Acta Odontologica Scandinavica, 2005; 63: 136–142

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ORIGINAL ARTICLE

In vitro effects of crude khat extracts on the growth, colonization, and glucosyltransferases of *Streptococcus mutans*

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Abstract

Millions of Yemenites, East Africans, and immigrants to Western countries chew khat daily for its amphetamine-like effects. There is little information in the literature concerning the possible effects of the habit on oral microbiota. Our objective was to study *in vitro* crude khat extract effects on *Streptococcus mutans* growth and sucrose-dependent colonization, and on its glucosyltransferase (GTF) activity and production. Three khat cultivars were used. Lyophilized crude aqueous khat extracts were applied to the different assays at concentrations of 0–1% (w/v). Sucrose-dependent colonization was assessed as the ability of *Streptococcus mutans* UA159 to form adherent biofilms in glass culture tubes. Colony forming units (CFUs) in the planktonic phase served as a measure of bacterial growth, while CFUs in the biofilm phase were used to quantify viability in the biofilms. GTFs activity was tested by incubating a crude GTFs preparation with sucrose and determining the amount of water-soluble and water-insoluble glucans formed. GTFs production was assayed by comparing intensities of GTF bands in Western blots of extracts from control and khat-containing cultures. The khat extracts effectively inhibited biofilm formation. The minimum biofilm inhibitory concentration (MBIC) varied among the cultivars (0.25–1%). The extracts also inhibited synthesis of both glucan types, particularly insoluble glucans (average 85% inhibition at 1%), with significant differences among the cultivars. However, khat increased bacterial growth and at sub-MBIC also viability within biofilms; there were no inter-cultivar differences. It is shown that khat leaves contain water-soluble constituents that inhibit some cariogenic properties of *S. mutans in vitro*.

Key Words: *Caries, Catha edulis, glucosyltransferase, khat, Streptococcus mutans*

Introduction

Streptococcus mutans, which is a principal cariogenic bacterium in humans [1,2], possesses several virulence factors that are associated with its cariogenicity [3]. An essential virulence factor of *S. mutans* is its sucrose-dependent, glucan-mediated colonization of teeth surfaces. Glucans are glucose polymers synthesized by extracellular glucosyltransferases (GTFs) [1]. There are three *S. mutans* glucosyltransferases: GTF B [4], GTF C [5], and GTF D [6]. GTF B and GTF C synthesize primarily water-insoluble glucans, while GTF D synthesizes only water-soluble glucans [7]. Water-insoluble glucans make a major contribution to plaque formation, and are essential for initiation of caries on smooth surfaces [8].

Interference with *S. mutans* ability to colonize teeth surfaces is an important strategy of dental caries prevention. In addition to being a vaccination target [9,10], inhibition of GTFs and sucrose-dependent *S. mutans* colonization has been a subject of many *in vitro* studies in which different agents, including monoclonal and polyclonal antibodies [11,12], plant extracts [13–15], natural substances [16,17], and chemical reagents [18], were shown to possess such inhibitory properties. Among active components present in plant extracts are tannins and other polyphenols [17,19–21].

Khat is the name generally used for *Catha edulis*, an evergreen shrub whose fresh leaves and twigs are habitually chewed for their stimulating amphetamine-like effects [22]. This habit is highly prevalent in Yemen and East Africa and has been introduced to Western countries by immigrants. The active substance responsible for the amphetamine-like effects is a phenylalkylamine called cathinone [23]. Khat has

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(Received 11 November 2004; accepted 10 February 2005)

ISSN 0001-6357 print/ISSN 1502-3850 online © 2005 Taylor & Francis
DOI: 10.1080/00016350510019838

a very complex alkaloid composition. Cathedulins, for example, comprise one group of alkaloids with up to 62 members [24]. Khat also contains vitamin C and tannins as well as small amounts of essential oils, sterols, triterpenes, thiamine, riboflavin, niacin, iron, and amino acids [25].

Despite the extensive literature about khat chewing, information concerning its effects on oral health is inadequate. A few studies have investigated the effects of khat chewing on oral hygiene, dentition, periodontium, oral mucosa, and temporomandibular joint, suggesting a number of detrimental or beneficial effects [26–34]. Concerning dental caries, the few studies published reported low caries prevalence among khat chewers [25,28].

During a khat-chewing session, oral bacteria are exposed for several hours to the various chemicals that are extracted from khat by saliva. Effects of such leachables on the growth and colonization patterns of oral bacteria, particularly those with relevance to dental and periodontal status, need to be elucidated. We previously investigated the effect of khat chewing on prevalence and levels of selected periodontal bacteria in sub- and supra-gingival plaque [35]. The objective of this study was to investigate *in vitro* effects of crude aqueous khat extracts on the growth and sucrose-dependent colonization of *S. mutans* as well as on its GTFs activity and production.

Material and methods

Khat extracts

Khat was purchased from a khat market in Sana'a city, Republic of Yemen. Three easily distinguishable Yemeni khat cultivars, referred to locally as *Thahla*, *Soti*, and *Hamdani*, were used. The fresh leaves and twigs were air-dried, packed in plastic bags, and transported to the Laboratory of Oral Microbiology, University of Bergen under permission from the Norwegian Medicines Agency in Oslo. Aliquots (10 g) of the dried material were each extracted with a total of 400 ml water over 4 h at 37°C. The water extracts were filtered using medium-grade filter papers (Schleicher & Schuell, Germany), lyophilized (Heto Drywinner, Heto-Holten, Denmark), and stored at −20°C. A fresh 10% (w/v) stock solution was prepared before each experiment by reconstitution of the freeze-dried extract in distilled water or potassium phosphate buffer (10 mM, pH 7.2) as appropriate and filter-sterilization using Acrodisc® syringe filters (Pall Corporation, USA). The extracts were also subjected to basic chemical analysis to quantify compounds with possible relevance to the experiments. Total carbohydrates were quantified using the phenol-sulfuric acid method [36], while total tannins were assessed using a colorimetric protein precipitation method [37].

Bacteria and culture medium

S. mutans UA159 (ATCC 700610) was used throughout and cultured in tryptic soy broth (TSB; Difco, USA) freshly buffered (FB) with 1 M $NaHCO_3$ to the final concentration of 0.1 M (pH 7.6). Hereafter, this medium is referred to as FB-TSB. All incubations were done at 37°C under microaerophilic conditions generated by the Anoxomat System™ (MART Microbiology BV, The Netherlands) connected to a gas source (80% N_2, 10% H_2, and 10% CO_2).

Anti-GTF antibodies

Rabbit polyclonal antibodies against GTFs were produced upon request by Eurogentec, Belgium. A 19-amino acid sequence, called GTF-P1, common to the different GTFs of *S. mutans* [11], was used as an immunogen with keyhole limpet hemocyanin as a carrier. It was previously shown that GTF-P1 contains a major B-cell epitope of GTFs [38] and that antibodies raised against it react with all GTFs of *S. mutans* [11].

GTFs preparation

A crude GTFs extract to be used in the enzyme activity assay was prepared according to the method described by Tomita et al. [39] with some modifications. *S. mutans* UA159 was grown in 1 l of FB-TSB for 24 h. The culture was divided into 125 ml aliquots and an equal amount of 100% ethanol chilled at −80°C for 30 min was added to each aliquot. The mixtures were kept cold at −20°C for half an hour and then centrifuged at 10,000*g* for 5 min to precipitate bacterial cells and proteins. The pellets were washed with acetone, suspended in a total of 30 ml of 8 M urea in potassium phosphate buffer, and vigorously stirred for 1 h. After centrifugation at 10,000*g* for 5 min, the supernatant containing the GTFs was dialyzed overnight against potassium phosphate buffer. Finally, the dialyzed GTFs extract was aliquoted into 1.5 ml Eppendorf tubes and stored at −20°C. Presence of the GTFs was confirmed by standard polyacrylamide gel electrophoresis (PAGE), activity staining [40], and Western blot (see Figure 1).

Biofilm formation and bacterial growth

A previously described biofilm model [39] was used with some modifications. In this model, the bacteria are grown in 2 ml FB-TSB supplemented with 5% sucrose and 0.01% filter-sterilized bovine submaxillary mucin (Sigma) in a sterile glass culture tube incubated at an angle of 30° to the horizontal. At the end of 10 h incubation a firmly adherent biofilm forms on the glass surface in contact with the bacterial culture. To test the effect of the khat extracts on *S. mutans* biofilm formation and growth, the model was run in a series of tubes as described above with the broth containing 2-fold

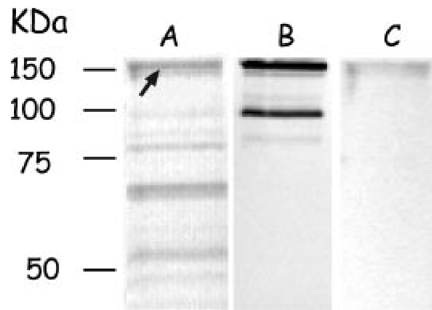

Figure 1. PAGE of the GTFs crude preparation. GTFs were visualized by (A) Coomassie blue staining (GTF bands arrowed), (B) PAS staining of glucans after incubation of the gel in sucrose-containing buffer (activity stain), and (C) Western blot. The band just below 100 kDa on the activity stain represents fructans. Additional bands represent proteins and glycoproteins present in the preparation.

serial dilutions of the khat extracts (1–0.01% w/v). The formed biofilms were challenged by vortexing for 10 s at 1200 rev/min (IKA mini-shaker, IKA-Werke Gmbh & Co., Germany). The planktonic and loosely adherent cell fraction was separated by decanting the 2 ml broth into another sterile tube, washing the biofilm for loose cells remnants with 2 ml phosphate buffered saline (PBS), and decanting them into the same tube. Finally, the biofilm was dispersed in 4 ml PBS (firm-cell fraction) by sonication for 10 s at 50 W (MSE 150 W Ultrasonic Disintegrator MK2; MSE Scientific Instruments). OD_{550} of the sonicate served as a measure of the amount of biofilm formed. Appropriate dilutions were made from both fractions and plated on Colombia blood agar (Acumedia Manufacturers, USA) for calculation of colony-forming units (CFUs). CFUs in the planktonic and loosely adherent cell fraction were used as a measure of bacterial growth, while those in the firm-cell fraction were used as a measure of viable cells in the biofilm. The experiment was performed six times, twice for each of the three khat cultivars.

GTFs activity

The glucan-synthesizing activity assay was performed as previously described [41], with some modifications. The reaction mixture (total volume 500 μl) consisted of 300 μl sodium acetate buffer (100 mM, pH 6.2) supplemented with 5% sucrose, 150 μl of the crude GTFs extract, and 50 μl potassium phosphate buffer (control) or 2-fold dilutions of the khat extracts (final concentration 1–0.01% w/v). The mixture was incubated at 37°C for 2 h. Water-insoluble glucans were precipitated by centrifugation at 10,000*g* for 5 min. The supernatant was then transferred to another tube and water-soluble glucans precipitated by adding 1 ml 100% ethanol followed by centrifugation as above. The glucan pellet was hydrolyzed by boiling in 300–400 μl of 1 M HCl for 30 min and then neutralized by addition of an equal amount of 1 M NaOH. Finally, glucans were quantified using the phenol-sulphuric acid method [36] with glucose as standard. To adjust for quantification errors due to khat carbohydrates that precipitated with the glucans, a parallel series of mixtures without the enzyme was prepared. Khat carbohydrates were then precipitated and hydrolyzed as for the glucan pellets and used to prepare spectrophotometeric blanks for the quantification procedure. The experiment was performed in duplicate for each cultivar.

GTFs production

Bacteria were grown on blood agar without khat extract (control) or with khat extract (0.25% w/v) for 2 days. Bacterial growth was harvested from 4 plates, suspended in 1 ml 0.1 M sodium dodecyl sulphate (SDS) in PBS, and vortexed for 1 h to extract extracellular and cell-bound proteins including GTFs. The cells were then pelleted by centrifugation at 10,000*g* for 5 min and the supernatant transferred to another tube. The protein concentration of the extracts was determined by the RC DC protein assay (Bio-Rad). Finally, Western blot analysis was performed using the anti-GTF antiserum. Relative amounts of GTFs produced were estimated by comparing intensities of GTFs bands on the Western blots of the different extracts using UVI Spot (UVItec Limited, UK).

Statistical analysis

For each outcome, data were summarized as means $\pm$SD of six determinations taking all khat cultivars together. Data were also summarized for individual cultivars as means of two determinations. To test for statistical significance of the effect of khat in each assay, linear regression analyses were performed with khat concentration as covariate. Khat cultivar was also included in the model as a categorical covariate to test for significant inter-cultivar effect differences. *P*-values $\leq$0.05 were considered statistically significant. Analyses were performed using SPSS 12.0 (SPSS Inc., USA).

Results

The khat extracts

The filtered aqueous extract of the *Soti* cultivar was yellowish-brown, while those of the other two cultivars were reddish-brown. All extracts had a pH of 6.0. The lyophilization process resulted in powders with colors corresponding to those of the aqueous extracts. The yield was 2–2.5 g extract per 10 g of the dry plant material. The total carbohydrate and tannins of the lyophilized extracts from the three cultivars are given in Table I.

Biofilm formation and bacterial growth

The effect of different khat extract concentrations on biofilm formation is shown in Figure 2. Khat effectively

Table I. Total carbohydrates and tannins in the lyophilized extracts from the different khat cultivars (mg/g)

	Sawti	Thahla	Hamdani
Carbohydrates	328	360	351
Tannins	18.6	17.3	8.2

inhibited formation of adherent biofilms. There was a threshold concentration at which total inhibition occurred. The threshold concentration, designated as minimum biofilm inhibitory concentration (MBIC), differed significantly among the cultivars. The *Soti* and *Thahla* cultivars were significantly more active biofilm inhibitors with a MBIC of 0.25% as opposed to 1% for the *Hamdani* cultivar.

Figure 3 shows viable cell counts in biofilms formed under sub-minimal biofilm inhibitory concentrations (sub-MBIC) at which the biofilm formation itself was not affected. Presence of khat significantly increased viable cell counts in the biofilms in a concentration-dependent manner. At the highest sub-MBIC (0.0625%) the increase was about 3-fold. There was no significant inter-cultivar effect difference. Similarly, the khat extracts significantly enhanced planktonic bacterial growth in a concentration-dependent manner (Figure 4). The increase was within the same log (3–4-fold at 1%). The effect did not significantly differ among the cultivars.

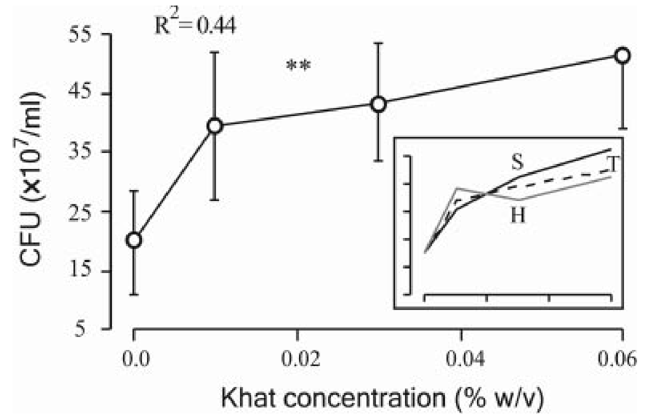

Figure 3. Effect of the khat extracts at sub-minimal biofilm inhibitory concentrations (sub-MBIC) on counts of viable cells (CFU) in the biofilm. For details, see Figure 2.

in a concentration-dependent manner. At the lowest concentration (0.0125%) there was an average of 20% and 24% inhibition of soluble and insoluble glucans synthesis, respectively. At 1% the average inhibition reached 73% for soluble glucans and 85% for insoluble glucans. There was a significant inter-cultivar effect difference particularly for water-insoluble glucans. As for the biofilm assay, the *Soti* and *Thahla* cultivars were more efficient, inhibiting 80% and 44% of insoluble and soluble glucans, respectively, at a concentration of 0.25%. A similar inhibition of insoluble glucans by the *Hamdani* cultivar occurred at 1%.

GTFs activity

Figure 5 summarizes the effect of the khat extracts on water-soluble and water-insoluble glucans synthesis by GTFs. Synthesis of both types of glucans was inhibited

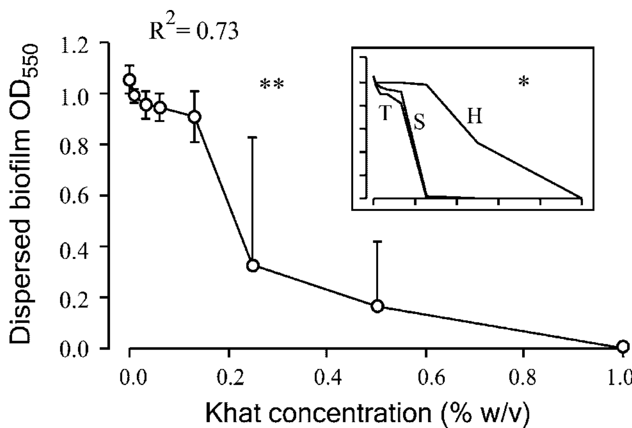

Figure 2. Formation of adherent biofilm by *S. mutans* UA 159 in the presence of various concentrations of the khat extract. The amount of formed biofilm was measured as OD_{550} after being dispersed. Data are presented as means $\pm$ SD of six determinations taking all cultivars together. The insert with a similar scale shows the same information for the individual khat cultivars (abbreviated T, S, and H) as means of duplicate determinations. Data were analyzed by multiple linear regression with khat concentration and cultivar type as covariates. $^*p<0.05$, $^{**}p<0.001$ (within the insert indicates significance of inter-cultivar effect difference). R^2 = correlation coefficient.

GTFs production

Contrary to their effect on GTFs activity, the khat extracts did not inhibit GTFs production but rather up-regulated it by 2–2.5-fold (Figure 6). Since GTFs B, C, and D have almost the same molecular weight, they were detected as a single band (i.e. total GTFs). The antiserum used did not show cross-reaction with other proteins in the preparation except for an occasional faint unidentified band at about 50 kDa.

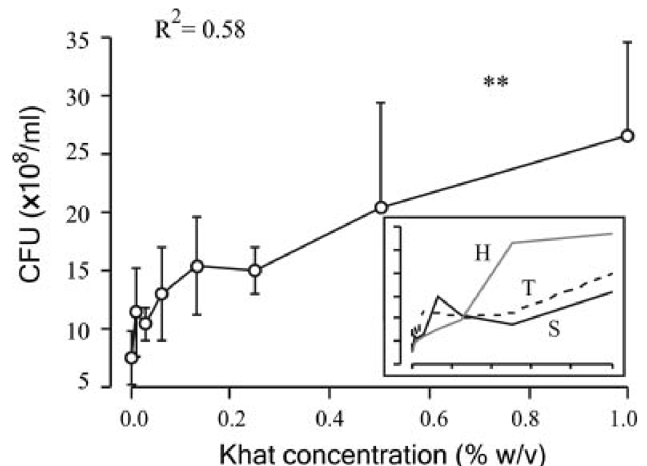

Figure 4. Effect of the khat extracts on planktonic bacterial growth measured as CFU. For details, see Figure 2.

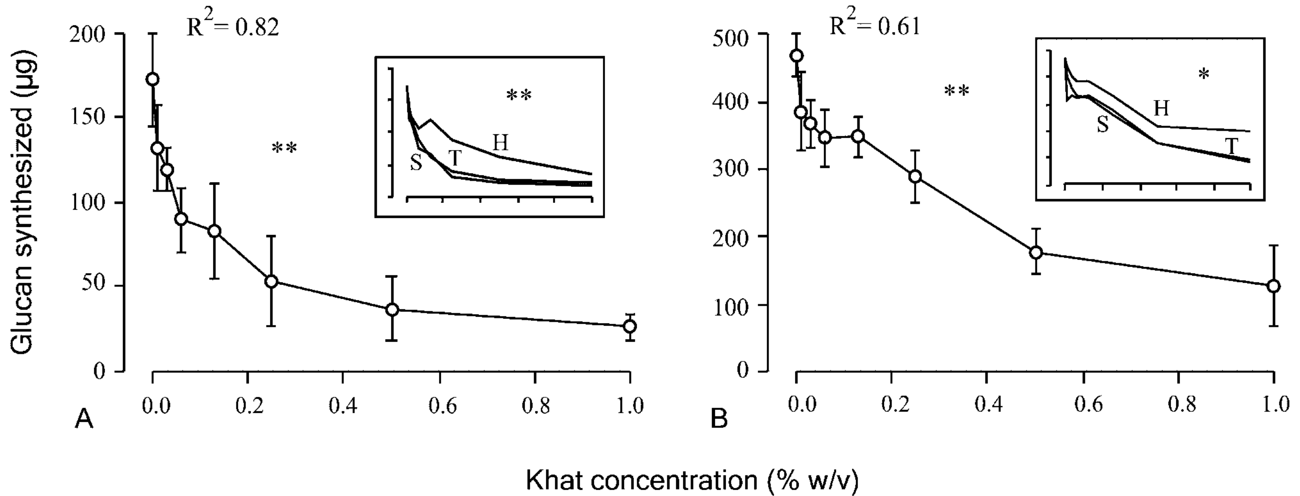

Figure 5. Effect of the khat extracts on synthesis of (A) water-insoluble and (B) water-soluble glucans. For details, see Figure 2.

While GTFs were up-regulated, a number of other extracellular and cell-bound proteins were down-regulated, as shown by one-dimensional PAGE (see Figure 7).

Discussion

Low caries prevalence among khat chewers has been reported [25,28]. However, no information is available whether this observation is a direct or indirect effect of khat chewing. *In vivo* and *in vitro* studies are therefore needed to elucidate the biological basis for the observed inverse association between khat chewing and dental caries. In line with this, *S. mutans* is a primary focus for such studies. Therefore, the purpose of the current study was to study *in vitro* effects of crude khat extracts on some cariogenic factors of *S. mutans* including growth, colonization, and GTFs activity and production. To be clinically relevant, extractions of khat were only made in water and at 37°C.

Biofilm formation on glass is a simple model that has been used by others to assess sucrose-dependent colonization of *S. mutans* [13,15,21,42]. In the current study, the biofilms were challenged by vortexing at 1200 rev/min for 10 s to show the gradual decrease in their adherence/integrity as the khat extract concentration increased. In fact, control biofilms withstood vortexing at 3000 rev/min while simple inversion of the glass tubes was enough to detach biofilms formed at 0.5–1% of the khat extract (data not shown). The khat extracts activity on adherence of *S. mutans* to glass was explained by subsequent results showing that the extracts inhibited glucans formation, particularly insoluble glucans, in a concentration-dependent manner. However, while GTFs activity was retarded by even the lowest concentration tested (0.0125%), biofilm formation was minimally affected at concentrations below 0.25%, suggesting that a certain amount of glucans-synthesizing activity inhibition was required (>40%) before biofilm formation was affected. The effect of the extracts on GTFs production as another possible mechanism of biofilm formation inhibition was ruled out.

The demonstrated properties of the khat extracts are not surprising since other plant extracts with such

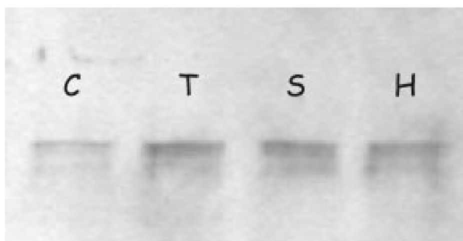

Figure 6. Effect of the khat extracts on production of GTFs by *S. mutans* UA 159 as shown by a Western blot of extracts from bacteria grown on control (C) and khat (T, S, and H)-containing blood agar, using rabbit anti-GTF antiserum. Bands were compared using UVI Spot (UVItec limited, UK). Degraded forms of GTFs appear as faint bands of lower molecular weight. T, S, and H are the name initials of the three khat cultivars used.

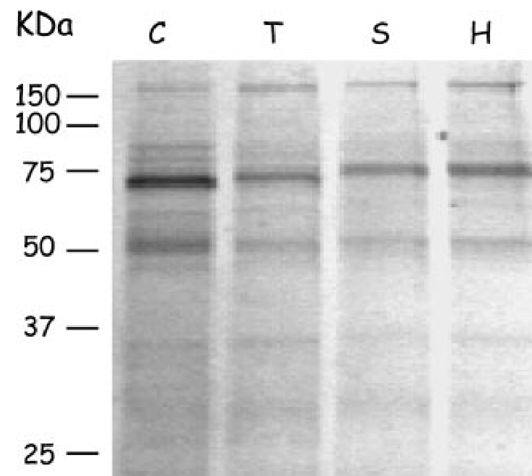

Figure 7. Coomassie blue-stained, one-dimensional PAGE of extracellular and cell-bound proteins extracted from *S. mutans* UA 159 grown on control (C) and khat (T, S, and H) containing blood agar. T, S, and H are the name initials of the three khat cultivars used.

properties have been reported previously [13,15,19, 21,43]. Tea (*Camellia sinensis*), for example, is one plant that has been extensively investigated for its anticariogenic properties [44]. Investigations have repeatedly implicated polyphenols, particularly tannins, as the active components of the plants investigated [17,20,21,45]. In the current study, the difference in total tannins present in the extracts from the 3 khat cultivars corresponded well to the differences in extract activity. In other words, the extract from the *Hamdani* cultivar, being the least active one, had about half the amount of tannins present in the extracts from the other two cultivars.

The extracts had no antibacterial effect against *S. mutans*. They rather favored planktonic bacterial growth and, at sub-MBIC, increased the number of viable cell counts in the biofilms. The presence of more viable acid-producing cells in the biofilm suggests an increase in its pH-lowering ability. However, this may be compensated for by the reduction in formation of glucans, which are known to modulate biofilm permeability and pH-lowering ability [46]. The growth enhancement may have been due to the high carbohydrate content of the extracts (about 35%). In addition, khat is rich in vitamin C (150 mg/100 g of fresh leaves) [25] and this, too, may have played a role.

The current study has limitations that have to be considered, particularly when extrapolating the results to the *in vivo* situation. Firstly, extractability of khat fresh leaves may differ from that of the dry plant material used in the study. Tannins, for example, may be less extractable from dry plant materials due to increased fixation *in situ* [47]. Some substances may be labile and thus degrade when plant leaves are being dried. Cathinone, which is the psychoactive component of khat, is a good example [22]. Secondly, in this study, exposure to the extracts was throughout the period of each assay, while in the oral cavity bacteria are exposed to the substances released by the chewing process for a maximum of 10 h per day. Thirdly, in addition to the sucrose-dependent colonization, *S. mutans* possesses a sucrose-independent colonization mechanism that involves interaction between cell surface-associated proteins and acquired dental pellicle [48], and this was not evaluated in the current study. Fourthly, since a crude GTF preparation was used, any inhibitory effect of the extracts on individual enzymes could not be examined. However, available results make some straightforward conclusions about the effect on individual enzymes possible; synthesis of insoluble glucans was more effectively inhibited, indicating that GTF-B and GTF-C were more susceptible to the extracts than GTF-D. Finally, fructosyltransferase was also present in the preparation (see Figure 1), and thus fructans must have formed and precipitated along with soluble glucans, which is a limitation that could not have been overcome unless purified enzymes or radioactively labeled substrates had been available. However, fructans are not important for *S. mutans* adhesion and they mainly act as an energy source [8].

In conclusion, the results show that khat leaves and twigs have water-soluble constituents, probably tannins, that are capable of inhibiting activity of GTFs from *S. mutans* and thus interfering with its ability to form adherent biofilms, suggesting that khat may have some anticariogenic properties. However, the effect of khat extracts on these virulence factors, as well as on others, has to be evaluated in a polymicrobial biofilm model if we are to better understand the situation *in vivo*.

Acknowledgments

We thank Professor George Francis for his help with the chemical analysis of khat extracts. The study was supported by the Norwegian Loan Fund for Education.

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