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In vitro antimicrobial and resistance-modifying activities of aqueous crude khat extracts against oral microorganisms

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Summary

Objectives: To assess antimicrobial activities of aqueous crude khat (*Catha edulis*) extracts against a panel of oral microorganisms and to test their ability to modify bacterial resistance to tetracycline and penicillin in vitro.
Design: Lyophilized aqueous extracts were prepared from three khat cultivars. The agar dilution method of the NCCLS was used to test the extracts, at concentrations of 20—1.25 mg/ml, against 33 oral strains. MIC was defined as the lowest concentration at which there was no visible growth. Slight growth was defined as marked growth reduction (MGR). The E-test was used to determine the MICs of tetracycline and penicillin-G for three resistant strains in absence and presence of a sub-MIC of the khat extracts (5 mg/ml).
Results: Eighteen strains (55%) were sensitive to the extracts (MICs 5—20 mg/ml). Most of these were periodontal pathogens with *Porphyromonas gingivalis* and *Tannerella forsythensis* being the most susceptible (MIC 5—10 mg/ml). *Veillonella parvula*, *Actinomyces israelii* and some streptococci were not sensitive. Except for *Lactobacillus acidophilus* that showed MGR at 1 mg/ml, cariogenic species were neither sensitive. The extracts were active against *Streptococcus pyogenes* (MIC 10—20 mg/ml) but not against *Candida albicans* and *Staphylococcus aureus*. The presence of the khat extracts at a sub-MIC resulted in a 2—4-fold potentiation of the tested antibiotics against the resistant strains.
Conclusions: Khat has water-soluble constituents possessing selective antibacterial activity against oral bacteria. There is preliminary evidence for presence of an antibiotic resistance-modifying component. Further investigation is needed to identify the active components and assess their clinical relevance.

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Introduction

The human oral cavity is a habitat for about 500 cultivable and non-cultivable bacterial species.[1] Up to 100 species can be present in a particular oral cavity.[2] While the majority of these species are commensals, a subset is opportunistic pathogens. Their key role of in the etiology of periodontitis and dental caries, the most prevalent diseases in the world, is well established.[3] They have also been implicated in the etiology of a number of systemic diseases like infective endocarditis,[4,5] respiratory infections,[6,7] cardiovascular diseases[3,8] and brain abscess.[9]

Oral bacterial isolates resistant to pencillins, metronidazole, tetracycline and macrolides have been reported by researchers from different countries.[10] Such resistant bacteria have also been isolated from infections at extra oral sites.[11] To cope with the wide-spread problem of antibiotic resistance, a number of strategies such as reduced antibiotic use and antibiotic alternatives have been proposed.[12] Among antibiotic alternatives are therapies derived from complementary and alternative medicine. In fact, there are an overwhelming number of studies on the antibacterial activities of plant and natural products derivatives. Essential oils are one such example.[13,14] Recently, some plant extracts have been shown to potentiate the activity of antibiotics against resistant bacterial strains, introducing the concept of resistance modification.[15,16]

Millions of Yemenites and East Africans as well as immigrants to the western countries habitually chew the fresh leaves and twigs of *Catha edulis*, an evergreen plant of the family Celestraceae commonly known as khat. Khat chewing produces stimulating amphetamine-like effects that are attributable to cathinone, a phenylalkylamine present in the fresh plant material.[17] Khat has a complex group of alkaloids (cathedulins).[18] It also contains vitamin C and tannins as well as small amounts of essential oils, sterols, triterpenes, thiamine, riboflavin, niacin, iron and amino acids.[19]

There is one report of antimicrobial activity of extracts from khat in which two isolated compounds (22 β-hydroxytingenone and tingenone) were shown to possess potent antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus durans* and *Mycobacterium* species but not against *Escherichia coli* and *Candida albicans*.[20] Oral bacteria are exposed to leachable khat constituents during the chewing process (4—10 h per day). In a previous in vivo study, it was shown that khat chewing influenced the prevalence and levels of some periodontal bacteria in sub- and supragingival plaque, inducing a microbial profile that was not incompatible with periodontal health.[21] In another study, aqueous khat extracts were shown to inhibit glucan synthesis and adherent biofilm formation by *Streptococcus mutans*, while favor its growth.[22] In vitro effects of khat extracts on oral bacteria other than *S. mutans* have never been reported. The purpose of the present investigation was therefore firstly, to assess in vitro antimicrobial properties of crude aqueous khat extracts against a panel of selected oral microorganisms that are relevant to periodontitis, dental caries and systemic diseases and secondly, to test the ability of these extracts to modify resistance of clinical oral bacterial isolates to tetracycline and penicillin.

Materials and methods

Khat extracts

Samples of three Yemeni khat cultivars, referred to locally as *Thahla*, *Soti*, and *Hamdani*, were purchased from a khat market in Sana'a city, Republic of Yemen. 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, Oslo. Specimens of the three cultivars were deposited at Herbarium BG, University of Bergen (no. ES 001001, 001002, and 001003).

Twenty-gram aliquots of the dried material were each extracted with a total of 500 ml distilled water over 5 h at 37 °C with shaking (200 rpm). The water extracts were filtered using medium grade filter papers (Schleicher & Schuell, Germany), lyophilized (Heto Drywinner, Heto-Holten, Denmark) and stored at −20 °C (extraction yield was 20—25%). Fresh stock solutions (200—12.5 mg/ml) were prepared by dissolving 5 g of the freeze-dried extract in 25 ml distilled water, filter-sterilization using Acrocap® syringe filters (Pall Corporation, USA) and then making serial two-fold dilutions in sterile distilled water.

Test strains

Thirty-three selected oral strains were tested against the extracts. These were categorized as cariogenic bacteria (4 strains),[23] periodontal disease-associated bacteria (14 strains), periodontal health-associated bacteria (12 strains),[2,24] and others (three strains). The selected strains were mostly laboratory strains, but some clinical strains were also included (Table 1). Clinical strains were initially isolated based on colony characteristics on appropriate selective media and then further iden-

Table 1 Antimicrobial activity of aqueous extracts of leaves and twigs of three Yemeni khat cultivars.

	MIC[a]				
	Thahla	*Soti*	*Hamdani*	TC[b]	AC[c]
Periodontal disease-associated bacteria					
Porphyromonas gingivalis ATCC 33277	5	5	5	0.016	<0.016
Porphyromonas gingivalis Clinical isolate	10	10	5	0.016	<0.016
Tannerella forsythensis FDS 2008	10	5–10	5	<0.016	<0.016
Actinobacillus actinomycetemcomitans ATCC 33384	20	20	10	0.125	0.064
Actinobacillus actinomycetemcomitans ATCC 43717	MGR[d]	MGR	10	0.125	0.125
Actinobacillus actinomycetemcomitans Clinical isolate	MGR	MGR	20	0.25	0.25
Prevotella intermedia VPI 4197	10–20	20	10	0.125	<0.016
Campylobacter rectus ATCC 33238	–	–	–	<0.016	0.125
Eubacterium nodatum CCUG 15996	10	10	10	0.064	<0.016
Peptostreptcoccus micros CCUG 17638	20	20	10–20	0.25	0.032
Fusobacterium nucleatum ATCC 25586	20	20	20	0.064	0.032
Fusobacterium nucleatum Clinical isolate 1	20	20	20	0.064	0.016
Fusobacterium nucleatum Clinical isolate 2	–	–	–	0.125	0.064
Streptococcus constellatus ATCC 27823	20	20	MGR	32[e]	0.125
Periodontal health-associated bacteria					
Veillonella parvula ATCC 10790	–	–	–	0.25	0.064
Actinomyces israelii CCUG 18307	–	–	–	0.016	<0.016
Actinomyces naeslundii CCUG 15310	20	20	20	0.064	0.032
Eikenella corrodens ATCC 23834	–	–	–	0.125	<0.016
Eikenella corrodens Clinical isolate	–	–	–	0.125	0.032
Capnocytophaga gingivalis ATCC 33624	10–20	20	10	0.016	0.032
Streptococcus intermedius ATCC 27335	–	–	–	1	0.032
Streptococcus mitis ATCC 9811	20	20	20	1	0.032
Streptococcus salivarius ATCC 13419	–	–	–	0.5	0.032
Streptococcus gordonii CCUG 33482	20	20	20	0.5	0.064
Streptococcus sanguis ATCC 10556	20	20	MGR	0.5	0.125
Streptococcus anginosus ATCC 33397	–	–	–	1	0.064
Cariogenic bacteria					
Streptococcus mutans ATCC 25175	–	–	–	1	0.032
Streptococcus sobrinous CUGG 25735	–	–	–	1	0.016
Lactobacillus acidophilus CCUG 5917	MGR	MGR	MGR	0.5	0.064
Lactobacillus fermentum CCUG 30318	–	–	–	1	0.032
Others					
Staphylococcus aureus ATCC 6538	–	–	–	1	0.064
Streptococcus pyogenes Clinical isolate	10–20	20	10–20	0.5	0.016
Candida albicans Clinical isolate	–	–	–	NT	NT

ATCC: American Type Culture Collection; FDC: Forsyth Dental Center; VIP: Virginia Polytechnic Institute and State University; CCUG: Culture Collection, University of Gothenburg; –: not sensitive at 20 mg/ml; NT: not tested; amphotericin B was used as standard (MIC = 0.125).

[a] Minimum inhibitory concentration: mg/ml for the extracts and μg/ml for the standards.
[b] Standards: TC, tetracycline.
[c] Standards: AC, amoxicillin.
[d] Marked growth reduction.
[e] Resistant.

tified using biochemical assays except for *Candida albicans* and *Streptococcus pyogenes* that were identified by the germ tube test and serotyping, respectively. All strains were sub cultured at least twice before being tested. For the resistance modification assay, strains available for testing were the tetracycline-resistant *Streptococcus oralis* TH-13 and *Streptococcus sanguis* SH-2 (originally provided by Professor Thae Horaud) and the penicillin-resistant *Fusobacterium nucleatum* 9911 (kindly provided by Prof. Eija Könönen).

Susceptibility assay

Susceptibility testing was performed using the agar dilution method according to the National Committee for Clinical Laboratory Standards (NCCLS).[25,26] Supplemented brucella agar (SBA; BBL) was used for

anaerobes while Muller–Hinton agar (Difco) supplemented with 5% defibrinated sheep blood (MHA-B) was used for aerobes. To prepare agar dilution plates, the khat extracts were incorporated at five concentrations (20–1.25 mg/ml) by diluting the fresh khat stock solutions 1:10 in warm (~50 °C) molten SBA or MHA-B and pouring into 9 cm Petri dishes. The plates were stored at 4 °C and used within 1 week. Suspensions of the test strains with a turbidity equivalent to McFarland standard no. 0.5 were prepared and further diluted 1:10 in normal saline. Finally, an automatic multipoint inoculator (MAST Group Ltd., UK) was used to spot 1 μl of each suspension onto the agar surface. Two khat-free plates were inoculated before and after each series of plates to serve as growth controls and to check for contamination. SBA plates were incubated anaerobically at 37 °C for 2 days while MHA-B plates were incubated microaerophilically at 37 °C for 1 day. Microaerophilic and anaerobic conditions were generated by an Anoxomat System™ (MART Microbiology BV, The Netherlands) connected to a gas source (80% N_2, 10% H_2 and 10% CO_2). Minimum inhibitory concentration (MIC) was defined as the minimum concentration of the khat extract at which there was no visible growth. Slight growth or multiple tiny colonies were described as marked growth reduction (MGR). In susceptibility testing according to NCCLS, reference strains with known MIC values are usually included for quality control. Since no such reference is available for khat, tests were done in duplicate in two experimental setups and MICs were reported as a range if readings differed. Tetracycline and amoxicillin were used as standards (amphotericin B was used with *C. albicans*).

Resistance modification assay

To test for resistance-modifying activities of the khat extracts, the MICs of tetracycline and penicillin-G were determined for the three test strains in absence and presence of a sub-MIC of the khat extracts (5 mg/ml) using the E-test (AB Biodisk, Sweden).

Results

Antimicrobial activity

The antimicrobial activity of the aqueous khat extracts against the 33 selected oral strains is shown in Table 1. The extracts demonstrated selective antimicrobial properties. Most periodontal disease-associated bacteria, particularly *Porphyromonas gingivalis* and *Tannerella forsythensis*, were sensitive with MICs of 5–20 mg/ml. Only *Campylobacter rectus* and one clinical strain of *F. nucleatum* were resistant. *Actinobacillus actinomycetemcomitans* ATCC 43717 and the clinical strain were only sensitive to the *Hamdani* cultivar extract but showed MGR at 20 mg/ml of the other two extracts. Periodontal health-associated bacteria were less susceptible with only five strains being sensitive at the highest concentration tested (20 mg/ml). None of the cariogenic bacteria were sensitive. However, *Lactobacillus acidophilus* showed MGR at 10 mg/ml. The extracts were active against *S. pyogenes* (MIC 10–20 mg/ml) but not against *C. albicans* and *Staphylococcus aureus*. We observed that *S. pyogenes* was not hemolytic in presence of even the lowest concentration (1.25 mg/ml) of the khat extracts.

Although the extracts of the three cultivars had generally the same antimicrobial profile, there were some differences in their MICs for some strains. However, no extract was consistently more active than the others. While the extract of the *Hamadani* cultivar was more active against bacteria like *P. gingivalis, T. forsythensis,* and *A. actinomycetemcomitans*, it showed less activity against *Streptococcus constellatus* and *S. sanguis*.

Resistance modification

Table 2 shows the effect of the khat extracts on the MICs of tetracycline and penicillin-G for the three test strains. Presence of the khat extracts at a sub-MIC (5 mg/ml) resulted in a 2- and 4-fold potentiation of tetracycline against *S. sanguis* TH-13 and *S. oralis* SH-2, respectively. Also, there was a 4-fold potentiation of the penicillin-G activity against *F.*

Table 2 Modification of antibacterial resistance by aqueous khat extracts as shown by change in the MICs for the test strains.

Test strain	Antibiotic	MIC (μg/ml)	
		No khat present	Khat present (5 mg/ml)
Streptococcus oralis SH-2	Tetracycline	16	4
Streptococcus sanguis TH-13	Tetracycline	128	64
Fusobacterium nucleatum 9911	Penicillin-G	64	16

nucleatum 9911. However, only for *S. oralis* SH-2 the potentiation resulted in a shift from ''resistant'' to ''intermediate''.[27] No differences among the three cultivars were observed.

Discussion

Oral bacteria have been recently tested for antimicrobial susceptibility to a number of plant extracts and natural substances.[14,28–30] To the best of our knowledge, this is the first report on antimicrobial activity of khat against oral bacteria. The test strains used in the study were selected and categorized from an oral health point of view; however, many of them like *P. gingivalis*, *A. actinomycetemcomitans* and streptococci are involved in systemic diseases particularly infective endocarditis.[4,5] For clinical relevance, since saliva is basically water, we tested only aqueous khat extracts made at 37 °C.

Due to the crude nature of the khat extracts, the MICs obtained were much higher than those of the antibiotics. However, the MICs are indeed comparable to or even less than those of some other crude plant extracts, e.g. garlic aqueous extracts.[31] Furthermore, for khat chewers, these concentrations are probably achievable in vivo because of the large quantities of khat chewed at a time (100–200 g). In fact, the concentration range of the khat extracts used in the current study was chosen on the basis of the quantity mentioned above and the presumed salivary rate while chewing.

While most of the periodontal pathogens were inhibited by the extracts, only a few periodontal health-associated bacteria were sensitive at 20 mg/ml, the highest concentration tested. In fact, the growth of some species like *Veillonella parvula* and *Actinomyces israelii* was not attenuated at all in the presence of the khat extracts. All streptococci showed better growth on plates with low concentrations of the khat extracts compared to control plates. This selectivity may provide an explanation, at least in part, for findings from a previous study on the effect of khat chewing on selected periodontal bacteria in sub- and supragingival plaque.[21] In that study, it was found that khat chewing significantly increased the prevalence and/or levels of periodontal health-associated bacteria while decreased those of some periodontal disease-associated bacteria.

Except for the marked growth reduction demonstrated by *L. acidophilus*, the extracts were inactive against the cariogenic bacteria. For *S. mutans*, this is consistent with a previous study in which aqueous khat extracts were shown to increase *S. mutans* planktonic growth.[22] However, possible anticariogenic properties of khat can not be ruled out because the same study showed that the extracts effectively inhibited glucan synthesis and biofilm formation by *S. mutans*.

A number of plant constituents with antimicrobial properties have been described. Tannins with its hydrolysable and condensed types are one such example.[32,33] Khat contains tannins and their total amount in the aqueous extracts used in the current study was previously measured to be 8–19 mg per g of the lyophilized material.[22] Alkaloids have also been shown to possess antibacterial properties against oral and non-oral bacteria.[34,35] Khat has a complex alkaloid composition and a recent study revealed the presence of 62 cathedulins in crude methanolic extracts of khat.[18] Other plant constituents that have been widely tested for antimicrobial activity are essential oils.[13,14] At least 11 compounds were identified in the essential oil from khat,[36] some of which like α-terpineol and α-pinene are antibacterial.[37] Differences in the antimicrobial activity among the cultivars suggest that there is more than one potentially active component, the concentration of which differs among the cultivars. Fractionation of the extracts to identify such components is required.

Interestingly, in addition to its selective antibacterial activity, the khat extracts at a sub-MIC (5 mg/ml) potentiated the activity of tetracycline and penicillin-G against the resistant test strains. Since no such potentiation was observed against sensitive or slightly resistant strains (data not shown), the activity was referred to as ''resistance modification'', an expression adopted from recent reports of compounds from *Lycopus europaeus* and *Rosmarinus officinalis* that modulate resistance of *S. aureus* to tetracycline and erythromycin.[15,16] However, this finding provides preliminary evidence for such an activity; purification of potentially active components and testing a larger panel of bacterial strains resistant to more antibiotics are indicated.

In conclusion, the results show that aqueous khat extracts exhibit selective antibacterial activities against oral bacteria, and provide preliminary evidence for presence of one or more water-soluble constituents with antibiotic resistance-modifying properties. Investigation is ongoing to identify the active component(s), to elucidate underlying mechanisms, and to assess the clinical relevance of the findings of the present study.

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