

Article

Cytotoxic Influence of Khat (*Catha edulis (Vahl) Forssk. ex Endl*) on Oral Fibroblasts, Squamous Carcinoma Cells, and Expression of α Smooth Muscle Actin

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Abstract: The aim was to determine the cytotoxicity of Khat (*Catha edulis (Vahl) Forssk. ex Endl*) on normal oral fibroblasts (NOFs) and SCC4 (squamous carcinoma cells) along with expression of α -smooth muscle actin (α -SMA) in fibroblasts. Khat filtrate was prepared to obtain a concentrated viscous solution. NOFs and SCC4 cells were cultured in biological cabinets and were grown in Dulbeccos' modified Eagles medium. Frozen cells were thawed at 37 °C and cell seeding was performed. NOFs and SCC4 cells were seeded on 96 well plates and allowed to attach. The medium was removed and a fresh medium containing different concentrations of Khat was added. The group without Khat served as a negative control and 4% paraformaldehyde as the positive control. Cell viability was assessed using the MTT assay and effect of Khat on fibroblast and SCC4 phenotypes was evaluated by immunostaining. Analysis of variance was used to assess data ($p < 0.05$). NOF 316 showed cell death in response to 4% paraformaldehyde, 12.5, 6.25, and 3.12 mg/mL of Khat. The highest concentration of Khat (25 mg/mL) failed to cause cytotoxicity of NOF 316. NOF 319 and NOF 26 displayed cell death at all concentrations of Khat, however, cytotoxicity was not dose dependent. NOF 18 and SCC4 cells showed dose-dependent cell death. NOF 316 showed α -SMA expression after 1 mg/mL of Khat exposure. Not all fibroblasts were α -SMA-positive, suggesting specific activation of a subset of fibroblasts. Khat is cytotoxic to NOF and SCC4 cells. Furthermore, it can also cause activation and phenotypic changes in oral fibroblasts, indicating a potential role in progression of oral squamous cell carcinoma.

Keywords: normal oral fibroblast; squamous carcinoma cell; Khat; myofibroblasts; cytotoxicity; cell death

1. Introduction

Oral cancer is considered to be a significant health problem, as around ninety percent of all oral cancer cases are oral squamous cell carcinomas (OSCC) [1]. It has a worldwide

distribution and is the sixth most commonly occurring cancer globally [2]. The major risk factors associated with oral cancer are tobacco smoking and alcohol consumption. Other established risk factors i.e., betel quid chewing, use of smokeless tobacco, human papilloma viruses (types 16 and 18), nutrient deficiency, exposure to solar radiation, and genetic predisposition also causes OSCC [3]. However, there exists a great difference in inter- and intra-country distribution of these causative factors, which possibly explains the different geographical pattern of the disease [1]. One such product was reported to cause malignant and pre malignant oral lesions and is routinely used in the Arabian Peninsula as Khat [4].

The increasing use of Khat (*Catha edulis* (Vahl) Forssk. ex Endl) is associated with grave health hazards [5]. Chronic consumption of Khat affects almost every organ of the human body. It also leads to oral histopathological changes i.e., hyperkeratosis, epithelial hyperplasia, and mild dysplasia [6]. Lukandu et al. revealed that Khat induces abnormal epithelial differentiation and decreases basal cell proliferation [7]. Similarly, a study conducted by Al-Ahdal et al. reported that the cytotoxicity and mutagenicity of Khat leaves extract on human cells [8]. Khat performs its genotoxic activity by inhibiting de novo synthesis of proteins, RNA, and DNA, to reduce free radical metabolizing enzymes, induces oxidative stress, and provokes caspase-dependent apoptosis in various leukemic cells [9,10]. However, studies on its toxicological potential remain scarce.

Previously, it was believed that epithelial genetic changes were the sole cause of oral pre-cancer and cancer, but recent reports showed that progression of carcinoma is derived from the epithelial component as well as mesenchymal tissue [11]. The connective tissue stroma in the vicinity of the tumor plays an important part in oral cancer progression [12]. Normal oral fibroblasts (NOFs) are cells of mesenchymal origin and are omnipresent in almost all tissues within the stroma or connective tissue [13]. In precancerous and cancerous lesions, NOF are modified or 'activated' into myofibroblasts (MFs) and express α -smooth muscle actin (α -SMA) due to mechanical cell stress and tumor stimulating factors [14]. These MFs tend to differentiate in the presence of the transforming growth factor (TGF)- β , another factor that increases in fibrotic lesions. Studies showed that the stroma surrounding the tumor assists in metastasis and malignant progression [15]. However, the role of mesenchymal tissue and NOFs in disease progression due to Khat is not yet determined.

Considering the available indexed literature, it was found that numerous studies showed adverse effects of Khat on oral mucosa. Whereas, its role in OSCC, particularly in relation to connective tissue/stromal changes is largely uncharacterized. Therefore, it is hypothesized that Khat is cytotoxic to the NOFs and SCC4 cells (squamous carcinoma cells). It is also hypothesized that it will exhibit positive expression for α -SMA in fibroblasts. Hence, the aim of the current study was to determine the cytotoxicity of Khat on NOFs and SCC4 cells, along with the expression of α -SMA in fibroblasts.

2. Materials and Methods

2.1. Khat Preparation

Khat (mature) used in the present study was commercially obtained from Sana, Yemen. The Khat extraction method was similar, as previously explained by Aziz et al. [16]. Small pieces of frozen Khat leaves (100 gm) were dissolved in 100 mL of 95% ethanol. The prepared solution was centrifuged at 5000 rpm for 5 min and filtered using Whatman filter paper (no.1). After filtration, 100 mL of ethanol were added to the remaining leaves and the procedure was repeated. Rotary evaporator (EYELA, N-1001S-W, Tokyo, Japan) at 70 rpm was used to concentrate ethanolic Khat at 30 °C, until 30% of ethanol was left. The concentrated viscous solution obtained was then diluted using 100 mL of distilled water and stirred at ambient temperature for 1 h. The filtrate was frozen for 24 h by keeping it at -80 °C and then dried by lyophilization (Labconco, Kansas City, MO, USA). Every 100 g of dried Khat leaves gives 8 g of Khat in powder form. Liquid chromatography was performed to confirm the presence of alkaloids, i.e., 80% cathine and 20% norephedrine. Cathinone was not discovered in the analysis. All materials and equipment used in the

study are presented in Appendix A. Dimethyl sulfoxide (DMSO) was used a vehicle for suspension of Khat extract.

2.2. Culture of NOFs and SCC4 Cells

2.2.1. Cell Lines and Culturing

NOFs in the present study were obtained after ethical approval from patients undergoing third molar extractions at Charles Clifford Dental Hospital. NOFs and SCC4 cells (American Type Culture Collection/ATCC CRL-1624) were cultured in Biological Class II sterile laminar flow cabinets. In order to maintain cell viability, the confluence medium was routinely changed and cells were maintained at 37 °C in a 5% CO₂ incubator. Details of cells used in this study are provided in Table 1.

Table 1. Cells used in the study.

Cell Line	Type	Origin
NOF 18	Primary cells	Normal Oral Fibroblasts
NOF 26	Primary cells	Normal Oral Fibroblasts
NOF 316	Primary cells	Normal Oral Fibroblasts
NOF 319	Primary cells	Normal Oral Fibroblasts
SCC4	Cell line	Squamous Cell Carcinoma

2.2.2. Cell Growth Medium

All cells were grown in Dulbeccos' Modified Eagles Medium (DMEM) (Invitrogen) with supplements (Table 2).

Table 2. Medium used for growing primary cells and SCC4 cell line.

Composition	Volume	Remarks
Dulbecco's Modified Eagles Medium (DMEM) supplemented by 4500 mg/L glucose, GlutaMAX™ I and Sodium Pyruvate	450 mL	Invitrogen, UK lot- 1250148 lot- 1122288 lot- 1369047
10% Fetal Bovine Serum (FBS)	50 mL	Biosera, East Sussex, UK
Pen/Strep (Antibiotics)	5 mL 100 IU/mL Penicillin, 100 µg/mL Streptomycin	Sigma Aldrich, Dorset, UK

2.2.3. Thawing and Seeding Cell Lines

Frozen cell vials were removed from liquid nitrogen and thawed in a water bath at 37 °C. Thawed cells were moved to a sterile universal tube containing 9 mL of pre-warmed medium and centrifuged at 1000 rpm for 5 min. The supernatant was discarded, the cell pellet was re-suspended in 10 mL fresh culture medium, and moved to a 75 cm² tissue culture flask.

2.2.4. Adherent Cell Sub-Culturing and Passaging

Inverted microscope (Axioinvert Carl Zeiss) was used to visualize the cell growth. Sub-culturing was performed when 80% of confluence was reached. To passage the cells, the medium was removed; cells were washed twice in a 10 mL solution of Ca²⁺ and Mg²⁺ free PBS. Trypsin/EDTA solution was added and flasks were placed in the incubator for 10 min. Trypsin was neutralized by fresh medium and the cells were centrifuged, resuspended, and counted using a hemocytometer.

2.3. MTT Assay

2.3.1. Seeding Cells in 96 Well Plates

1.5×10^4 NOFs and 1×10^4 SCC4 cells (in 100 μ L per well) were seeded on 96 well plates and allowed to get attached for 24 h.

2.3.2. Khat Exposure

Khat at a concentration of 25 mg/mL was used throughout this study, which was diluted accordingly. The extract was filtered using 0.2 μ m Merck Millipore express filters in a liquid form. After 24 h, 96 well plates were checked under the inverted microscope to ensure cell attachment. The medium was removed and a fresh medium containing 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, and 0.19 mg/mL of Khat was added. The group without Khat served as a negative control and 4% paraformaldehyde as the positive control. Paraformaldehyde was dissolved in DMSO and the control wells were also treated with it. The 96 well plate was incubated for a further 24 h at 37 °C in a 5% CO₂ incubator. In addition, a triplicate of each concentration was run and the average values were taken.

2.3.3. Determination of Cytotoxicity/Metabolic Activity

Cell viability was assessed using the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay, a yellow tetrazole. MTT solution (Sigma Aldrich) (0.5 mg/mL in PBS–100 μ L/well) was added after removing the medium and washing the cell, using PBS solution. The unreacted MTT solution was removed after one hour and newly formed purple intracellular formazan salt was solubilized using acidified isopropanol (50 μ L/well). Absorbance was measured at 540 nm with a reference at 630 nm, using a spectrophotometer (Tecan) and Magellan software. Background subtraction was done and optical densities were normalized to a control sample of the untreated cells. Data were entered and analyzed through Microsoft Excel. Analysis of variance (ANOVA) was used to determine the significance of data obtained from MTT assays.

2.4. Effect of Khat on Fibroblast Phenotype

2.4.1. Immunostaining Preparation

2×10^4 cells per well were seeded on sterile coverslips in 24 well plates. The plate was incubated for 24 h in 5% CO₂ at 37 °C. the old medium was removed from all wells and the cells were washed with 1 mL of medium. Cells were then exposed to Khat concentrations of 1 mg/mL and 0.5 mg/mL; and the 40 μ g/mL of Transforming Growth Factor- β (TGF- β) acted as a positive control. A total of 500 μ L of medium without Khat or TGF- β acted as negative control.

2.4.2. Immunofluorescence for SMA

The medium from the 24 well plate was removed and the cells were washed twice in PBS solution, followed by fixation in 100% 1 mL methanol for 10 min. The coverslips were washed once in 0.5 mL of 4 mM sodium deoxyclate in PBS and permeabilized in the same solution for 10 min. Sodium deoxyclate was removed and the coverslips were blocked using 500 μ L of 2.5% Bovine Serum Albumin (BSA) in PBS for 30 min, followed by an incubation with 0.5 mL of FITC-conjugated anti-alpha smooth muscle actin antibody (Sigma, Clone1A.4, dilution 1:1000) for one hour at 37 °C, in dark. An IgG isotype antibody was used as a negative control. After 1 h, the coverslips were washed in PBS and placed on glass slides containing 50 μ L DAPI (vectorized). Glass slides were kept at 3–4 °C for a day and were viewed using a fluorescence microscope (Zeiss Axioplan 2, imaging with software Proplus 7.0.1. Presence of a green staining was considered to be positive.

3. Results

3.1. MTT Assay

3.1.1. NOF 316

MTT Assay was performed to determine the viability of NOF 316 primary cells, after being exposed to different concentrations of Khat. Cell death was seen in response to 4% paraformaldehyde (positive control), 12.5, 6.25, and 3.12 mg/mL concentrations of Khat, as compared to the negative control ($p < 0.05$). However, the lower doses of Khat (1.56, 0.781, 0.39, and 0.195 mg/mL) did not display significant cytotoxicity. Interestingly, at the highest dose, i.e., 25 mg/mL, Khat did not display a significant increase in cell death (Figure 1).

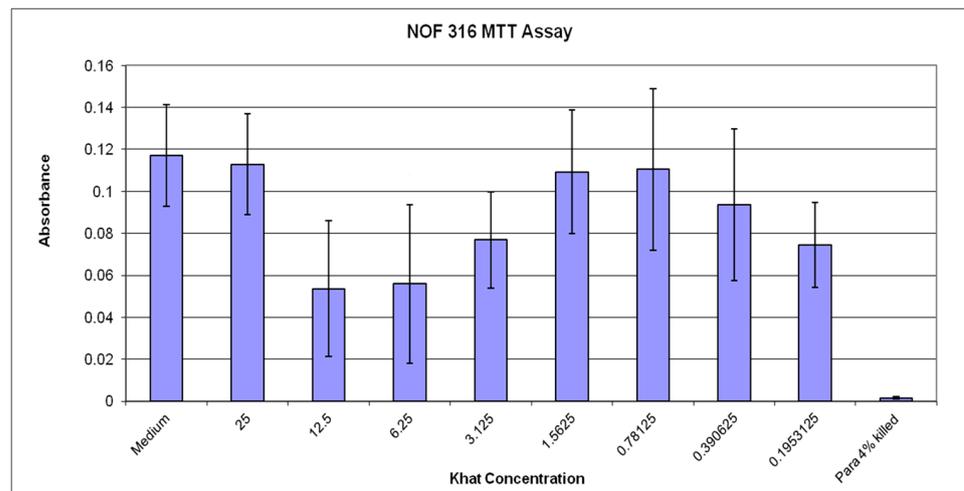


Figure 1. Absorbance from MTT Assay for NOF 316 cells treated with different concentrations of Khat solution (25 mg/mL to 0.195 mg/mL). Medium with only NOF 316 was used as a negative control while 4% paraformaldehyde was used as a positive control showing complete cell death.

3.1.2. NOF 26

Results for NOF 26 were somewhat different from NOF316, as nearly all concentrations of Khat caused a significant increase in cell death, as compared to the negative control ($p < 0.01$). A similar response was observed in the positive control and cytotoxicity appeared to be dose dependent (Figure 2).

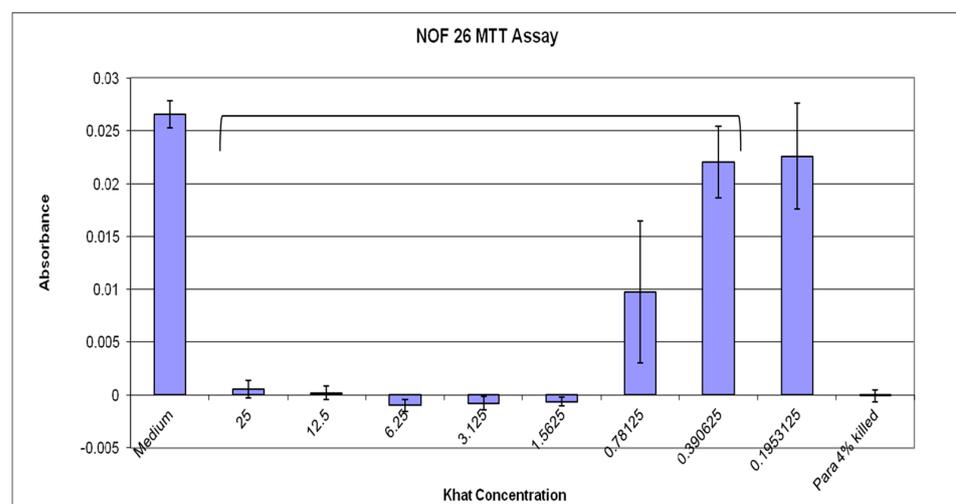


Figure 2. Absorbance from MTT Assay for NOF 26 cells treated with different concentrations of Khat solution (25 mg/mL to 0.195 mg/mL). Medium with only NOF 26 was used as a negative control, while 4% paraformaldehyde was used as a positive control showing complete cell death.

3.1.3. NOF 319

NOF 319 cells showed a similar pattern to NOF 26 cells, as all concentrations of Khat caused significant cell death ($p < 0.01$). However, cytotoxicity did not appear to be dose-dependent. However, the positive control caused significantly more cell death, as compared to Khat ($p < 0.01$) (Figure 3).

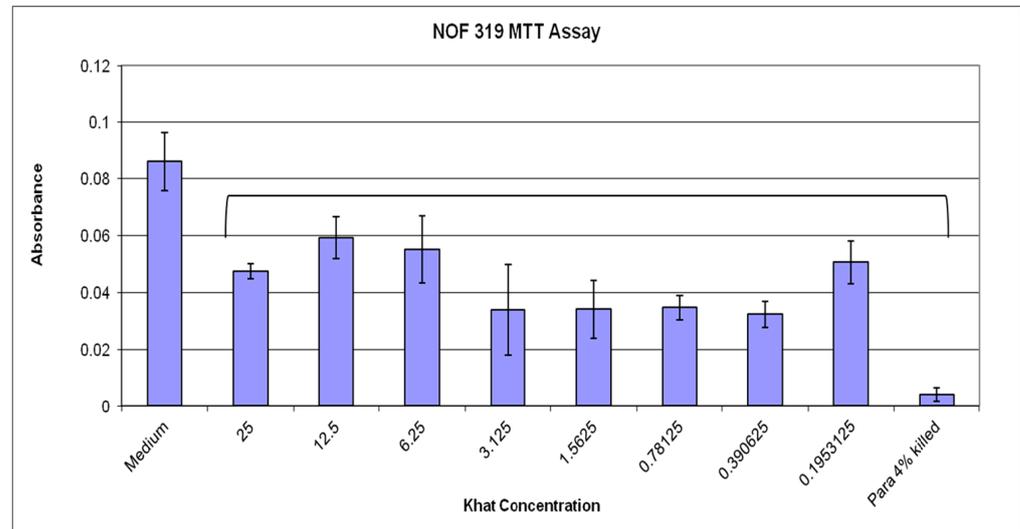


Figure 3. Absorbance from MTT Assay for NOF 319 cells treated with different concentrations of Khat solution (25 mg/mL to 0.195 mg/mL). Medium with only NOF 319 was used as a negative control, while 4% paraformaldehyde was used as a positive control showing complete cell death.

3.1.4. NOF 18

Exposure of NOF 18 cells to different Khat concentrations resulted in a significant increase in cell death, as compared to the negative control ($p < 0.01$). In addition, the extent of cell cytotoxicity was dependent on Khat concentration, showing a dose-dependent response (Figure 4).

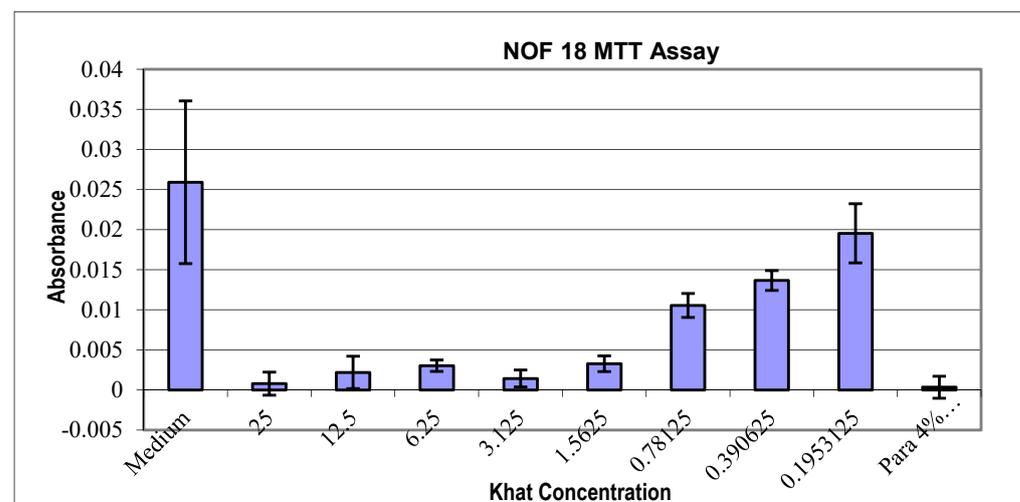


Figure 4. Absorbance from MTT Assay for NOF 18 cells treated with different concentrations of Khat solution (25 mg/mL to 0.195 mg/mL). Medium with only NOF 18 was used as a negative control while 4% paraformaldehyde was used as a positive control showing complete cell death.

3.1.5. Cytotoxicity of Different NOF's

A comparison of the different batches of NOFs used showed a somewhat similar trend with the highest Khat toxicity seen between 1.56 to 12.5 mg/mL concentrations. Surprisingly, in some cells, the highest concentration (25 mg/mL) failed to cause cell death. However, a significant variation between the different cells was observed (Figure 5).

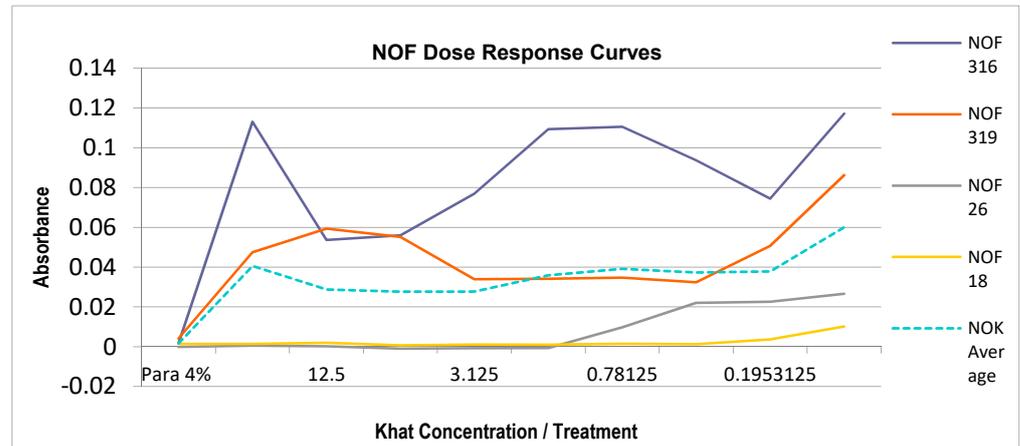


Figure 5. Comparison of absorbance values from MTT Assay for NOF 18, NOF 26, NOF 319, and NOF 316 cells treated with different concentrations of the Khat solution (25 mg/mL to 0.195 mg/mL). Medium only with cells specific for each experiment was used as a negative control, while 4% paraformaldehyde was used as a positive control showing complete cell death. Aqua dotted curve = average of the 4 assays.

3.2. SCC4 Cells

The SCC4 cells also displayed concentration-dependent cytotoxicity. The highest cytotoxicity was seen after cells were incubated with 25–3.125 mg/mL of Khat, which was significantly different from the negative control ($p < 0.005$). However, lower doses of Khat (1.56–0.19 mg/mL) failed to elicit a significant response, with an increase in number of cells seen with two concentrations (1.5625 and 0.1953125) (Figure 6).

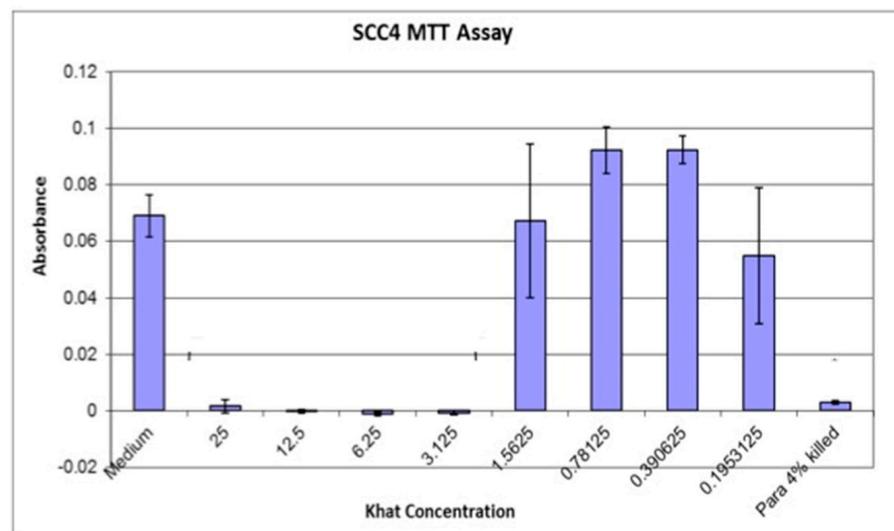


Figure 6. Absorbance from MTT Assay for SCC4 cells treated with different concentrations of Khat solution (25 mg/mL to 0.195 mg/mL). Medium with only SCC4 cells was used as a negative control while 4% paraformaldehyde was used as a positive control, showing complete cell death.

SCC4 (Late Passage)

Somewhat different results were obtained when late passage of SCC4 cells were performed. A significant reduction in absorbance was seen with all doses of Khat (except 1.56 mg/mL) indicating toxicity ($p < 0.05$). No obvious difference was seen between the different doses used, suggesting variability in cell behavior in late passages (Figure 7).

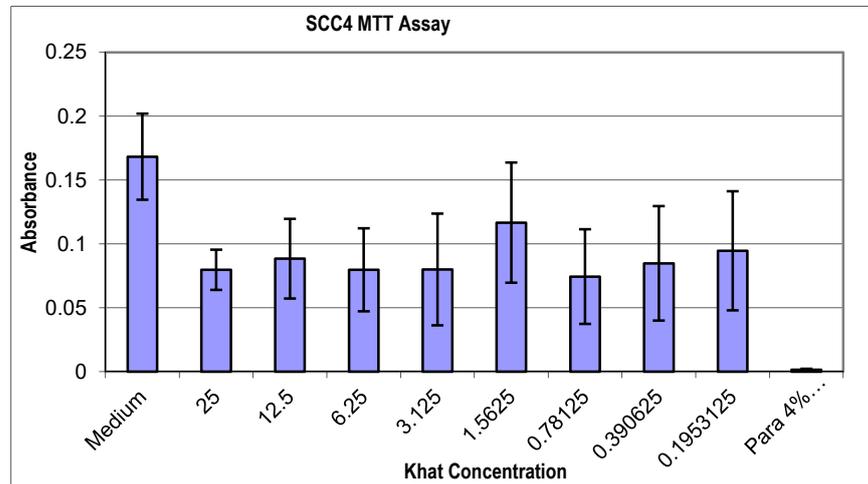


Figure 7. Absorbance from MTT Assay for SCC4 cells (late passage) treated with different concentrations of Khat solution (25 mg/mL to 0.195 mg/mL). Medium with only SCC4 cells was used as a negative control, while 4% paraformaldehyde was used as a positive control showing complete cell death.

3.3. Comparison of NOF's and SCC4 Assays

Comparison between the average cell number for all NOFs and repeat SCC4 assays showed no significant difference between the two cell types, with a similar dose response curve (Figure 8).

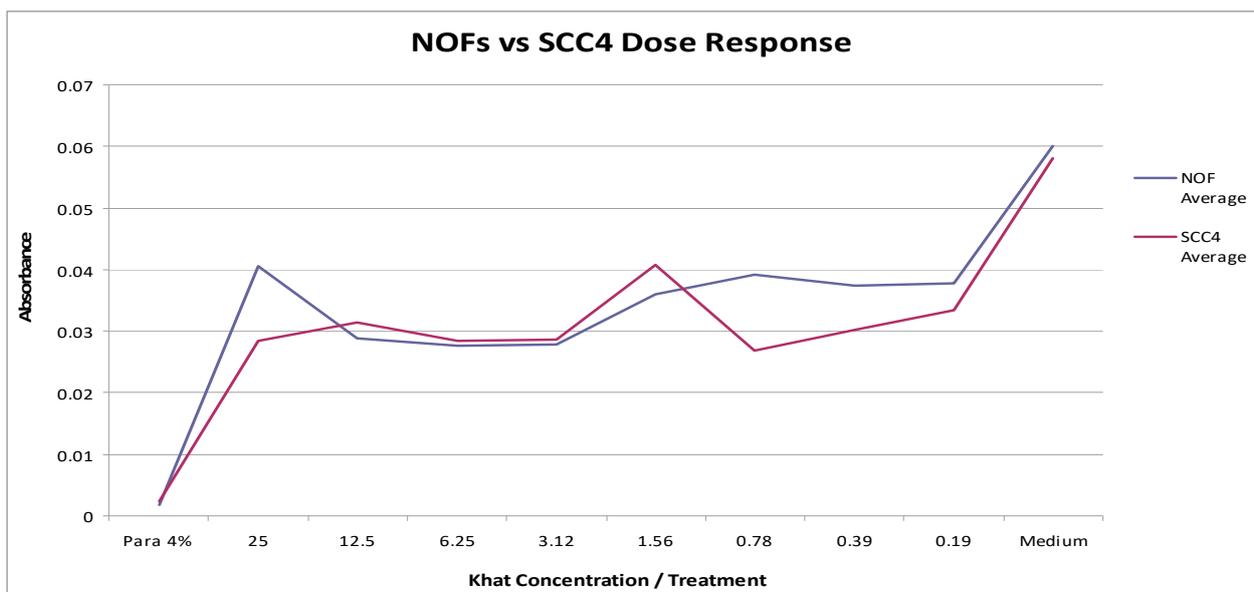


Figure 8. Comparison of dose response curves (MTT Assay) for NOFs and SCC4 cells treated with different concentrations of Khat solution (25 mg/mL to 0.195 mg/mL). Medium was used as a negative control while 4% paraformaldehyde acted as a positive control.

3.4. Detection of α -SMA

No α -SMA staining was seen with the negative control IgG isotype antibodies for NOF 316 (Figure 9). This suggested that 1 mg/mL of Khat concentration was sufficient to alter the fibroblast phenotype. Figure 10 shows a clear positive staining of α -SMA fibers running as vertical strands throughout the cell body of a fibroblast. Not all fibroblasts were α -SMA-positive, suggesting specific activation of a subset of fibroblasts. Similar results were seen when cells were stimulated with TGF- β , as this is known to increase the expression of α -SMA. SMA staining was seen in NOF 316, after incubation with 40 μ g/mL of TGF- β (Figure 11). TGF- β is a protein that regulates proliferation and cellular differentiation, and causes apoptosis in tumor cells.

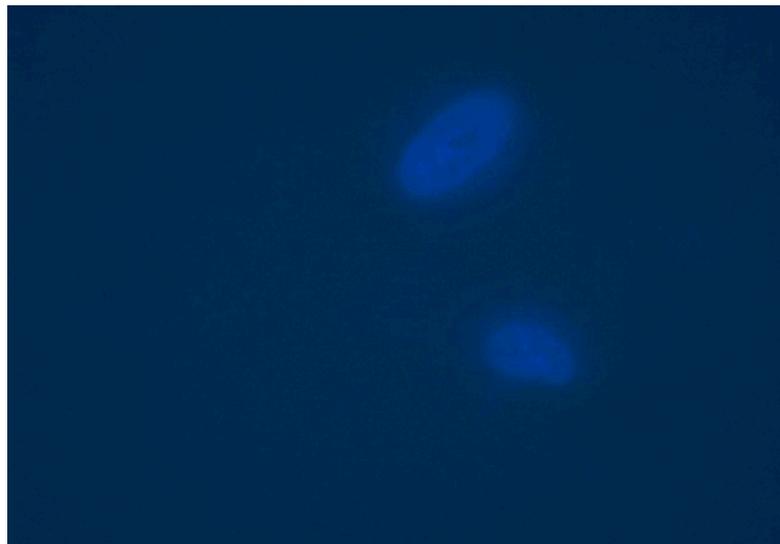


Figure 9. NOF 316 showing nuclear DAPI staining with the IgG antibody. (magnification $\times 100$, Fluorescence at 1.3 s, DAPI at 0.07 s).

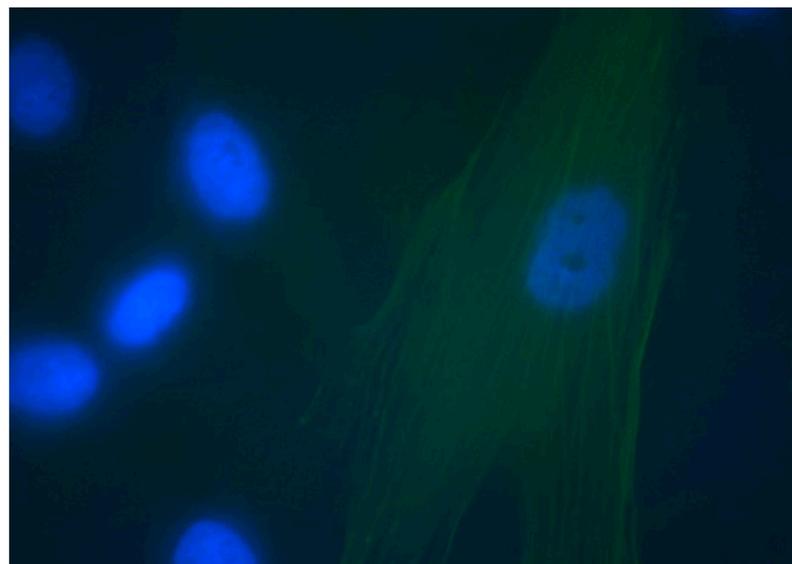


Figure 10. NOF 316 showing SMA expression after stimulation with 1 mg/mL Khat (magnification $\times 100$, Fluorescence at 1.3 s, DAPI at 0.07 s).

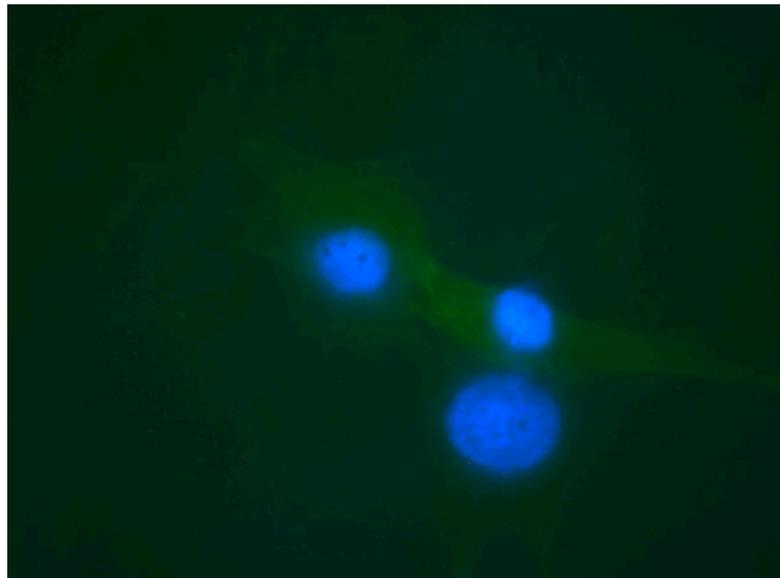


Figure 11. SMA expression in NOF 316 after incubation with 40 µg/mL of TGFβ (magnification ×100, Fluorescence at 1.6 s, DAPI at 0.100 s).

4. Discussion

The present study was based on the hypothesis that Khat is cytotoxic to NOFs and SSC4 cells. It was also hypothesized that Khat would stimulate positive expression of α -SMA in fibroblasts. Interestingly, the study revealed the cytotoxicity of Khat for both NOFs and SSC4 cells, and increased cell death occurred as compared to the negative control. Moreover, fibroblasts also stained positive for α -SMA after Khat exposure. Therefore, both postulated hypotheses were accepted. For cytotoxicity assessment, MTT assay was employed, as the technique was convenient, rapid, and reproducible [17]. Similarly, immunofluorescence was used to determine α -SMA positive staining in fibroblastic cells, as it showed high sensitivity and specificity over other techniques [18].

Khat was studied in relation to its damaging effects on oral mucosal keratinocytes, but its potential influence on the oral fibroblasts and cancer cell line was not studied. Multiple studies stated that Khat chewing is associated with oral hyperplasia, hyperkeratinization, and oral cancers [19]. The results of the present study showed that Khat caused cell death of both NOFs and SCC4 cells. The exact mechanism for Khat-induced cell death is not known. However, the available literature focused on the presence of alkaloids (cathine and other phytochemicals) as a cause of its cytotoxicity and suggests that Khat could induce cell death even in short tissue exposure [6]. In addition, Khat also triggers intracellular generation of free radical, i.e., reactive oxygen species (ROS) and Glutathione (GSH) depletion, which initiates programmed cell death (apoptosis) by causing injury to the DNA [20]. Furthermore, in the present study, it was also observed that higher concentrations (25 mg/mL) of Khat displayed variable outcomes of cytotoxicity, not showing significant cell death of NOF 316 cells, as compared to the other tested cells.

Interestingly, Khat showed dose-dependent cell death of NOF 18, NOF 26, and SCC4 cells, indicating higher toxicity at higher doses. In contrast, NOF 316 and NOF 319 did not exhibit dose-dependent cytotoxicity due to Khat. A possible explanation for this might be derived from the fact that Khat (25 mg/mL) used in the present study was in the frozen form and might have lost some of its activity [21]. Moreover, the variations in backgrounds and oral habits of the patients from which NOFs were obtained, could have resulted in such outcomes [22]. Similar findings were also reported in studies by Abderrahman and Modallal [23] and Lakundu et al. [24]. Furthermore, it was reported that Khat caused genetic material damage and its genotoxic effect on normal keratinocytes and fibroblasts increased with higher dose [25]. Their findings supported the outcomes of the present study. Therefore, clinically speaking, oral lesions including mucosal keratosis,

pigmentation, and leukoplakia were not only induced by Khat, but could also progress and differentiate due to increased frequency and the extent of Khat chewing habit. Hence, patients should be educated that the Khat chewing increases the risk and incidence of oral lesions and a cessation of the habit should be encouraged. The present study reports a positive expression for α -SMA staining in fibroblast, indicating a phenotypic change. SMA is a well appreciated marker for MFs differentiation in tumor stroma, particularly in relation to OSCC [26]. MFs display intermediate physical characteristics between fibroblasts and smooth muscle cells [27]. Various laboratory-based studies demonstrate the role of cancer cell line in acquisition of fibroblast transformation in MFs, by secreting various extracellular matrix proteins, matrix-degrading enzymes, and growth factors [28]. These MFs modulate the growth, adhesion, migration, invasion, and differentiation of cancer cells. MFs tend to differentiate in the presence of TGF- β , another factor that increases in fibrotic lesions [29]. Recently, studies showed that suppression of α -SMA stops the connective tissue growth factor (CTGF) activity linked with reduced nuclear factor kappa B (NF- κ B) translocation in different areas of progenicity, indicating the importance of MFs in tissue fibrosis and cell death [30]. In addition, a study conducted by Etemad-Moghadam et al., reported that MFs appear more frequently in OSCCs and are detected more towards the invasive front [31]. These findings indicate that Khat influences the progression and development of pre-malignant and malignant lesions, as it express α -SMA in fibroblasts. Weak and anecdotal evidence exists in relation to Khat chewing and oral cancer, in light of findings from retrospective and descriptive studies [32,33]. Our findings further the clinical notion, that Khat usage (expression of α -SMA) facilitates differentiation and progression of pre-malignant lesions. Therefore, Khat users (with tobacco use) should be warned of the risk of possible development of pre-malignant lesions and their progress to malignancy. Hence, further randomized controlled trials investigating the effect Khat usage with and without tobacco smoking, on the incidence of malignant and pre-malignant lesions are recommended.

Khat showed cytotoxicity at almost all concentrations, as compared to the negative control but there are certain limitations, which should be considered in the interpretation of the outcomes of the present study. This investigation was in-vitro, and cell cytotoxicity was performed under ideal conditions. In the present study, decreased cell viability was observed, which indicated induction of differentiation or cell cycle arrest not necessarily indicating cell death. Therefore, for strictly assessing cell death, other assays including trypan blue exclusion assay is recommended in future studies. For the cytotoxicity of cells, MTT assay, and for α -SMA, immunofluorescence was performed. However, VELscope[®] (Visually Enhanced Lesion scope) is a relatively new method to screen oral lesions and is recognized as an efficient tool by World Health Organization (WHO) in oral cancer prevention [34]. Therefore, further clinical trials investigating the influence of Khat on inducing oral lesions using VELscope[®] as a tool are recommended. In addition, confounding factors, i.e., smoking, tobacco chewing, oral hygiene, and chronic diseases are critical in the development of OSSC, and result in accelerated cell death in the presence of Khat. However, these factors were not assessed in the present study. Moreover, this study used control, Khat, and TGF- β (as a positive control), therefore, an interaction of Khat and TGF- β by incubating cells pretreated with TGF- β with Khat should be performed in future studies. Additionally, the effect of dose-, duration-, and friction-associated trauma in Khat chewers is of great importance in assessing its clinical influence on mucosal differentiation. Therefore, further clinical randomized controlled trials assessing the oral cellular changes among Khat users in oral epithelial and mesenchymal cells with the associated factors (smoking, tobacco chewing, oral hygiene, chronic diseases, and trauma) are recommended to clinically translate the findings of the existing study.

5. Conclusions

Results from the present study showed that Khat is cytotoxic to oral fibroblasts. It also causes cell death of oral cancer cells (i.e., SCC4 cells). Furthermore, it can also

cause activation and phenotypic changes in oral fibroblasts, indicating a potential role in progression of OSCC.

Author Contributions: A.U.Y.S., M.A.A. and E.I.A., data collection, study design, data assessment, and manuscript writing, manuscript revision. M.A.-A., A.M.A. and R.J., data inference, experiment performance (SEM and RS), study design, manuscript drafting, data analysis, and manuscript revision. A.R.A. and S.A.M., experiment (MTBS and DC), data collection, funding, resources, data interpretation, writing, revise, editing, and final manuscript approval. N.A., F.V. and T.A., experiment (MTBS and DC), data collection, data interpretation, funding, resources, software, writing, revise, manuscript revisions, and final manuscript approval. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The study data is available from the corresponding author on request.

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Conflicts of Interest: The authors declare that they have no conflict of interest and all authors have read and approved the final draft.

Appendix A

Table A1. Materials and equipment used in the study.

Materials/Equipment	Company
Dulbecco’s Modified Eagles Medium, Cell culture medium	Gibco by Life technologies Invitrogen
PBS solution (without Ca ²⁺ and Mg ²⁺)	Sigma Aldrich
Trypsin/EDTA	Invitrogen
Acidified Isopropanol	Fisher Scientific, Leicestershire, UK
MTT, Thiazolyl Blue Tetrazolium Bromide	Sigma Aldrich
Galaxy R CO ₂ Incubator	Brunswick lab, (UK)
MSE Harrier 18/80 Refrigerated Centrifuge	MSE, UK
25 cm ² and 75 cm ² tissue culture flasks (Cellstar®)	Greiner Bio-One Ltd. (Gloucestershire, UK)
96 well tissue culture plates	Fisher Scientific UK Ltd. (Loughborough, UK)
Integra Biosciences PIPETBOYpro	Thermo Scientific, UK Ltd.
Disposable sterile pipettes (Costar®)	Corning Incorporated (USA)
Micropipettes	Thermo Scientific and Gilson lab (UK)
Labpette pipettes	Manufactured by Labnet (NJ, USA)
Pipette tips	STARLAB, Ltd. and Gilson. (UK)
Universal containers	SARSTEDT Ltd. (Leicester, UK) & Sterilin UK
Manual Desktop Counter	Ryman, UK
Cell counting chamber slides/Haemocytometer	Invitrogen
Zeiss Axiovert 200 M inverted microscope	Carl Zeiss Ltd. (Hertfordshire, UK)
Syringe and Its filter units	Millex®GP, Ireland
Eppendorf centrifuge tubes	Eppendorf, UK
24 well tissue culture plates	Fisher Scientific UK Ltd. (Loughborough, UK)
Glass slides	Thermo, UK
Axioplan 2 Imagin software	Operated by University of Sheffield

Table A1. Cont.

Materials/Equipment	Company
Spectrophotometer plate reader, Infinite M200	Tecan, UK
Magellan™ data analysis software	Tecan, UK
100% Methanol	Provided by tissue culture lab, Dental School, University of Sheffield
Sodium deoxycholate	Provided by tissue culture lab, Dental School, University of Sheffield
Bovine Serum Albumin (BSA)	Sigma Aldrich, UK
Monoclonal Anti-Actin, α -Smooth Muscle - FITC antibody produced in mouse	C2 Sigma Aldrich, UK
clone 1A4, purified immunoglobulin, buffered aqueous solution	
DAPI nuclear stain	Life Technologies, Invitrogen
Monoclonal Anti-Human IgG1–FITC antibody produced in mouse	Sigma Aldrich, UK
TGF- β	Sigma Aldrich, UK

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