



Review article

Khat (*Catha Edulis* Forsk) induced apoptosis and cytotoxicity in cultured cells: A scoping reviewGamilah Al-Qadhi^{a,*}, Marwan Mansoor Ali Mohammed^b, Mohammed Al-Ak'hali^{c,d}, Essam Ahmed Al-Moraissi^e^a Department of Basic Dental Sciences, Faculty of Dentistry, University of Science and Technology, Yemen^b Department of Oral and Craniofacial Health Sciences, College of Dental Medicine, University of Sharjah, United Arab Emirates^c Department of Preventive Dental Sciences, College of Dentistry, Jazan University, Jazan, Saudi Arabia^d Department of Periodontology, Faculty of Dentistry, Sana'a University, Sana'a, Yemen^e Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Thamar University, Yemen

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ABSTRACT

Background: Khat (*Catha edulis* Forsk) leaves are chewed by people in certain regions of East Africa and the Middle East for their stimulating amphetamine-like effects. The purpose of this scoping review is to systematically map the current *in vitro* publications that investigated the toxicological potential effects of khat on cultured human or animal cells in terms of cellular viability and activity.**Methods:** A comprehensive electronic database search was undertaken up to December 2020 without starting date or language restrictions in accordance with the PRISMA extension for scoping review guideline and methodological quality evaluation based on the guidelines for reporting pre-clinical *in vitro* studies on dental materials. All *in vitro* studies that investigated the effect of khat plant extract (*Catha Edulis*) on the cultured human or animal cells were included.**Results:** The initial search yielded 599 articles and 16 articles were finally selected to be included. The treatment of cells with khat produced different degrees of cellular changes, including decreased cellular survival, induction of apoptosis, increased ROS production, alteration of cell phenotype, and of arrest cell cycle. In this contest, khat-exposed cells expressed higher levels of pro-apoptotic protein Bax and lower levels of anti-apoptotic Bcl-2, up-regulated p38, p53, p16, and p21 proteins, as well as premature expression of differentiation markers.**Conclusion:** Based on the current scoping review, khat induced apoptosis and cytotoxicity in cultured human cells, including oral cells.

1. Introduction

Khat (*Catha edulis* Forsk) is an evergreen shrub belonging to the Celastraceae family. Due to their psychostimulatory effects, people who live in Eastern Africa and the Arabian Peninsula, particularly Yemen, have chewed the khat plant for centuries [1]. The main active chemical components of the khat are cathinone and cathine, which are structurally related to amphetamine. Chewing khat leaves release cathinone, a stimulant that gives the feeling of euphoria. When cathinone is broken down in the body, it produces chemicals including cathine and norephedrine, which have a similar structure to amphetamine and adrenaline (epinephrine) [2].

The potential adverse effects of khat are spread over many organs of the body. It was reported that khat chewing is associated with some mental positive experiences such as euphoria, enhanced imagination, the liberation of ideas and improved self-confidence in the first part of the session; however, these are followed by negative symptoms including anxiety, nervousness, depression, and insomnia [3]. In addition to the central stimulant action of cathinone, chewing of khat leads to several peripheral effects that include the development of a dry mouth, blurred vision and mydriasis [3, 4]. Cardiovascular effects appear rapidly after the use of khat and they include tachycardia, palpitations, vasoconstriction, and an increase in blood pressure depending on the amount and potency of the material absorbed [5].

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Likewise, khat chewing is frequently associated with numerous irritative disorders of the gastrointestinal tract, including gastritis, constipation, and delayed gastric emptying due to the presence of tannins and the sympathomimetic action of cathinone [6, 7]. The adverse effects of khat on reproductive organs were reported in several studies. In males, the semen volume, sperm motility and count, as well as plasma testosterone levels were significantly declined in khat chewers. In females, it reduced the placental blood flow due to its vasoconstrictive effect [8], which leads to low birth weight infants [9].

Regarding oro-dental tissues, the studies showed a controversial effect of khat concerning dental caries, Hill and Gibson (1987) showed that khat chewers have a lower dental caries rate [10], and the fluoride content in khat appears relatively low to effect on this finding [11]. Nezar et al. showed that khat extract can inhibit some cariogenic properties of *Streptococcus mutans* in vitro [12]. Khat is considered non-cariogenic, but the habit is usually associated with high consumption of sugary and soft drinks and the sugar tablets to counteract the bitter taste of khat which can lead to cervical caries [13].

The controversy is stronger in the relation of khat chewing with periodontal diseases, where some studies showed that khat has detrimental effects on the periodontium [14, 15, 16], others showed khat has possibly none or some beneficial effect on the periodontium [17, 18]. Strong evidence indicates that gingival recession was significantly higher in khat chewers [19, 20], and some case reports that khat induced plasma cell gingivitis [21]. Many studies showed that khat cause histopathological changes on the oral mucosa, including acanthosis, rete ridges, hyperkeratosis, and oral white lesions [22, 23] as well as ultrastructural changes in the salivary glands [24]. More importantly, the long-term khat chewing might contribute to the high incidence of oral cancer [25, 26, 27].

Most alkaloids, which are the main toxic substances in khat, are absorbed through the oral mucosa after its release from the khat during the session of khat chewing. Thus, oral mucosa would be in contact with a higher concentration of alkaloids with possible toxic effects [28, 29]. At the cellular and molecular levels, several in vitro studies showed that fresh khat leaves or khat extract induced apoptosis, cytotoxicity, and reactive oxygen species (ROS) production [30, 31, 32, 33, 34]. As far as we know, no previous review has addressed the toxicological potential of khat on cells. Therefore, the purpose of this scoping review was to systematically analyze the existing research in this area, particularly in vitro studies that investigated the toxicological potential effects of khat on human or animal cells in terms of cellular viability and response and to address the molecular mechanism underlying these effects. The current scoping review was designed based on the following PCC elements: population: human or animal cells, concept: the potential cytotoxic effect of khat, and context: in vitro studies.

2. Methods

2.1. Protocol registration

This scoping review adhered to the Preferred Reporting Items for Systematic reviews and Meta-Analyses extension for Scoping Reviews (PRISMA-ScR) guideline [35] (Supplementary material I: PRISMA-ScR Checklists). The protocol was registered in the Open Science Framework for scoping review protocol registration at the following link <https://osf.io/wzhh8/>

2.2. PCC question

What are the effects of khat (*Catha Edulis* (Val) Forsk) on cell viability and response in *in vitro* studies? The research question was designed based on PCC items, population: human or animal cells, concept: potential cytotoxic effects of khat (*Catha Edulis*), and context: *in vitro* studies.

2.3. Eligibility criteria

To be included in this review, studies should be *in vitro* original studies that investigated the effect of khat leaves extract (*Catha Edulis*) on human or animal cells under the culture condition regardless of the cell type or the way of extraction. Reviews, case reports, and expert opinions were excluded during the initial phase of screening. Studies that used irrelevant study designs or synthetic cathinone or drugs that have similar pharmacological actions to khat were also excluded during the full texts screening stage.

2.4. Information sources and search strategy

In December 2020, the initial search was implemented in three electronic databases (PubMed, Scopus, and Web of Science) without any date or language restrictions. A combination of the keywords and index terms based on PCC was used, including P: khat OR qat OR gat OR gaat OR jaad OR chat OR catha edulis OR khat extract, AND C: cell viability OR cellular viability OR cell survival OR cellular survival OR cell cycle OR cell toxicity OR cytotoxicity OR cell damage OR cell death OR apoptosis OR programmed cell death OR genotoxicity, AND C: *in vitro* study OR *in vitro* OR *in vitro* technique OR cell culture technique OR cell culture method OR culture technique OR preclinical study OR *in vitro* model.

For each database, retrieved citations were documented and imported into Mendeley reference manager software. The folder then was exported to Rayyan® web application and duplicated articles were deleted. The process of literature screening was performed using Rayyan QCRI online tool, which provides independent access for retrieved publications, so each author worked independently.

2.5. Selection of sources of evidence

The selection of studies was carried out by two authors (GA, MMAM) independently. During the first level of screening, the title and abstract of retrieved papers were screened to identify the potentially eligible papers. References that did not fulfill the inclusion criteria were excluded. In the second phase, full-text papers were further screened to decide which study was appropriate for inclusion. Any discrepancy relating to the study selection was resolved by the discussion. Reasons for exclusion were reported.

2.6. Data charting process and data items

Two researchers (GA, MMAM) developed and refined the charting table. Data were extracted independently, including study ID (Author and year of publication), study design (cell line/type, source of cells/tissue, validation of identity, sex of human/animal, species, exposure, dose, frequency and duration of dosing, number of replicates/groups, % serum/plasma in medium), and outcome (outcome measures, method of analysis, result note and overall endpoint).

2.7. Critical appraisal of individual sources of evidence

Since the risk of bias tool is not available for *in vitro* studies, the methodological quality assessment was conducted using the guidelines for reporting pre-clinical in vitro studies on dental materials, based on the modification of the CONSORT checklist with some modification [36]. The critical appraisal in scoping review summarizes the existing evidence regardless of methodological quality or risk of bias [35], and the assessment was independently done by two authors (GA, MMAM). The grading system was (1) clearly inadequate, (2) possibly accurate, and (3) clearly accurate responses, or (yes or no response), which were scored with (0), (1) [37]. The rating of overall quality was as the following: 1–9: low, 10–19: moderate, and 20–29: high.

2.8. Synthesis of results

Due to the wide range of heterogeneity, the results were summarized, categorized, and reported in a narrative, qualitative way.

3. Results

3.1. Study selection

The initial search yielded 595 potentially relevant citations from the electronic databases (PubMed = 301, Web of Science = 280, Scopus = 5), and four articles were detected during the hand search of the retrieved references. Overall, 578 were identified after the elimination of duplicates and subjected to the first step of screening (titles and abstracts). Out of these, 22 articles were screened further in terms of full texts. Six records were excluded as they did not meet the inclusion criteria and reasons for exclusion were reported and the remaining sixteen references were included (Figure 1). (S1 search strategy and list of excluded studies).

3.2. Characteristics of included studies

To investigate the potential effects of khat on cells, different cultured cells or cell lines were exposed to the khat extract, including leukemia cell lines [38, 39, 40], blood cells [34, 41, 42], oral keratinocytes and fibroblasts [33, 43, 44, 45], cancer cells [46, 47], interstitial cells [48], and hepatic, renal and cardiac cells [30, 32, 49], respectively. The majority of studies have reported the source of cells, except three studies [32, 38, 47]. Regarding the gender of humans or the origin of animals, five of sixteen studies provided this information [33, 42, 44, 48, 49]. The human was the most widely used species, while mice were used in three

studies [30, 42, 48], and Bovine in one study conducted by Ageely et al. [49]. Cells were treated with different forms of khat extract, including fresh leaves [41, 46, 48], alcoholic extract [30, 32, 38, 40, 42, 43, 47], organic and/or aqueous extract [33, 34, 44, 45, 49].

Considering the dose and duration of exposure, various doses starting from 1 to 2000 $\mu\text{g}/\text{ml}$, as well as different durations from 0.5 h to 72 h were reported. In most studies, khat-treated cells showed statistically significant alterations in cell function in a dose- and time-dependent manner. Particularly, there was a dose-dependent significant increase in cell death and a decrease in cell viability upon exposure to increasing concentrations of khat. The dose (200–400 $\mu\text{g}/\text{ml}$) induced cytotoxic and genotoxic effects in the human T lymphoblastoid cell line. The increasing DNA damage and reduction in cell viability were statistically significant after exposure to 200 and 400 $\mu\text{g}/\text{ml}$, respectively [34]. Similarly, the concentration of 200 $\mu\text{g}/\text{ml}$ induced features of apoptosis [39, 40], and 400 $\mu\text{g}/\text{ml}$ also induced apoptosis in breast cancer cells [47].

In human oral keratinocytes and fibroblasts, a significant reduction in cell viability was observed in the 100 $\mu\text{g}/\text{ml}$ concentration of khat [30, 44], and ≥ 100 $\mu\text{g}/\text{ml}$ concentration caused profound cell death compared to controls [32, 33, 41]. Collectively, khat concentration at or above 100 $\mu\text{g}/\text{ml}$ was mainly responsible for the observed cellular effects. The 24 h duration of exposure was most often used, while 72 h exposure was used in two studies in some experiments [30, 44], and crucial exposure duration was between 8 and 24 h [32].

All studies indicated that the experiments were run in triplicate, except one study by Al-Ahdal et al. [46] did not provide enough information about this item. Fetal bovine serum (FBS) was used as a serum supplement for cell cultures in most studies, whereas 2% bovine serum albumin and 2% human AB serum were used in two studies [41, 48], respectively (Table 1).

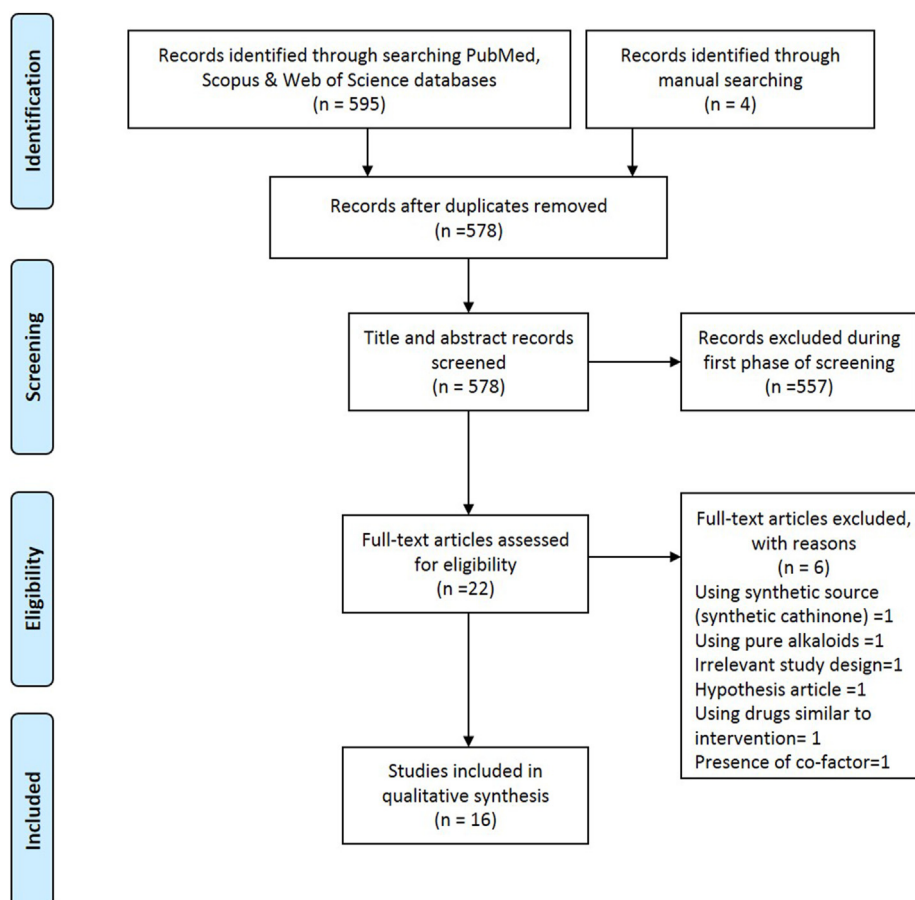


Figure 1. Flow diagram showing the different phases of literature screening for the scoping review process (Editable file: PRISMA Flow Diagram, Liberati et al., 2009).

Table 1. Characteristics of included studies.

No.	Author and year of publication	Cell line/type	Source of cells/tissue	Sex of human or origin of animal	Species Strain	Exposure	Dose	Frequency and duration of dosing	Number of replicates per group	% serum/plasma in medium
1.	Al-Ahdal et al., 1988	Epidermoid carcinoma cells (KB), normal human fibroblasts (1BR.3) & xeroderma pigmentosum fibroblasts (XP2Bi)	documented	NR	human	Fresh khat leaves	20–200 ng/ml	2,4,8,10 & 25h	NM	10% fetal bovine serum (FBS)
2.	Dimba et al., 2003	Leukemia HL-60, Jurkat, NB4 cell lines	not documented	NR	not relevant	Methanolic khat extract	NM	2 & 8 h	Triplicate & more	10% FBS
3.	Barkwan et al., 2004	Human T lymphocyte cell line	documented	NR	human	Aqueous khat extract	1–2000 µg/ml	12 h	Triplicate	10% FBS
4.	Dimba et al., 2004	Leukemia HL-60, Jurkat, NB4 cell lines & primary peripheral leukocytes	documented	NR	human	Methanolic Khat extract	6.3–200 µg ml ⁻¹	0.5–8 h	Triplicate & more	10% FBS
5.	Nyongesa et al., 2006	Mouse testes & interstitial cells	documented	35 male	Swiss Mice	Fresh khat leaves	0.06, 0.6, 6 30, 60 mg/ml	0.5–3 h	Triplicates	2% Bovine Serum Albumin
6.	Lukandu et al., 2008a	Normal human oral keratinocytes & fibroblasts	documented	16 female 19 male	human	Organic khat extract	0, 3.16, 10, 31.6, & 100 µg/ml	24 & 72 h (for 7 days)	Triplicate	10 % FBS
7.	Lukandu et al., 2008b	Normal human oral keratinocytes & fibroblasts	documented	16 females 19 males	human	Organic & Aqueous khat extracts	10, 31.6, 100 & 316 µg/ml	4, 8 & 24 h	Triplicate & more	10 % FBS
8.	Lukandu et al., 2009	Normal human oral keratinocytes & fibroblasts	documented	NR	human	Organic & Aqueous khat extracts	10, 31.6, 100 & 316 µg/ml	0.5–24 h	Triplicate	10% FBS
9.	Bredholt et al., 2009	Monocytic Acute Myeloid Leukemia cell line (MOLM-13) & acute biphenotypic leukemia (MV-4-11)	documented	NR	human	Methanolic Khat extract	200 µg/ml	4, 6, 8 & 24 h	Triplicate	10% FBS
10.	Lukandu et al., 2010	Normal human oral keratinocytes & fibroblasts	documented	NR	human	Methanolic Khat extract	0, 3.16, 10, 31.6, & 100 µg/ml	6 h (for 7 days)	Triplicate	10% FBS
11.	Murdoch et al., 2011	Peripheral blood mononuclear cells (PBMCs)	documented	NR	human	Fresh khat leaves	1–100 µg/ml	4–24 h	Triplicate	2% human AB serum
12.	Abid et al., 2013	L02 Hepatic cells	not documented	NR	human	Ethanollic khat extract	10, 50 and 100 µg/ml	4, 8, 16, 24 h	Triplicate	10% FBS
13.	Ageely et al., 2016	Madin-Darby Bovine Kidney cell line	documented	United state	Bovine	Organic khat extract	10, 5, 2.5, 1.25, 0.62, 0.31, & 0.15 mg/ml	4 & 48 h	Triplicate	10% FBS
14.	Mohen et al., 2016	H9c2 (2–1) Rat cardiomyoblast cell lines	documented	NR	mice	Methanolic khat extract	86.5 µg/ml	0, 24, 48 & 72 h	Triplicate	10% FBS
15.	Lu et al., 2017	human breast cancer MDA-MB-231 cell	not documented	Female	human	Ethanollic khat extract	20, 200, 300 & 400 µg/ml	4, 8, 16 & 24 h.	Triplicate	10% FBS
16.	Abdelwahab et al., 2018	RAW 264.7 cells (monocytic macrophages)	documented	NR	Murine mice	Methanolic khat extract	30, 15, 7.5, 3.75 µg/ml		Triplicate	10% FBS

3.3. Results of individual sources of evidence

The following outcomes were reported:

3.3.1. Cellular viability

Various assays were used to determine the survival and number of khat-exposed cells, such as trypan blue dye exclusion test, hemocytometer, chromatin condensation assay, WST-1/proliferation assay, and Microculture Tetrazolium Assay (MTT). Most included articles clearly indicated that the treatment of cells with khat reduced cellular survival compared to untreated cells and this inhibitory effect was more significant at high concentration and long duration [30, 32, 34, 40, 42, 44, 45, 46, 47, 48, 49]. Likewise, khat decreased cell proliferation [43, 49].

3.3.2. Apoptosis

3.3.2.1. Morphological evaluation. Several staining and microscopic (phase-contrast, light, and transmission electron) techniques were used to display the morphological and ultrastructural features of khat-treated cells. The results showed that khat induced typical features of apoptosis, such as loss of microvilli, blebbing or budding of the plasma membrane, cell shrinkage or rounding up, organelle separation, cytoplasmic vacuoles, chromatin condensation and nuclear fragmentation [32, 33, 38, 40, 44, 47, 49]. Moreover, degeneration of mitochondrial inner membrane was a distinct feature of khat-treated cells [40, 45], and this impairment was further confirmed by measurement of cellular O₂ consumption rate [40], nuclear and cytosolic re-localization of apoptosis-inducing factor (AIF) in mitochondria [45]. Also, the reduction of total epithelial thickness was observed in khat-exposed oral cells showing premature differentiation of cells [43].

For further identification of apoptosis, cells were labeled with Annexin-V/propidium iodide (PI) double staining [32, 39, 41, 47], Annexin-V/biotin and Tunnel assay [33], Acridine Orange (AO)/Ethidium Bromide (EB) staining [49], and AO/PI staining [30] and analyzed by flow cytometry or fluorescence microscopy. The finding showed that the probing of apoptosis in khat-exposed cells yielded a high rate of apoptosis.

At the genetic level, nuclear morphology and function were assessed in five of sixteen articles. In particular, khat inhibited deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein synthesis with RNA being more vulnerable to this toxic effect of khat [46], and induced DNA damage and fragmentation [33, 34], and increased micronuclei frequencies [34], while khat-exposed cells exhibited less sensitivity to nuclear changes under epifluorescent microscope/Hoechst staining [40]. Furthermore, khat induced cell cycle arrest in the G1 phase [44].

3.3.2.2. Expression of apoptotic-related proteins

3.3.2.2.1. Bax and Bcl-2. The expression or activation of several proteins regulated the pathway involved in khat-induced apoptosis. Specifically, the pro-apoptotic protein Bax, which increased significantly after treatment of cells with khat, while the anti-apoptotic Bcl-2 was decreased [30, 32, 47]. In contrast, in another study [40], no changes observed in Bax and Bcl-2 protein levels in khat-treated cells, but there was a decline in levels of anti-apoptotic Mcl-1 protein.

3.3.2.2.2. Cysteine proteases (Caspases). Khat was associated with the activation of caspase 3 [39], caspase -8 and c-FLIPL, particularly in MOLM-13 cells [40], caspases-8 and -9 [32], caspases-3 and -7 [30], while caspase-9 and caspase-3 levels were not affected by khat-exposure in two studies [45, 47] respectively.

3.3.2.2.3. Cell cycle regulatory proteins. Khat exposure increased the expression of p53, p16, and p21 in primary oral cells [44], and number of p21-positive cells and levels of p38 that were reversed by the p38 inhibition, while p16 was not affected [43]. Khat also activated transcription factors p38 Mitogen-Activated Protein Kinase (MAPK) and Hypoxia Inducible Factor-1 Alpha (HIF-1 α), but inhibited NF κ B p65 [41],

increased phosphorylation of c-Jun Amino-Terminal Kinases (JNKs) and Extracellular Signal-Regulated Kinases (ERKs) [32, 47], while p38 was stable [32].

3.3.2.2.4. Differentiation markers. Exposure to khat induced premature expression of transglutaminases, involucrin, and flaggrin in oral cells [43].

3.3.3. ROS production

By using cellular Dichlorofluorescein Diacetate (DCF-DA) assay, excessive ROS production was detected following the treatment of cells with khat [30, 32, 33, 41], and inhibition of Glutathione (GSH) [33]. However, decreased ROS generation was reported in two studies [42, 47].

3.3.4. Antimicrobial and anti-inflammatory activities

Khat altered the antimicrobial and phagocytosis activity of cells. Certainly, it inhibited the production of inflammatory cytokines tumor necrosis factor-alpha (TNF- α), interleukin (IL-6) and chemokine ligands CCL5, CXCL8 and increased production of anti-inflammatory cytokines IL-2, IL-10, interferon-gamma (IFN- γ), and Fas Ligand, as well as inhibited phagocytosis [41]. Khat extract modulated the inflammatory process of monocytic macrophage cell line of mice through inhibition of nitric oxide (NO) proinflammatory production [42].

3.4. Critical appraisal within sources of evidence

Although *in vitro* studies had moderate to high-quality rate, there were missing important factors that reduced quality, including randomization, blinding, and reporting the excluded data. It is critical to report how many culture samples were excluded due to failure of culturing, to randomize the cultured cells into wells of the plate as well as to impose blinding, at least during the outcome assessment. On the positive side, all studies conducted the experiment in triplicate or more, which is important to validate data. There was also detailed documentation of cell sources in most studies except three. The quality of the *in vitro* studies selected in this scoping review is shown in S3 table.

3.5. Synthesis of results

Since this scoping review included different outcome measures, we categorized and described the results following narrative qualitative research approaches (Table 2 and Figure 2).

4. Discussion

Because of euphoric properties, khat (*Catha edulis* Forsk) leaves are commonly chewed by people in parts of Africa and some of the Middle East countries such as Yemen [50]. Several publications have proposed the negative effects of khat-chewing habits on general medical and dental aspects of khat chewers, while the toxicological effects of khat extract require more attention in terms of cellular and molecular aspects. Therefore, this scoping review aimed to explore the existing evidence and mechanisms that mediate the toxicological actions of khat on human and animal cells in *in vitro* models.

In this scoping review, we identified 16 *in vitro* eligible studies addressing the association between khat and cytotoxicity. Cell viability is one of the most essential indicators of the biological status and health of cells. The assays to determine the levels of cell viability and/or proliferation reflect the cytotoxicity of certain drugs or chemical agents and include dye exclusion (e.g. trypan blue) and colorimetric assays (e.g. MTT assay, WST-1 assay) [51, 52]. In this review, most studies have used trypan blue exclusion, MTT, and WST-1 methods to assess cellular viability. The finding showed that khat treatment inhibited the viability of many cell types and it markedly increased this inhibition at higher doses and after long-time exposure.

Table 2. Results of included studies.

No.	Author and year of publication	Method of analysis	Outcome measures	Effect of khat	Endpoint
1.	Al-Ahdal et al., 1988	Colony-formation assay	Cellular viability	Reduced survival cells	Cytotoxicity
		Assays for macro-molecule biosynthesis	DNA, RNA & protein biosynthesis	Inhibited DNA, RNA and protein synthesis, RNA was more vulnerable to the toxic effects of khat	
2.	Dimba et al., 2003	Trypan blue dye exclusion test, chromatin condensation assay & Haemocytometer	Cellular viability	Reduced survival cells	Apoptosis
		Transmission Electron Microscope (TEM)	Cellular ultrastructure	Induced structural changes resembling apoptosis	
		Western blot analysis	Caspase 3- cleavage	Data not shown	
3.	Barkwan et al., 2004	Trypan blue dye exclusion test	Cellular viability	Reduced survival cells	Cytotoxicity Genotoxicity
		Micronuclei assay	Clastogenic potential	Increased micronuclei frequencies	
		Alkaline comet assay	Detection of DNA damage	Increased DNA damage	
4.	Dimba et al., 2004	Trypan blue dye exclusion test, chromatin condensation assay & Haemocytometer	Cellular viability	Reduced survival cells	Apoptosis
		TEM	Cellular ultrastructure	Induced structural changes resembling apoptosis	
		Annexin V/Propidium Iodide (PI) staining + flow cytometry	Probe apoptotic fractions	Data not shown	
		Western blot analysis	Caspase 3- cleavage	Activation of caspase-3	
5.	Nyongesa et al., 2006	Haemocytometer	Cellular viability	Reduced survival cells	Different production of testosterone according to the dose
		Radio-immune assay	Testosterone concentration	Impairment in reproductive function at high dose, while low concentration had a converse effect	
6.	Lukandu et al., 2008a	Trypan blue dye exclusion test & Haemocytometer	Cellular viability	Reduced survival cells	Cytotoxicity
		Phase-contrast microscopy	Cell morphology	Induced structural changes resembling apoptosis	
		Flow cytometry	Cell cycle analysis	Cell cycle arrest in G1 phase	
		Western blot analysis	Primary antibodies: p53, p21, & p16	Increased the expression of p53, p16, and p21	
7.	Lukandu et al., 2008b	Trypan blue dye exclusion test, Chromatin condensation assay & Haemocytometer	Cellular viability	Reduced survival cells	Apoptosis & ROS production
		Phase-contrast microscopy	Cell morphology	Induced structural changes resembling apoptosis	
		TEM	Cellular ultrastructure	Induced structural changes resembling apoptosis	
		Annexin V- biotin & TUNEL assay	Apoptotic fraction	Induced biochemical features resembling apoptosis	
		Fluorescent DNA stains	Plasma membrane permeability	DNA fragmentation	
		Fluorescence-activated cell sorter	Intracellular ROS & GSH	Increased ROS production and depletion of GSH	
8.	Lukandu et al., 2009	WST-1 viability/proliferation assay Phase-contrast microscopy	Cellular viability	Reduced survival cells	Apoptosis
		Haemocytometer	Cell attachment & number	Irreversible changes at high dose and long time	
		Immunofluorescence	Detection of apoptosis inducing factors (AIF)	Induced nuclear translocation Of AIF	
		Fluorescence-activated cell sorter	membrane potential	Decreased mitochondrial inner transmembrane potential	
		Western blot analysis	Expression of: anti-pro-caspase-3, anti Bcl-2 anti-AIF anti-cytochrome c	Caspase 3 not affected Decreased Bcl-2 AIF translocated from nuclear to cytosolic	

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Table 2 (continued)

No.	Author and year of publication	Method of analysis	Outcome measures	Effect of khat	Endpoint
9.	Bredholt et al., 2009	WST-1 viability/proliferation assay	Cellular viability	Reduced survival cells, MV-4-11 cell line exhibited more resistance to khat	Apoptosis
		Epifluorescent microscope/Hoechst staining	Nuclear Morphology	Exhibited less sensitive to changes	
		TEM	Cellular ultrastructure	Degenerated mitochondria and morphological features of autophagy	
		High-resolution respirometry	Cellular O2 consumption rate	Impairment of mitochondrial function in MOLM-13 cells, whereas MV-4-11 cells were less affected	
		Western blot analysis	Protein levels of Bcl-2 and Bax	Not change	
		Mcl-1, Caspase-8 colorimetric kit	Caspase-8 activity	Activated caspase -8, particularly in MOLM-13 cells Reduced levels of anti-apoptotic Mcl-1 protein	
		RT-PCR	Expression of c-FLIP _L	Induced c-FLIPL cleavage in MOLM-13, whereas no significant changes in MV-4-11 and HL-60	
10.	Lukandu et al., 2010	Light microscope (H&E)	Structural changes, differentiation & degree of keratinization	Decreased total epithelial thickness	Alteration of cell phenotype
		Histomorphometric analysis	Cell proliferation	Decreased cell proliferation	
		immunohistochemical analyses	p21 expression	Increased p21 positive cells (premature expression)	
		Transglutaminase Colorimetric Microassay	Transglutaminase activity	Induced transglutaminases	
		Western blot analysis	Expression of: Filaggrin, Involucrin, p38, p-p38, p16	Induced premature expression of involucrin & filaggrin Increased levels of p38 and were reversed by p38 inhibition p16 not affected	
11.	Murdoch et al., 2011	FL2 PI channel + Flow cytometry	Cellular viability	Reduced survival cells	Cytotoxicity Apoptosis Alteration of cell phenotype and antimicrobial activity of PBMCs
		Annexin-V/PI staining + Flow cytometry	Probe apoptotic fractions	Features of apoptosis	
		Immunoblotting assay	Cellular metabolic function, total cellular protein	Activated transcription factors p38 MAPK and HIF-1 α but inhibited NF κ B p65	
		Flow cytometry	CD markers	Increased expression of PBMC surface receptors	
		ROS detection reagents	Level of ROS	Increased ROS production	
		FACS Array Bio-analyzer	Cytokine secretion	Inhibited the production of inflammatory cytokines TNF- α , IL-6 & chemokines CCL5, CXCL8 Increased production of anti-inflammatory cytokines IL-2, IL-10 IFN- γ and Fas Ligand	
		Flow cytometry	Phagocytic activity (dextran uptake)	Inhibited phagocytosis	
12.	Abid et al., 2013	Trypan blue dye exclusion test	Cellular viability	Reduced survival cells	Apoptosis ROS production
		TEM	Cellular ultrastructure	Induced structural changes resembling apoptosis	
		Hoechst 33258 staining, Annexin V/PI staining + flow cytometry	Probe apoptotic fractions	Features of apoptosis	
		Western blot analysis	Apoptosis-related proteins expression	Increased Bax Decreased Bcl-2 Activated caspase 8&9 Stable FAS	
		Western blot analysis	MAPKs activation	Increased phosphorylation of JNK & ERK Stable p38	
		Cellular DCF-DA assay	Levels of ROS	Increased ROS production	
13.	Ageely et al., 2016	Trypan blue dye exclusion test & Haemocytometer	Cellular viability	Reduced survival cells	Apoptosis
		Clonogenic survival assay	Cell proliferation	Inhibited cell proliferation	
		Phase-contrast microscopy	Cell morphology	Rounding up of cells	
		Light microscope/H&E staining	Cell morphology	Cells shrinkage, chromatin condensation, nucleus fragmentation	
		Acridine Orange (AO)/Ethidium Bromide (EB) staining + fluorescence microscope	Plasma membrane alterations & apoptotic cells	Induced morphological changes resembling apoptosis	
		Annexin V/PI staining + flow cytometry	Probe apoptotic fractions	Increased apoptotic bodies	
		MTT (Microculture tetrazolium assay)	Cellular viability	Reduced survival cells	
14.	Mohen et al., 2016	AO/PI staining + Fluorescence microscope	Formation of apoptotic bodies	Features of apoptosis (DNA denaturation, nuclear chromatin condensation)	Cytotoxicity Apoptosis ROS production
		ELISA assay	Expression of Bax & Bcl-2 proteins	Increased Bax Decreased Bcl-2	
		luminescence-based assay	Activity of capases 3,7	Increased activity of capases 3&7	
		Cellular DCF-DA assay	Levels of ROS	Increased ROS production	

(continued on next page)

Table 2 (continued)

No.	Author and year of publication	Method of analysis	Outcome measures	Effect of khat	Endpoint
15.	Lu et al., 2017	Trypan blue dye exclusion test Annexin V/PI staining + flow cytometry Hoechst 33258/light fluorescent microscope TEM Western blot analysis	Cellular viability Probe apoptotic fraction Assessment of apoptotic features Cellular ultrastructure Expression of Caspase-9, Bcl-2, Bax proteins & the activation of MAPKs	Reduced survival cells Features of apoptosis (nuclear fragmentation & chromatin condensation). Features of apoptosis Induced morphological changes resembling apoptosis Caspase -9 not detected Increased Bax Decreased Bcl-2 Increased JNK & ERK	Apoptosis
16.	Abdelwahab et al., 2018	Cellular DCF-DA assay MTT assay DPPH, ORAC & FRAP assays Griss reaction Cellular DCF-DA assay	Levels of ROS Cellular viability Antioxidant activity Inflammation/Nitric oxide (NO) production Levels of ROS	Decreased ROS production Reduced survival cells High antioxidant potency. Inhibited NO pro-inflammatory production Decreased ROS production	Cytotoxicity

Khat-treated cells underwent morphological changes that resemble apoptosis features. Apoptosis is a programmed, regulated energy-dependent cell death process, characterized by certain morphological and biochemical features in which caspase activation and key apoptotic proteins play a significant role [53]. Khat-exposed cells showed distinct features of apoptosis under light and electron microscopes, including microvilli loss, plasma membrane blebbing, pyknosis because of chromatin condensation, cytoplasmic vacuolization and breaking up cells into apoptotic bodies. The intercalating staining methods were used to confirm the previous finding and to identify the early and late stages of apoptosis. For instance, Annexin V/Propidium Iodide (PI) and Acridine Orange (AO)/Ethidium Bromide (EB) staining were commonly used to probe apoptotic fractions. Untreated cells showed no Annexin-V binding, but khat-treated cells showed an increase in the number of annexin-V positive cells [32, 41, 47]. Annexin-V can bind specifically to the inner layer of the plasma membrane phosphatidylserine (PS), so the staining targets any loss on the integrity of the phospholipid layer of the plasma membrane and subsequently identify apoptotic cells [54].

Kumar et al. stated that phytoconstituents may act as apoptosis-inducing agents through different mechanisms, including caspases activation, ROS induction, cell cycle inhibition, NF- κ B inhibition, and AP-1 (activating protein-1) inhibition [55]. From among these mechanisms, it has been confirmed that the first three mechanisms induce apoptosis in cells treated with khat.

The extracts of khat revealed substantial cytotoxicity on cancer cells, with IC₅₀ ranging from 22–59 μ g/mL compared to previous claims (IC₅₀: ranging from 33–200 μ g/mL). The cytotoxic effect on normal cells may have several risks to the health of khat users since khat extract demonstrated more cytotoxicity against normal cells (IC₅₀: 6–41 μ g/mL) [56].

Overall, three mechanisms are involved in the apoptosis process and eventually activate the caspases cascade, which is responsible for these distinct morphological alterations observed during cell death. The primary mechanisms include receptor-ligand binding, a mitochondrial-mediated pathway and stress in the endoplasmic reticulum. These mechanisms are associated with the activation of caspases 8, 9, and 12, respectively [57, 58].

To understand the underlying mechanism of cell death induced by khat, caspase activity and Bcl-2/Bax protein expression have been evaluated in many studies. The induction of apoptosis by khat simultaneously affected most of the cells and critically depended on the dose and duration. The caspase-8 and -9 were involved in the cascade of cellular events leading to khat-induced cell death. Note that the Fas expression was stable during the same experiment, suggesting that activation of caspases was stimulated by death signaling molecules rather than Fas [32].

Similarly, the activation of caspase -1, -3 and -8 was detected and involved in the cellular events leading to khat-induced apoptosis. The pretreatment of khat-exposed cells with caspase inhibitor pan-caspase (ZVAD-fmk) results in selectively blocking caspase -1 and -8 and counteracts the apoptotic morphological changes [39]. In khat-exposed cardiomyoblast cells, the activity of caspase-3 and -7 also increased [30], and they are considered effector caspases that are involved in the final execution of cell death in both intrinsic and extrinsic pathways, mitochondrial and death receptors, respectively [53]. In contrast, Lu et al. stated that the caspase-9 expression was not detected in khat-exposed breast cancer cells, indicating the classical cell death pathway did not regulate the process of khat-induced apoptosis [47]. Nevertheless, the observed apoptosis was explained in terms of classical cell death pathway.

Three studies indicated khat-induced apoptosis through the mitochondria-mediated pathway and modulated the Bax/Bcl-2 ratio. This finding was based on up-regulation of Bax and down regulation of Bcl-2 proteins, which increased the Bax/Bcl-2 ratio in terms of apoptosis coordination [30, 32, 47]. Proteins of the Bcl-2 family as regulators in the apoptosis of the mitochondrial pathway and can be divided into anti-apoptotic (e.g. Bcl-2, Bcl-XL, and Mcl-1) or pro-apoptotic (e.g. Bax,

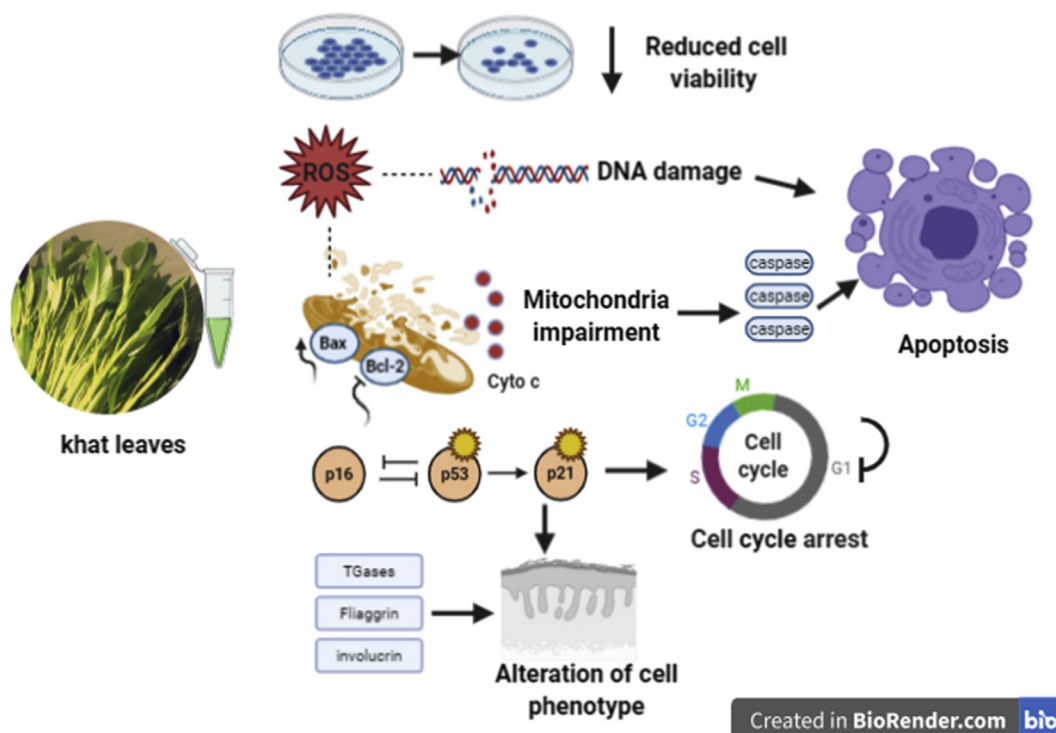


Figure 2. Possible toxicological effects of khat on cultured cells.

Bad, Bak, Bcl-Xs, and NOXA) members. The balance or ratio between anti-apoptotic and pro-apoptotic proteins is crucial for the survival or death of cells [59].

In contrast to the above results, Bredholt et al., reported that there was no change in Bax and Bcl-2 levels, however, there was a reduction in anti-apoptotic Mcl-1 proteins [40]. In response to pro-apoptotic members of the Bcl-2 family, loss of integrity of the outer mitochondrial membrane occurs and the intermembrane space proteins known as cytochrome c are released consequently activate caspase cascade in the cytosol [60]. A possible explanation for these inconsistent findings might be the presence of some heterogeneity among the studies in terms of the type of exposed cell, a type of culture medium, method of analysis, dose, and duration of the experiment.

Along the same line, khat exposure results in degeneration of mitochondria of normal human oral cells accompanied by the early loss of inner transmembrane potential, which occurred before all other morphological and biochemical changes, and a substantial decline in cellular viability. Subsequently, Apoptosis-Inducing Factors (AIF) were translocated from the cytoplasm to the nucleus of mitochondria and could play a key role in khat-induced toxicity, particularly the changes related to nuclear morphology [45].

Increasing the generation of ROS has been a mechanism by which khat induces cell death in several cell lines, including hepatic, oral, cardiomyoblast cells [30, 32, 33, 41]. Also, pretreatment of cardiomyoblasts with N-Acetyl cysteine (NAC) significantly reduced the ROS-mediated oxidative stress and subsequently the incidence of apoptosis [30]. Therefore, this is a further confirmation of the role of ROS in the induction of apoptosis in khat-treated cells.

The mitochondrial respiratory chain is a significant source of intracellular ROS production and simultaneously an important target for the adverse effects of ROS. Increasing ROS production can lead to oxidation of macromolecules and has been implicated in DNA damage, senescence, and apoptosis. In particular, mitochondria-generated ROS triggers the release of cytochrome c and other pro-apoptotic proteins, which can activate caspase cascade and apoptosis [61]. Hence, the results of the former studies further show that khat-induced apoptosis is regulated by

the mitochondrial-mediated pathway. However, decreased ROS level has been observed in breast cancer cells and monocytic macrophages [42, 47]. Notably, different cell lines could affect the findings.

The MAPK signaling cascades are the basic signaling pathway that regulates cellular activities such as proliferation, differentiation, apoptosis and stress responses [62]. This pathway includes ERKs, JNKs and p38 protein kinase, in which ERK is involved in cell proliferation and cell cycle regulation, while JNK and p38 are activated in response to environmental stress, inflammatory cytokines and apoptosis [63]. In the current review, hepatic cells and breast cancer cells treated with khat demonstrated apoptosis that primarily mediated by sustained activation of the JNK pathway and partially by the ERK pathway [32, 47].

Peripheral blood mononuclear cells exposed to khat showed an increase in the expression and phosphorylation of p38 signaling molecules and transcription factor HIF-1 α [41]. Hypoxia activates HIF-1 α and prolonged hypoxia induces apoptosis through the direct association of HIF-1 α and p53 and p53-induced gene expression [64]. A similar pattern of results was reported in human buccal oral mucosa treated with khat. Premature and abnormal differentiation and keratinization of buccal mucosa were associated with up-regulated levels of p38 and reversed by specific p38 MAPK inhibitors, indicating p38 as the key player in khat-induced phenotype alteration [43]. This was consistent with the premature induction of differentiation markers, such as transglutaminases, involucrin, and filaggrin.

The cell cycle arrest observed in khat-exposed oral cells may be attributed to increased levels of p53 and p16 in response to oncogenic and genotoxic stress. Note that the oral keratinocytes and fibroblasts had differential protein expression and consequently different responses to khat exposure, and permanent or temporary cell cycle arrest, respectively [44]. The p53 and p21 mediated growth suppression and senescence, where p21 plays a critical role as a mediator for the p53-dependent G1 arrest, is associated with DNA damage [65]. Some suggest an association between genetic injury and khat chewing or exposure, as khat increases micronuclei in human oral cells and T lymphocyte cell lines, compared with non-khat chewers or non-exposed [29, 34].

The khat exhibited selective antimicrobial properties, Nezar et al. showed that khat has an antibacterial effect against eighteen strains of oral microbiota out of 33 tested strains. The sensitive strains include periodontal disease-associated bacteria like *Porphyromonas gingivalis* and *Prevotella intermedia* [66]. Similarly, Ruqaiyyah et al. found the selective antibacterial, antifungal and possible anti-acanthamoebic properties of khat [67]. The isolated active compounds were 2 β -hydroxytingenone and tingenone, which were identified by various spectral methods. Both compounds exhibited significant activity against different bacterial strains [68].

Taken together, khat is a potent agent that inhibits the survival and proliferation of many cultured cells. This inhibition is most pronounced when the khat concentration was equal to or more than 100 $\mu\text{g/ml}$ and the exposure time is between 8 and 24 h.

5. Conclusion

Based on the available results, we can conclude that the khat inhibited the viability and proliferation of numerous cell types in *in vitro* studies. Cells exposed to khat exhibited ultrastructural changes that resemble to apoptosis features. This inhibition was probably mediated via the mitochondrial pathway of apoptosis and associated with an alteration in the mitochondria phenotype as well as increased Bax and decreased Bcl-2 protein expression. In like manner, cell death in khat-exposed cells was associated with increased ROS production and eventual activation of caspase cascades. Further studies are required to compare the phytochemistry of khat with phytoconstituents from other plants and *in vivo* and clinical reviews are highly recommended.

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