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Subgingival plaque microbiota in Saudi Arabians after use of miswak chewing stick and toothbrush

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Abstract

Background: The chewing stick, the miswak, is used in many developing countries as the traditional means for oral hygiene. It is prepared from the roots, twigs and stem of *Salvadora persica* or other alternative local plants.

Objectives: To compare the effects of the chewing stick miswak (from *S. persica*) and toothbrush on subgingival plaque microflora among Saudi Arabian individuals. Further, to investigate whether components extracted from *S. persica* may interfere with the subgingival plaque micro-organisms.

Material and Methods: Fifteen healthy Saudi Arabian male volunteers aged 21–36 years were included in a single-blind, randomized cross-over study. The participants were taught how to use each device properly. Plaque sampling for DNA test was performed at the baseline, 1 week after professional tooth cleaning, and after 3 weeks of either miswak or toothbrush use. Identification and quantification of microbial species were performed by the checkerboard method, using whole genomic, digoxigenin-labelled DNA probes. Inhibition zones around miswak were examined on agar plates with *Actinobacillus actinomycetemcomitans* and the leukotoxicity of this bacterium was analyzed in a bioassay with macrophages ± extracts of miswak.

Results: Miswak and toothbrushing had a similar influence on the levels of the subgingival microbiota. However, *A. actinomycetemcomitans* was significantly more reduced by miswak ($p < 0.05$) than by toothbrushing. These results were supported by our in vitro results which, indicated that extracts from *S. persica* might interfere with the growth and leukotoxicity of *A. actinomycetemcomitans*.

Conclusions: In contrast to toothbrush use, miswak use significantly reduced the amount of *A. actinomycetemcomitans* in the subgingival plaque.

Key words: DNA probe; oral microbiota; *Salvadora persica*; subgingival plaque

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The chewing stick, the miswak, is used in many developing countries as a traditional means for oral hygiene. The most common chewing stick is prepared from the roots, twigs and stem of *Salvadora persica* or other alternative local plants. Chewing sticks may play a role in the promotion of oral hygiene, and evaluation of the effectiveness of chewing sticks requires further research according to the Consensus Statement on Oral Hygiene (2000). The World Health Organization (WHO) has also recommended and encouraged the use of these sticks as an effective tool for oral hygiene (WHO 1984). A recent study in Saudi Arabia disclosed major variations in oral hygiene habits among people from different age and socio-economic levels (Al-Otaibi et al. 2003a). Among 50–60-year olds, 44% of those with lower education never used a toothbrush but all were regular miswak users. Several reports have claimed that chewing sticks are effective in reducing plaque and gingival inflammation. It has been reported that the periodontal status of miswak users in a Sudanese population was similar to that of toothbrush users (Darout et al. 2000). Recently, the results of a single-blind cross-over clinical study indicated that the miswak was more effective than toothbrushing

for reducing plaque and gingivitis in a sample of male Saudi Arabians, when the experimental period was preceded by professional instruction of the proper use of miswak and toothbrush (Al-Otaibi et al. 2003b).

The value of chewing sticks is believed to be in their mechanical cleansing action. However, the use of miswak has also been reported to inhibit the formation of dental plaque chemically, and exert antimicrobial effect against many oral bacteria (Al-Lafi & Ababneh 1995). In vitro studies have demonstrated that aqueous extracts of miswak have growth-inhibitory effects on several micro-organisms (Homer et al. 1992, Al-Lafi & Ababneh 1995, Almas & Al-Bagieh 1999).

Dental plaque is a biofilm containing approximately 500 different microbial species (Moore & Moore 1994, Paster et al. 2001). Certain species such as *A. actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia* and *Treponema denticola* have been suggested to be etiological agents in destructive periodontal diseases (van der Weijden et al. 1994). The periodontal disease with the clearest association with an oral bacterium is a condition known as localized aggressive periodontitis (LAP), previously known as localized juvenile periodontitis (LJP) (Armitage 1999). The major causative agent of LAP is *A. actinomycetemcomitans* (Mandell et al. 1987, Slots & Ting 1999) and this bacterium has also been implicated in adult forms of destructive periodontal diseases (Rodenburg et al. 1990). This bacterium has been reported to be the causative agent of a number of infections including bacterial endocarditis, meningitis, septicaemia, absesses and osteomyelitis (Page & King 1966, Muhle et al. 1979, van Winkelhoff & Slots 1999). *A. actinomycetemcomitans* produces a leukotoxin that is considered to be an important virulance factor implicated in the pathogenesis of periodontal disease (Newman et al. 1996). This toxin can protect a population of bacteria from phagocytic killing (Johansson et al. 2000a). Moreover, leukotoxin of *A. actinomycetemcomitans* induces extracellular release of granule enzymes from polymorphonuclear leukocytes (PMNs) (Johansson et al. 2000a, Claesson et al. 2002). Recently, it has been reported that leukotoxin of *A. actinomycetemcomitans* kills human macrophages in a mechanism that induces caspase-1 activation and a rapid secretion of bioactive IL-1β (Kelk et al. 2003).

It has been demonstrated in vitro that the aqueous extract of miswak has growth-inhibitory effects on several oral micro-organisms (Homer et al. 1992, Al-Lafi & Ababneh 1995, Almas & Al-Bagieh 1999). Using the checkerboard DNA–DNA hybridization (CKB) method, Darout et al. (2002) stated that miswak may have a selective inhibitory effect on the levels of certain bacteria in saliva, particularly several oral streptococci species.

The main aim of the present study was to compare the effects of the use of the miswak and toothbrushing on subgingival plaque microflora in male Saudi Arabian individuals. Furthermore, and as a follow-up of the interesting clinical findings, we extended our aim to investigate whether components extracted from *S. persica* (miswak) may interfere with the growth and leukotoxicity of *A. actinomycetemcomitans*.

Material and Methods

Experimental design

The study was approved by the Ethics Committee at Huddinge University Hospital and by the General Medical Affair Administration at Makkah City. The study was performed according to a single-blind, randomized cross-over design. During the miswak period, subjects were instructed to use the miswak before prayer (in accordance with the religious tradition) five times a day and to refrain from using a toothbrush. During the toothbrush period, they were instructed to brush their teeth two times per day without toothpaste, in the morning and in the evening before going to the bed, and to refrain from using a miswak during this period.

One week before study start, intraoral examination and scaling+professional tooth cleaning were performed. Participants were informed about the study and informed consent was obtained. Oral hygiene habits were recorded by a structured interview based on a prepared questionnaire. Subjects were then instructed to perform according to their normal tooth cleaning habits for the next week.

One week later, sampling of plaque micro-organisms by paper points for identification and quantification by specific whole genomic DNA probes (DNA test) was carried out. No professional tooth cleaning was performed at this visit. Subjects were then instructed to use either miswak or toothbrush (without toothpaste) during the following 3 weeks. The assistant dentist instructed the subjects on how to use either miswak or toothbrush and recorded the order, while the examining dentist (M. A.-H.) did not know which (single-blind). Each subject was given a new toothbrush (regular, straight-handled Oral-B toothbrush) to be used without toothpaste. Each subject was also given four fresh sticks of miswak (20 cm in length and 7 mm in width). They were instructed to save the unused sticks in a refrigerator. Randomized assignment of the subjects regarding the order of miswak and toothbrush was carried out, by a random binary outcome of a dice, even or odd numbers.

Three weeks later, sampling of plaque micro-organisms by paper points for DNA test was performed. Professional tooth cleaning was performed. Subjects were then instructed to perform according to their normal tooth cleaning habits for the next week.

One week later, sampling of plaque micro-organisms by paper points for DNA test was carried out. No professional tooth cleaning was performed at this visit. Subjects were instructed to use either toothbrush or miswak (in contrast to the first period of the experiment) for the next 3 weeks.

Three weeks later, sampling of plaque micro-organisms by paper points for DNA test was carried out.

Subjects

A total of 15 male subjects aged 21–36 years participated in this study. They were regular dental patients visiting the Dental Centre at Al-Noor Specialist Hospital at Makkah City in Saudi Arabia, who volunteered to participate in the study. One of the authors (M. A.-H.) informed each participant about the study. All patients were interviewed regarding their oral hygiene habits and use of miswak. The inclusion criteria were: subjects without evidence of periodontitis, having $\geqslant$24 teeth, being healthy (not having any diagnosed disease), non-smoking. They were current users of miswak and toothbrush or had used miswak earlier, right-handed and without orthodontic appliances.

Analysis of plaque micro-organisms

The clinical examination comprised sampling of plaque micro-organisms

by paper points for DNA test. Sterile paper points (endodontic medium paper points) were used to sample plaque micro-organisms, from the distobuccal aspects of the first molars, 26 and 46, and the canines, 23 and 43. Before use, the paper points were autoclaved two to three times, making them more rigid and easy to handle. The samples were collected from each subject at each visit and stored in clean plastic Eppendorf tubes (Elkay, Costelloe, Ireland) for subsequent analysis by the checkerboard DNA–DNA technique (Socransky et al. 1994) at the Department of Oral Microbiology, University of Gothenburg.

The following 12 bacterial species were tested: *P. gingivalis*, *P. intermedia*, *Prevotella nigrescens*, *Tannerella forsythensis*, *A. actinomycetemcomitans*, *Fusobacterium nucleatum*, *T. denticola*, *Peptostreptococcus micros*, *Campylobacter rectus*, *Eikenella corrodens*, *Selenomonas noxia* and *Streptococcus intermedius*.

Checkerboard DNA–DNA hybridization

Digoxinen-labeled, whole-genomic DNA-probes were prepared by using the High-Prime labelling kit (Boehringer Mannheim, Mannheim, Germany). The detailed procedure of CKB was described by Papapanou et al. (1997). Evaluation of the number of bacteria in the samples was performed by comparing the obtained signals with the ones generated by pooled standard samples containing 10^6 and 10^5 of each of the species. The signals were coded on a scale from 0 to 5, where 0 indicated no signal; 1, signal density weaker than the one of the low standard (i.e. $<10^5$ bacteria); 2, signal density equal to the one of the low standard ($=10^5$ bacteria); 3, signal density higher than the one of the low standard but lower than that of the high standard ($>10^5$ but $<10^6$ bacteria); 4, signal density equal to the one of the high standard ($=10^6$ bacteria) and 5, signal density higher than the one of the high standard ($>10^6$ bacteria).

Analysis of in vitro effects of miswak extracts

Miswak extract

Fresh chewing sticks (miswak) were sent from Saudi Arabia and kept in a refrigerator for about 1 month before analysis at the Division of Oral Microbiology, Department of Odontology, Umeå University.

Miswak fragments were extracted in RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA) with 10% fetal bovine serum (Sigma-Aldrich) (20 mg/ml) under gentle agitation at room temperature for 1 h. The extract was passed through a filter with a pore size of 45 µm.

Bacterial culture and leukotoxin purification

A. actinomycetemcomitans (strain HK1519, JP2-like clone) was cultured on blood agar and leukotoxin was purified from cells of this bacterium in accordance with the previously described protocol (Johansson et al. 2000b). Briefly, the purification procedure included removal of outer membrane proteins by cell treatment with a 100 mM NaCl solution, extraction of leukotoxin with a 300 mM NaCl solution and purification of leukotoxin from the extract by liquid chromatography.

Preparation of monocytes

Mononuclear leukocytes (MNLs) were isolated from an enriched leukocyte fraction (buffy coat) obtained from 450 ml venous blood. The blood was taken from donors visiting the University Hospital blood bank in Umeå. Informed consent was obtained from all subjects. MNL were isolated by isopycnic centrifugation in Lymphoprep® (Nycomed AB, Lidingö, Sweden) as described (Kelk et al., 2003). The fraction containing MNL was collected and the cells were washed three times ($250 \times g$, 10 min) with phosphate-buffered saline (PBS) to remove the platelets. The cell pellet was then re-suspended in culture medium RPMI-1640 containing 10% fetal bovine serum (Sigma-Aldrich) to yield 5×10^6 cells/ml. This suspension was distributed into 35-mm Petri dishes (NUNC A/S, Roskilde, Denmark) at 8 ml/dish and incubated at 37°C in 5% CO_2 for 2 h to allow the monocytes to adhere. The non-adherent lymphocytes were removed by two rinses with 1 ml PBS. The adherent cells were detached from the dish surface by treating with 0.1% trypsin in PBS for 1 min, washed once with PBS and re-suspended in culture medium to yield 10^6 cells/ml. The suspension containing approximately 95% monocytes was distributed onto a 24-well microtiter plate (NUNC) at 1 ml/well and cultured for 20 h at 37°C in 5% CO_2 to equilibrate the cells. Prior to experimentation, the culture medium was replaced by 0.25 ml fresh medium/well.

Analysis of leukotoxic activity

The monocytes were cultured in RPMI-1640 with 10% fetal bovine serum in the presence or absence of miswak extract (80%). The leukotoxic activity in mixtures of purified leukocyte fractions and leukotoxin was quantified by analysis of lactate dehydrogenase (LDH) activity released from damaged cells (Johansson & Kalfas 1998).

Antibacterial effect of miswak extract

A suspension of *A. actinomycetemcomitans* (200 µl 10^6 bacteria/ml) was spread on a blood agar plate (Johansson et al. 2000a). Thereafter, a piece of miswak was cleaned from bark and inserted into the agar. The agar plate was incubated for 48 h at 37°C to allow bacterial growth at the surface. The area around the miswak without visible bacterial growth was examined optically and documented by a digital camera.

Data analysis

The CKB signals were coded on a scale from 0 to 5 as described previously. The mean values of levels of the 12 subgingival microbial species of the participating subjects were computed before and after the miswak and toothbrushing periods. The non-parametric Wilcoxon matched pairs test was applied to evaluate statistical differences between the two oral hygiene devices. In this analysis, $p \leqslant 0.05$ was considered as statistically significant. Statistical analysis was performed using the STATISTICA 6 version software program (StatSoft Scandinavia AB, Tulsa, OK, USA).

Paired *t*-test, two-sample equal variance was used to test the following hypothesis: ''the leukotoxic activity of *A. actinomycetemcomitans* is decreased in a sample with miswak extract''.

Results

Analysis of plaque micro-organisms

The analysis of the plaque samples for the presence of 12 subgingival microbial species using whole genomic DNA

probes and the CKB method showed that the subjects after the miswak period had significantly lower levels of *A. actinomycetemcomitans* in the subgingival plaque samples. There were no significant differences between miswak and toothbrush in levels of the remaining 11 bacterial species. The use of miswak reduced *A. actinomycetemcomitans* significantly more ($p<0.05$) than the ordinary toothbrush.

Tables 1 and 2 show the frequencies and numbers of 12 micro-organisms from the selected subgingival sites, before and after the miswak and toothbrush periods. Each number represents one positive sample. Table 1 presents the results from analysis before and after the miswak period, while Table 2 gives the corresponding data for the toothbrush period.

Effects of miswak extracts

Miswak fragments exerted an antibacterial effect against *A. actinomycetemcomitans* as presented in Fig. 1. In an incubated blood glucose agar plate containing a suspension of *A. actinomycetemcomitans*, a zone of about 10 mm around the piece of miswak stick was completely protected from bacterial growth.

The presence of miswak extract (80%) for 3 h in cultures of monocytes (MØs) did not interfere with the number of viable cells, as analyzed by quantification of neutral red uptake (data not shown).

Fig. 2 shows that the miswak extracts protected human MØ from leukotoxin-induced lysis. MØs were exposed to

Table 1. Frequencies and numbers of 12 micro-organisms from the selected subgingival sites, before and after the miswak period

Bacteria	Before miswak period ($n=15$)	After miswak period ($n=15$)	*p*-value
Campylobacter rectus	1	1 1	NS
Selenomonas noxia	1 1	1	NS
Eikenella corrodens	1 1 1 1	1	NS
Porphyromonas gingivalis	1 3	1 1 1 1	NS
Fusobacterium nucleatum	1 1 1 1 1 2	3 1 1 1 1	NS
Actinobacillus actinomycetemcomitans	1 1 1 1 2 2	1 1	<0.05
Treponema denticola	1 1 1 1 1 1	2 2 1 1 1 1 1	NS
Tannerella forsythensis	1 1 1 1 1 1 2 2	2 1 1 1 1 1 1	NS
Streptococcus intermedius	1 1 1 1 1 1 1 1 3	3 3 1 1 1 1	NS
Prevotella intermedia	1 1 1 1 2 3 3 3 3	3 2 2 2 1 1 1 1 1 1 1 1	NS
Prevotella nigrecens	1 1 1 2 2 2 2 3 3 3 3	3 3 3 3 2 2 2 2 2 1 1 1	NS
Peptostreptococcus micros	1 2 2 2 2 3 3 3 3 3 3 3 3 3	3 3 3 3 3 3 3 3 2 2 1 1 1 1	NS
no. of positive samples	15 10 5	5 10 15	

Each number represents one positive sample. The numbers 1–5 indicate the approximate number of bacteria in each sample. 1 = $<10^5$ bacteria; 2 = 10^5 bacteria; 3 = $>10^5$ bacteria; 4 = 10^6 bacteria and 5 = $>10^6$ bacteria. NS, not significant.

Table 2. Frequencies and numbers of 12 micro-organisms from the selected subgingival sites, before and after the toothbrush period

Bacteria	Before toothbrush period ($n=15$)	After toothbrush period ($n=15$)	*p*-value
Campylobacter rectus	1	1	NS
Selenomonas noxia	1	1	NS
Eikenella corrodens	1 1 1 2	2 1	NS
Porphyromonas gingivalis	1 1 1 2	2 1 1 1	NS
Fusobacterium nucleatum	1 1 1 1 2	1 1 1 1 1 1	NS
Actinobacillus actinomycetemcomitans	1 1 1 2 3	2 1 1 1	NS
Treponema denticola	1 2	1 1 1	NS
Tannerella forsythensis	1 1 1 1 1 1 1 1 1 2	3 3 3 1 1 1 1 1 1	NS
Streptococcus intermedius	1 1 1 1 1 2	3 1 1 1 1 1 1	NS
Prevotella intermedia	1 1 1 1 1 1 1 3 3	3 2 2 1 1 1 1 1	NS
Prevotella nigrecens	1 1 1 1 1 1 2 2 2 3 3 3 3 4	3 3 3 2 1 1 1 1 1 1 1 1	NS
Peptostreptococcus micros	1 1 1 1 2 2 3 3 3 3 3 3 3 3	3 3 3 3 3 3 3 3 3 3 2 1 1 1	NS
no. of positive samples	15 10 5	5 10 15	

Each number represents one positive sample. The numbers 1–5 indicate the approximate number of bacteria in each sample. 1 = $<10^5$ bacteria; 2 = 10^5 bacteria; 3 = $>10^5$ bacteria; 4 = 10^6 bacteria and 5 = $>10^6$ bacteria. NS, not significant.

Fig. 1. Digital photograph showing the inhibition of *Actinobacillus actinomycetemcomitans* growth on a blood glucose agar plate in an area around a piece of miswak without visible bacterial growth. The agar plate was incubated for 48 h at 37°C. A zone of about 10 mm around the miswak stick was completely protected from bacterial growth.

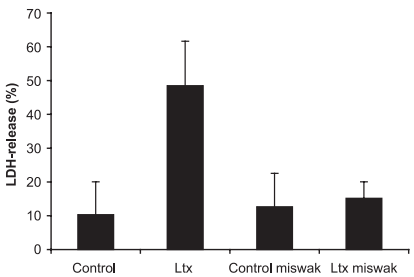

Fig. 2. Monocytes (MØs) were exposed ''for 60 min'' for leukotoxin (10 ng/ml) – in the presence of miswak (80%) in MØ or absence of miswak extract. The leukotoxic activity was quantified by analysis of lactate dehydrogenase (LDH) activity released from the damaged MØs. (*Control* indicates MØs cultured only in RPMI, *Ltx* indicates MØs cultured in the presence of RPMI supplemented with leukotoxin, *Control Ltx* indicates MØs cultured in RPMI supplemented with miswak extract, and *Ltx miswak* indicates MØs cultured in the presence of RPMI supplemented with miswak extract and leukotoxin).

leukotoxin (10 ng/ml) in the presence (miswak) or absence (RPMI) of miswak extract (80%) for 60 min. The leukotoxic activity was quantified by analysis of LDH-activity released from damaged MØ. The leukotoxic activity of *A. actinomycetemcomitans* was abolished in the sample with miswak extract ($p < 0.05$). There was no significant difference between control and miswak control.

Discussion

The present study compared the effect of the chewing stick miswak and toothbrush on subgingival microbiota in a sample of male adult Saudi Arabians who were habitual miswak and toothbrush users. To standardize the experimental conditions, all subjects were issued with identical conventional toothbrushes and chewing sticks of fairly uniform length and width, and were instructed in efficient use of both devices. Twelve selected bacterial species were identified from subgingival plaque samples and the levels were quantified by using validated whole genomic DNA probes and the CKB method.

After the miswak period, the subjects showed a statistically significant reduction of the levels of *A. actinomycetemcomitans* in the subgingival plaque samples. The remaining 11 bacterial species showed no significant changes after the use of either of the two devices.

In comparing CKB and cultural analysis of subgingival plaque samples for a small number of organisms, Papapanou et al. (1997) suggested that stringency and sensitivity were key areas of CKB technology that could lead to disparity between this and other methodologies such as cultural analysis. They concluded that checkerboard results were probably more reliable.

It has earlier been demonstrated that extracts of miswak have inhibitory effects on the growth of several oral micro-organisms, such as *Streptococcus mutans*, *Streptococcus faecalis*, *P. gingivalis*, *P. intermedia*, *T. denticola*, *F. nucleatum*, *E. corrodens* and *C. rectus* (Homer et al. 1992, Al-Lafi & Ababneh 1995, Sote & Wilson 1995, Almas 1999, Almas & Al-Bagieh 1999).

Recently, Darout et al. (2002) compared the salivary levels of 25 oral bacteria between habitual miswak and toothbrush users among adult Sudanese subjects, and their findings suggested that the miswak might have selective inhibitory effects on the level of certain bacteria in saliva, particularly several oral streptococci species. In that study, *A. actinomycetemcomitans* and other bacterial species were present in significantly higher numbers in the saliva of miswak users than in the toothbrush users. Furthermore, recent data reported that miswak users harbored significantly higher plaque levels of *S. intermedius*, *A. actinomycetemcomitans*, *Veillonella parvula*, *Actinomyces israelii* and *Capnocytophaga gingivalis* and significantly lower *Selenomonas sputigena*, *Streptococcus salivarius*, *Actinomyces naeslundii* and *S. oralis* than did toothbrush users (Darout et al. 2003). The findings from our study, however, showed a statistically significant reduction of the levels of *A. actinomycetemcomitans* in the subgingival plaque samples after the miswak period. These different findings may be attributed to the different design of the studies; the present study having a single-blind, randomized cross-over outline, investigating the effects in the same subjects.

The antimicrobial activity of chewing stick extracts against a variety of human pathogens was tested in the present study. However, there is limited information on the active compounds that contribute to the oral health benefits of *S. persica*. Darout et al. (2000) interpreted the reduction of some bacterial species by the presence of several anionic components of miswak, which had potent promoter effects of salivary peroxidase thiocyanate and hydrogen peroxide antimicrobial system.

The present results should be interpreted with some caution, however, considering the limited number of participants in the study. An increased number of individuals and a longer experimental time might have revealed more significant differences than were found among those 15 subjects. Further, all the participants were males. It has been shown, however, that gender did not have a significant effect on salivary levels of most or all of the species assessed in several studies and no significant differences were found in the subgingival microbiota between males and females from different ethnic groups (Schenkein et al. 1993, Darout et al. 2002).

It has been suggested that not all strains of *A. actinomycetemcomitans* have the potential to cause periodontal disease. The bacterium produces a potent leukotoxin that can destroy PMNs and is assumed to be an important virulence factor (Brogan et al. 1994, Macheleidt et al. 1999). The present study shows that compounds released from *S. persica* have the capacity to inhibit the growth of *A. actinomycetemcomitans* (strain HK1519, JP2 clone). Furthermore, these compounds protect human leukocytes from leukotoxin-induced cell lysis. Pre-incubation of MØ with miswak extract inhibited leukotoxin-induced cell lysis also when the extract was removed before the addition of toxin. This indicates a protective effect on the target cells rather than an effect on the bacterial toxin.

Furthermore, possible antibacterial effects of the miswak should also be investigated on other periodontal pathogens such as *Porphyromonas* and *Prevotella* species.

The effect of miswak use on the level of *A. actinomycetemcomitans* as demonstrated in the present study to our knowledge, has, not been documented earlier in the literature. These findings should be confirmed and investigated in a larger group of subjects. Identification of the mechanisms should be explored in vitro, and such studies have recently been initiated. Further, possible pharmacological benefits from the miswak extracts should be explored in combination with conventional treatment of LJP and other infections caused by *A. actinomycetemcomitans*.

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