habit [Table 2 and Figure 1].

Effect of short-term chewing (≤5 years) and long-term chewing (>10 years) on the levels of superoxide dismutase, glutathione reductase, and 8-hydroxydeoxyguanosine

These effects are examined by categorizing the chewing group into three levels [Figures 1-3]. The level of each enzyme is compared between non-khat and khat chewing individuals according to their longtime chewing. The data show that the level of SOD in individuals with a long time (more than 10 years) chewing gets to increase again, however, not reaching the same level of non-chewers [Figure 1].

The levels of GR increased in the first 10 years for chewers but again decreased slightly after 10 years. This reduction in the level of GR still differs, however, for non-khat chewers [Figure 2]. The data also show the same behavior for the enzyme 8-OhdG [Figure 3].

Table 1: Basic and biochemical characteristics of khat chewers and non-chewers

Characteristics	Mean±SD		Differences	*P*
	Khat chewers (*n*=92)	Non-chewers (*n*=30)		
Age (years)	28.6±6.2	26.1±4.5	2.5	0.045
Weight (kg)	70.6±18.1	65.8±16.6	4.8	0.201
Height (cm)	168.7±6.5	169.1±7.6	−0.4	0.772
BMI (kg/m^2)	24.8±6.3	22.9±5.1	1.9	0.136
Khat/week	4.4±2.1	0		
Used for (years)	12.3±6.6	0		
SOD (IU/L)	10.5±4.2	13.2±3.8	−2.7	0.002
GR (mU/ml)	4.5±3.4	2.8±1.9	1.7	0.001*
8-OHdG (ng/ml)	0.8±0.8	0.6±0.6	0.2	0.056*

*Mann-Whiney U-test. This test is conducted since the normality condition is not satisfied. For other measurements *t*-test is used. SOD: Superoxide dismutase; BMI: Body mass index; GR: Glutathione reductase; 8-OHdG: 8-hydroxydeoxyguanosine; SD: Standard deviation

Table 2: Comparison of chemical characteristics between khat chewers and non-chewers regarding the smoker status

Characteristics	Status	Khat chewers (*n*=92)		Non-chewers (*n*=30)		Differences	*P*
		Median	Minimum-maximum	Median	Minimum-maximum		
SOD							
Currently a smoker	No	8.9	1.4-19.8	13.8	5.3-19.6	−3.2	0.045*
	Yes	10.1	2.7-20.3	13.2	13.2-5.3	−2.4	0.021*
GR							
Currently a smoker	No	3.5	1.6-13.6	2.0	1.0-7.3	1.5	0.009
	Yes	3.3	1.0-14.3	2.2	1.1-7.3	1.1	0.028
8-OHdG							
Currently a smoker	No	0.2	0.1-2.5	0.1	0.1-1.5	0.1	0.386
	Yes	0.4	0.1-2.6	0.3	0.1-1.7	0.1	0.125

*Mean that *t*-test is used. The other *P* value are for the Mann-Whiney U-test. This test is conducted since the normality condition is not satisfied. SOD: Superoxide dismutase; GR: Glutathione reductase; 8-OHdG: 8-hydroxydeoxyguanosine

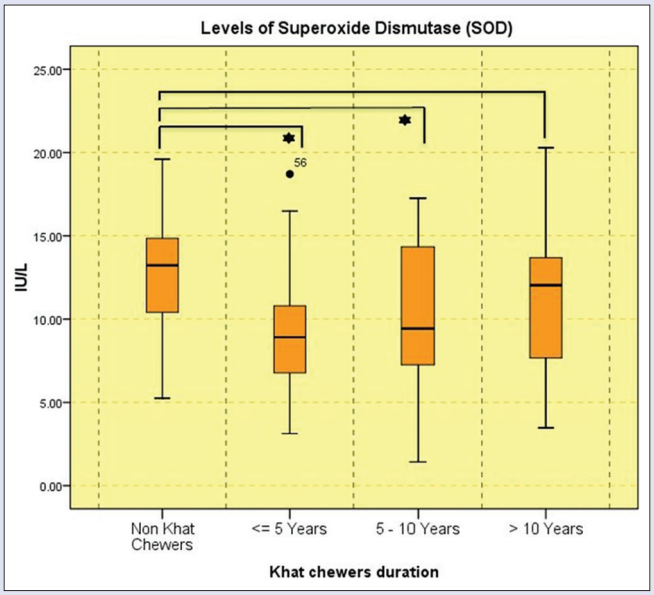

Figure 1: Box plot graph for the levels of superoxide dismutase, representing the highest, lowest, and median values in the non-khat chewers group and khat groups according to short- and long-term chewing. The symbol "*" indicates that the mentioned differences is statistically significant according to the adjusted Bonferroni, $P < (0.05/3 = 0.017)$

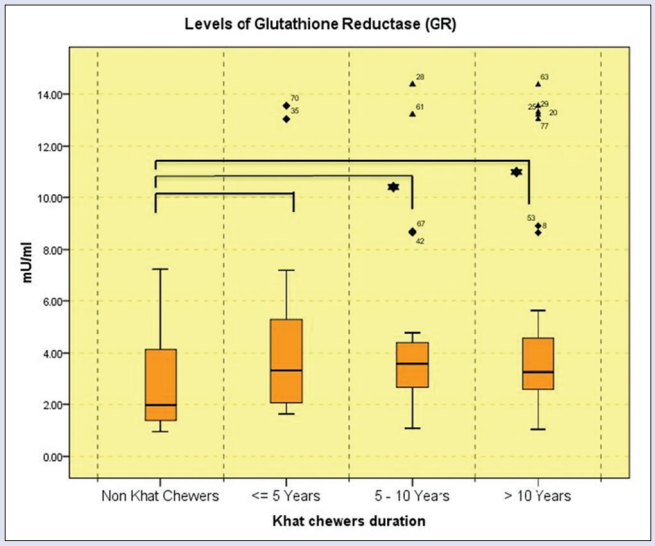

Figure 2: Box plot graph for the levels of glutathione reductase, representing the highest, lowest, and median values in the non-khat chewers group and khat groups according to the short- and long-term chewing. The symbol "*" indicates that the mentioned differences are statistically significant according to the U-test using adjusted Bonferroni, $P < (0.05/3 = 0.017)$

There was no significant effect of fruit/vegetable consumption, fast food dependent diet, energy drink consumption, and soft drinks consumption on the level of any of the investigated enzymes in the studied group.

DISCUSSION

In the current study, we investigated the possible influence of CKC on the antioxidant status by assessing the impact of this habit on antioxidant enzymes SOD and GR. In addition to this, the potential oxidative destruction of the DNA has been explored by measuring a surrogate biomarker, 8-OHdG. The results had showed that CKC has significantly reduced the levels of SOD in comparison to the corresponding control group. Meanwhile, the level of GR was significantly elevated, and a marginal, yet, a significant elevation in the level of 8-OHdG was observed in the khat chewers group. In this context, a recent study from Yemen reported a similar reduction, in the SOD and catalase activities, which agrees with our findings. This was explained as an indicative of cellular protein damage caused by an increased ROS production. The authors have also found an increase of GST, claiming that this could be explained by the leakage of cellular GST to the bloodstream.[19]

However, the drop in SOD activity may be due to an increase in the production of ROS, which could be resulting from the increase in lipid hydroperoxides. GR is the enzyme facilitating the reduction of GSSG to the reduced form of glutathione (GSH). The link between GR and oxidative stress was proven by its role in ROS-releasing macrophage in oxidative response. It was observed that higher persistent response was present in cells with no GR.[20]

The significant elevation in GR activity reflects the presence of highly toxic compounds. These compounds would be either exogenous as pesticides that are commonly sprayed in large amounts on khat trees or endogenous as a result of increased production of ROS. In a previous study, we were able to detect eight pesticide multiresidues in khat leaves consumed in the Jazan Region.[21]

GSH, which is present in most organs and cellular systems, is significantly protecting cells from oxidative injury, that is, why the decline in the GSH levels is associated with some pathological conditions, for example, chromosomal DNA fragmentation, cataract, and cystic fibrosis.[22] A recent report has even described free radical formation and GSH depletion as a possible early cause of khat cytotoxicity.[22,23] In addition, it was reported that there is a significant drop in the levels of erythrocyte SOD and catalase in khat chewers; on the other hand, an elevation in serum GST was observed. Moreover, there were depletions of GSH and Vitamin C along with an elevation of malondialdehyde (MDA) in khat chewers in comparison to controls. This might reflect an obvious oxidative status.[19]

Earlier studies have showed that rats treated with alkaloids fraction of khat extract significantly reduced the capacity of free radical metabolizing/scavenging enzymes and led to increased free radical concentration and followed by higher oxidative stress.[11] The possible effects of the sustained oxidative stress induced by khat consumption may lead to the development of several pathologies, including liver toxicity, cardiovascular toxicity, neurodegenerative disorders, and cancer.[24] On the other hand, another study has also reported that CKCs had higher levels of free radicals observed in their serum samples.[10] This increase in the levels of free radicals could either be due to khat consumption or the pesticide contamination of khat. In this regard, we have already reported the contamination of khat leaves with many pesticides as mentioned previously.[21]

Usually, the oxidative stress encompasses DNA damage, which is associated with the release of derivatives of nucleoside oxidation. 8-OHdG is one of the oxidized nucleosides that are excreted in body fluids during DNA repair. Its levels have been positively correlated with neurodegenerative disorders, cancers, and chronic inflammatory conditions.[25] Other literature showed that continuous reparation of the oxidized DNA is followed by the excretion of excised deoxyribonucleotides in the serum and urine. Multiple studies have reported the presence of 8-OHdG as an important biomarker of oxidative stress in bodily fluids.[26] 8-OHdG has been reported as the predominant biomarker of free radical-induced

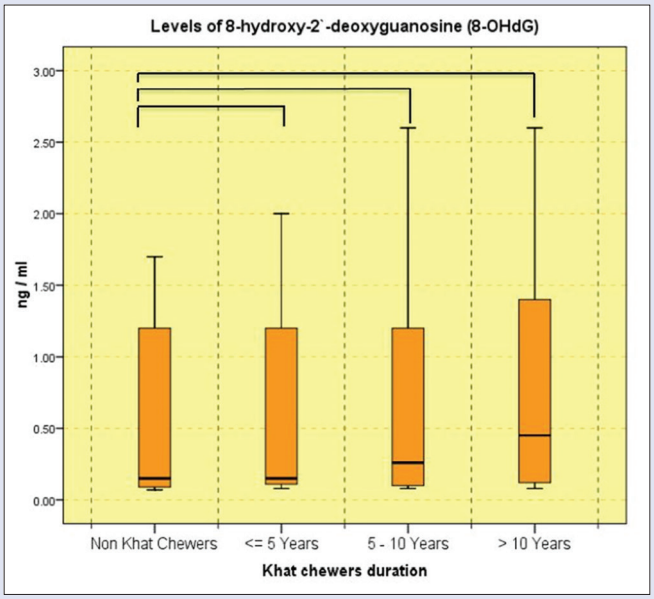

Figure 3: Box plot graph for the levels of 8-hydroxydeoxyguanosine, representing the highest, lowest, and median values in the non-khat chewers group and khat groups according to the short- and long-term chewing. U-test has showed no significance results between the non-khat chewers and others mentioned categories

oxidative damage of both mitochondrial and nuclear DNA. In addition, it is extensively utilized as a biomarker for assessing the chances of various neoplasms and degenerative diseases in clinics where it functions as an initiation and promotion factor of carcinogenesis. Recently, 8-OHdG started being used as a biomarker for the quantification of oxidative DNA impairment and to assess the risk of developing many diseases and cancers, especially after exposure to environmental carcinogens endogenously.[27]

Although our study presented a marginally significant rise in the levels of 8-OHdG in CKCs, it may still explain, at least in part, the possible correlation between CKC and the oxidative DNA damage through the negative impact on the levels of the antioxidant enzymes. To the best of our knowledge, no previous study has reported the relationship between CKC and the level of 8-OHdG as a surrogate biomarker of DNA damage in human blood.

Studies have showed that the consumption of khat is not always alone; sometimes, it is chewed together with tobacco and or alcohol. It has been, however, reported that khat consumption, especially if accompanied by alcohol and/or tobacco, might be a potential cause of oral neoplastic events and genotoxic in nature. This notion was consolidated by the 8-fold increase in the micronucleated buccal mucosal cells among khat chewers.[28] However, this observation was concluded by highlighting the influence of smoking effects, which is a definite cause of increasing micronuclei.[22]

In line with our findings, a very recent study has demonstrated that treatment with 400 μg/ml of khat extract was able to induce cell death via apoptosis mechanism in breast cancer, with an increase in the expression of the proapoptotic mitochondrial protein, Bax, and a decrease in the expression of B-cell lymphoma.[2] In addition, a decrease in ROS levels in a time-dependent manner was also reported. The authors have suggested that khat induces cell death in MDA-MB-231 cells via MAPK activation and triggering the mitochondrial (intrinsic) pathway of apoptosis. MAPK is an enzyme which regulates cell proliferation, survival, and cell differentiation.[29]

One limitation we faced during this study was the availability of nonchewer individuals. It was difficult to obtain a khat nonchewing subject (never chew khat) from our study region since khat chewing is a very popular habit. Accordingly, the age matching was not perfect between the CKCs and non-chewers groups.

CONCLUSION

Our study showed that that chronic daily chewing of khat certainly induces ROS production, potentially caused oxidative toxicity. Both enzymatic and nonenzymatic antioxidants were responsible for protection against this oxidative toxicity. ROS endogenous formation increased in CKCs in correlation with a decreased SOD level. Meanwhile, GR activity elevation reflects the presence of highly toxic compounds. Habitually, long-term khat chewers will be susceptible to oxidative toxicity and possible to DNA damage; therefore, they are strongly recommended giving up khat chewing**.** This study showed an increase in the levels of 8-OHdG in CKCs that may have been statistically insignificant, yet it may still explain an important role played by CKC in the DNA damage process.

CKCs found to be at high risk of oxidative stress with DNA damage. The endogenous oxidative damage to DNA initiates and promotes the carcinogenic process. This result adds to the role of CKC as a potential risk for health. Khat chewers are highly recommended to quit this habit for better health and quality of life. More studies are required to investigate the role of khat chewing short and long duration on the genotoxicity and antioxidant defense mechanism.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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