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SALVADORA PERSICA (MISWAK)

An effective way of killing
oral pathogens

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*To my father Hamed, and to the memory
of my mother Abla,
who was the greatest inspiration of my life.*

ABSTRACT

Mechanical removal of dental plaque is regarded as an effective means of controlling the progression of dental caries and periodontal disease. The habitual use of chewing sticks (Miswak) as an oral hygiene method is still widely spread throughout parts of Asia, Africa, and the Middle East. Thus, the World Health Organization recommends and encourages the use of chewing sticks as an effective oral hygiene tool in areas where it is customary. As *Salvadora persica* (Arak) is one of the most important species still used around the world, the main aim of this thesis was to test the hypothesis that *Salvadora persica* Miswak offers a unique combination of mechanical and chemical supragingival plaque control.

This thesis was based on two parts. A *clinical part* (Papers I & IV) investigated the effect of rinsing with Miswak extract on plaque pH after acidogenic challenge, and the effect of fresh and deactivated Miswak sticks on dental plaque and gingival inflammation in patients with gingivitis. A *laboratory part* (Papers II & III) tested the antibacterial activity of Miswak pieces and Miswak essential oil obtained by steam distillation against some Gram positive/negative bacteria. Through Gas Chromatography-Mass Spectrometry and Medium Pressure Liquid Chromatography the main antimicrobial component/s of *Salvadora persica* Miswak was specified and characterised.

The main findings were both fresh Miswak and essential oil Miswak extract had a strong antibacterial activity against Gram negative bacteria including some oral pathogens such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. Benzyl isothiocyanate (BITC) was the main antimicrobial component of Miswak. Bacterial cell membrane exhibited protrusions upon treatment with Miswak oil and commercially available BITC. Rinsing with Miswak extract, compared with water rinsing, resulted in protracted elevation of plaque pH (>6.0). Both fresh and deactivated Miswak reduced dental plaque and gingival inflammation. The decrease was statistically significant with active Miswak but was not with deactivated Miswak.

The positive clinical effects of Miswak on plaque pH, plaque and gingival indices, as well as the immediate strong antibacterial activity demonstrated in the laboratory experiments suggested that Miswak could be beneficial for oral health. Further laboratory and clinical investigations of antiviral and antifungal activities together with the effect on periodontal inflammation need to be performed. The cytotoxic activities of fresh Miswak and Miswak oil need to be evaluated before the development of oral applications becomes a future reality.

Key words: Miswak, *Salvadora persica*, chewing stick, traditional medicine, gingivitis, periodontitis, dental plaque, plaque pH, salivary flow, acid production, microelectrodes, oral hygiene, subgingival microbiota, antibacterial agent, isothiocyanate, chromatography, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*.

LIST OF PUBLICATIONS

This thesis is based upon the following papers, which will be referred to in the text by their Roman numerals:

- I. Sofrata, A., Lingström, P., Baljoon. M., Gustafsson, A., 2007. The effect of Miswak extract on plaque pH. *Caries Research* 41(6): 451-454. (Clinical Paper)
- II. Sofrata, A.H., Claesson, R.L., Lingström, P.K., Gustafsson, A.K., 2008. Strong antibacterial effect of Miswak against oral microorganisms associated with periodontitis and caries. *Journal of Periodontology* 79, 1474-1479. (Laboratory Paper)
- III. Sofrata, A., Santangelo, E., Azeem, M., Borg-Karlson, A.-K., Gustafsson, A., Pütsep, K., 2010. Benzyl isothiocyanate the main antibacterial component of Miswak sticks is highly active against the Gram-negative proteobacteria. (Laboratory Paper). Submitted
- IV. Sofrata, A.H., Brito, F., Al-Otaibi, M., Gustafsson, A., 2010. Short term clinical effect of active and inactive *Salvadora persica* Miswak on dental plaque and gingivitis. (Clinical Paper). Manuscript

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LIST OF ABBREVIATIONS

AP	Approximal plaque
BHI	Brain heart infusion
BITC	Benzyl isothiocyanate
CFU	Colony forming units
DMC	Dichloromethane
DMFT	Decayed, Missing, and Filled Teeth
DMSO	Dimethyl sulfoxide
Dsb	Disulfide bond (protein family)
EtOAc	Ethyl acetate
EtOH	Ethanol
GC-MS	Gas Chromatograph-Mass Spectrometry
GI	Gingival index
H ₂ O ₂	Hydrogen peroxide
HCl	Hydrochloric acid
He	Helium, Carrier gas
hMPO	Human myeloperoxidase
hSPO	Human salivary peroxidase
Mg ₂ SO ₄	Magnesium sulphate
MPLC	Medium Pressure Liquid Chromatography
NA	Not applicable
NIST	National Institute of Standards and Technology library
PBS	Phosphate Buffer Solution
pH	Potential for hydrogen ion concentration
PI	Plaque index
PYG	Peptone yeast glucose medium
Rf	Retardation factor
<i>S. persica</i>	<i>Salvadora persica</i>
SCN ⁻	Thiocyanate
SPME	Solid phase microextraction
TLC	Thin layer chromatography

INTRODUCTION

HISTORY OF TOOTH CLEANING AIDS

Man has long been interested in his appearance and maintaining a clean, pleasant appearing mouth and a nice smile. Tooth cleaning aids and toothpicks are traceable back in history (Hyson, 2003). Chewing sticks were used by the Babylonians as early as 3500 BC. Ancient Greek and Roman literature discusses toothpicks that were chewed to help cleaning the teeth and the mouth (Wu et al., 2001). Hippocrates (355 BC) recommended a round woollen ball dipped in honey as a toothbrush. Romans also used toothpicks from the mastic tree to clean their teeth (Hyson, 2003). Ancient Arabs were accustomed to Miswak to get their teeth white and shiny (Bos, 1993).

This kind of toothbrush was not just used by the Arabs, others used something similar, for example, the Japanese called it Koyoji and the Jews used a kind of wooden stick called Qesam in Hebrew and was mentioned in the Talmud. Even in the 1920s in some rural areas of the United States, chewing stick made of dogwood was still used (Hyson, 2003). In comparison with the chewing stick, the conventional toothbrush is relatively modern. According to the 17th century Chinese encyclopaedia, the first toothbrush was made in China in 1498. Even as late as the 1750s in Europe, the toothbrush appears relatively uncommon, although the toothpick is mentioned in the literature of the day. The toothbrush was strictly a "novelty sold in Paris for the cleaning of the teeth" (Hyson, 2003). Moreover, there is no illustration of an American toothbrush dated before 1818 (McCauley, 1946).

Today, manual toothbrushes are available at affordable prices for almost everyone, although many people in Afro-Asian communities cannot afford to purchase commercial toothbrushes and dentifrices. Miswak not only offers economical cleaning of the teeth, but has been traditionally used by many cultural groups for centuries, and religion reinforced these traditions. The influence of Islam on the spread and use of chewing sticks in different parts of the world is important (Khoory, 1983). Islam reinforced the use of Miswak as an aspect of dental care. Muslims follow the example of their prophet, and according to him, the Miswak should be used five times a day before each prayer (Bos, 1993). A national health survey in Pakistan found more than half of the rural population use chewing sticks as an oral hygiene tool (Asadi and Asadi, 1997). Several studies investigating oral hygiene habits in Saudi Arabia highlight the use of Miswak is popular as an oral hygiene aid in different population categories (Almas et al., 2003a; 2003b; 2003c; 2003d). However, among Saudi Arabians, there are large differences in current oral hygiene habits, which are mainly related to age and socio-economic level, and to a lesser extent gender (Al-Otaibi et al., 2003). In Africa, the use of chewing sticks is still wide spread, and chewing sticks are widely used in Sudan, Nigeria, and Namibia: many studies have been based on chewing sticks widely used in these countries (Wolinsky and Sote, 1983; Cai et al., 2000; Darout et al., 2000a).

MISWAK: THE NATURAL TOOTHBRUSH

Miswak: Definition and use

The name Miswak, also called miswaak, Miswaki, siwak, siwaki depending on the Arabic dialect and the country, is known in English as the natural toothbrush (Elvin-Lewis, 1980; Hattab, 1997; Al-Sadhan and Almas, 1999). Throughout the world, 182 species of plants have been used as chewing sticks, with 158 known to Africa alone. In Ghana and Nigeria, *Teclea vardoordniana*, *Garcinia*, and *Acacia* species are preferred. *Azadirachta indica* (Neem tree) is the most popular species used in India, Pakistan, and Nepal. In the Americas, *Cornus florida* (Dogwood) was used for many dental purposes. The most important species is *Salvadora persica* (*S. persica*), also known as Arak, as it is the main plant used as chewing sticks from East Africa through to the Asian subcontinent, including Saudi Arabia (Elvin-Lewis, 1980; 1982; Almas and Al-Lafi, 1995).

S. persica is a small tree or a shrub with a crooked trunk; usually one foot in diameter [Figure 1], the roots are spongy and easy to crush between the teeth. Pieces of the root usually swell and become soft when soaked in water (Eid et al., 1990b). For the root to be prepared for cleaning teeth, the stick is typically cut into 15 cm lengths. The width of the stick is usually from 0.7 to 1 cm wide. The stick is washed with water to free it from sand and before use, the bark is removed from the functioning end (about 1 cm long) of the stick, beaten hard or chewed until it becomes frayed [Figure 2]. The techniques employed to mechanically remove plaque are similar to that of the toothbrush. The cleaning movement should always be directed away from the gingival margin of the teeth on both buccal and lingual surfaces. Circular movements can be used to massage the gum, but care should be taken not to damage the soft tissue of the mouth. Fresh soft Miswak is preferred: if the Miswak is dry, the end of the Miswak should be soaked in fresh water for 24 hours before use (Almas and Al-Lafi, 1995; Almas et al., 1997). After use another brush is made from the rest of the stick. It is recommended to renew the head of the Miswak and use a fresh head as often as possible. The optimal way is to use the crushed part until it loses its taste and odour. At that point, the overused end has to be cut off because the presence of odorous components is considered a good indicator for tooth brushing efficiency (Bader et al., 2002). The same head is not recommended to be used for more than 24 hours because of cytotoxic activity (Mohammad and Turner, 1983). Usually, the teeth are brushed for 5 to 10 minutes, but some users chew on the stick for several hours while involved in other activities: this habit led to the name “chewing stick” (Hattab, 1997). However, the term chewing stick is considered misleading as the stick is chewed only briefly to fray its fibres before its common use as a toothbrush (Gazi et al., 1990).



Figure 1: *Salvadora persica* (Arak) shrub growing naturally in the desert.

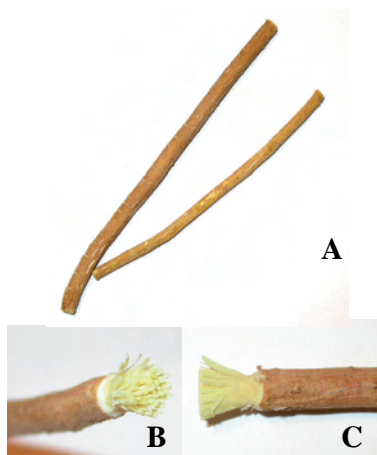


Figure 2: Miswak chewing sticks. A) Miswak before preparation for use. B) Frontal view of the Miswak bristles used for brushing teeth. C) Lateral view of the Miswak showing the prepared head for brushing teeth.

Different Miswak extracts and reported antibacterial activity

Miswak from *S. persica* chewing sticks has been tested for its antibacterial activity by different antimicrobial testing methods. The forms where Miswak was extracted are also different, including water, ethanol, methanol, ethyl acetate, and acetic acid extracts (AbdElRahman et al., 2002; Noumi et al., 2010). The usual method of crude Miswak extract preparation is by sun drying the sticks for few days. The sticks are then cut into small pieces and ground to powder with a mill or a household grinding machine. The powder is then mixed with sterile deionised water (or one of the previous mentioned solvents). The extract is allowed to soak for 48 hours. Then the mixture is centrifuged and filtered. Different dilutions of this extract are then prepared and tested against

different microorganisms (Al-Lafi and Ababneh, 1995; Almas et al., 1997; Almas, 1999; Almas, 2001).

In vitro studies testing this crude Miswak extract conclude Miswak has considerable antibacterial effect, which increases with extract concentrations (Al-Lafi and Ababneh, 1995; Almas et al., 1997). Water Miswak extract has an inhibition zone of 20 mm on *Streptococcus mutans* and 24 mm on *Staphylococcus aureus* (Al-Lafi and Ababneh, 1995). In other studies with water extract, inhibition zones on *Streptococcus faecalis* of 1 mm (5% Miswak extract), 4 mm (10% Miswak extract), and 7 mm (50% Miswak extract) are reported: the 50% extract had 3 mm inhibition zone on *S. mutans* (Almas, 1999; Almas and Al-Bagieh, 1999; Almas, 2001). Aqueous Miswak extracts of 15% and 20% concentrations have a fungistatic effect on *Candida albicans* for up to 48 hours (Al-Bagieh et al., 1994). Different alcohol extracts of *S. persica* have potent antifungal activity on different *Candidal* species. In a study on different extraction solutions, ethanol extract of *Salvadora persica* root is the most potent and *S. mutans* is the most susceptible strain (AbdElRahman et al., 2002). However, the results from these studies cannot be compared as the Miswak sources and the concentrations predations are different.

A volatile oil has been obtained form Jordanian *Salvadora persica* leaves, stem, and root. Gas Chromatography-Mass Spectrometry (GC-MS) analyses of the oil revealed different main components in different parts of the plant. The leaves have Benzyl nitrile (54%) and the stem has Cineole (46%) as the main components with reported antibacterial activity (Alali and Al-Lafi, 2003; Alali et al., 2004). The roots contain Glucotropaeolin, where Benzyl isothiocyanate and benzyl nitrile are separated by the enzymatic hydrolysis of the glucosinolate (Ezmirly and Seif-Elnasr, 1981; Bader et al., 2002). Synthetic Benzyl isothiocyanate has virucidal activity against *Herpes simplex* virus, inhibits the growth and acid production of *Streptococcus mutans*, and is fungistatic to *Candida albicans* (Al-Bagieh and Weinberg, 1988; Al-Bagieh, 1992; Al-Bagieh, 1998). However, the chemical compositions of *S. persica* roots and the exact amounts of each component are contradictory (Ezmirly and Seif-Elnasr, 1981; Abdel-Wahab et al., 1990; Bader et al., 2002).

The clinical effects of Miswak on oral health

When researchers realised large numbers of communities depend on chewing sticks as the primary oral hygiene method, the urge to identify its effect on dental health arose. Africa was the first area of focus. Elvin-Lewis et al (1980) evaluated the dental health of 887 habitual Miswak users in southern Ghana for habits and type of chewing stick preference. He summaries the findings that the presence of antibacterial and anticariogenic substances in many of the sticks suggests the low caries rates among Miswak users may not be related just to efficient plaque removal. A pilot cross-sectional study in West Africa evaluating the chewing stick compared to the toothbrush in 163 participants indicated plaque and bleeding scores were higher in chewing stick users and concluded chewing stick appears to be less efficient than the toothbrush in plaque removal, and the antimicrobial agents contained in the sticks offer no advantages over tooth brushing with toothpaste (Norton and Addy, 1989). Later on, several studies were published about oral health effects of Miswak in different parts of

the world. In Saudi Arabia, studies reported Miswak use reduces bleeding and plaque scores, and Miswak users have similar pocket depths to toothbrush users. However, Miswak users had significantly higher gingival recession than toothbrush users. The results of these studies indicate the use of Miswak influences plaque accumulation and periodontal health. The findings also reveal that the level of need for periodontal care in the study samples was lower than for toothbrush users in the same communities (Eid et al., 1990a; Eid et al., 1990b; Al-Khateeb et al., 1991; Eid et al., 1991; Guile, 1992). In a sample of Sudanese population, the periodontal status of Miswak users is better than that of toothbrush users, suggesting the efficiency of Miswak use for oral hygiene in groups familiar with its use, and that Miswak is comparable or slightly better than the toothbrush (Darout et al., 2000a).

Clinical studies in children have evaluated the efficiency of traditional Miswak as an oral hygiene tool among various populations and find Miswak as effective as the toothbrush in removing oral deposits. Miswak reduces plaque and gingival scores to the same extent as the toothbrush; however, the use of toothpaste with the Miswak does not increase Miswak effectiveness (Olsson, 1978; Danielsen et al., 1989; Helderman et al., 1992). Other clinical studies in adults determine Miswak is more effective than the toothbrush for reducing plaque and gingivitis, and appears more effective in removing plaque from embrasures, thus, enhancing interproximal health (Gazi et al., 1990; Al-Otaibi et al., 2003). Miswak has an effect on salivary and subgingival microbiota levels. Darout et al. (2002; 2003) found that Miswak users have higher levels of some oral bacterial strains including *A. actinomycetemcomitans* and lower levels of other oral bacterial strains than the toothbrush users. However, Al-Otaibi et al. (2004) found Miswak users had lower *A. actinomycetemcomitans* levels than toothbrush users. These findings suggest Miswak may have a selective inhibitory effect on the level of certain bacterial strains in saliva and plaque. Miswak has an immediate effect on the composition of mixed saliva and there is a significant increase in calcium and chloride concentrations when a piece of Miswak is chewed rather than rubber while pH and phosphate values are significantly lowered (Gazi et al., 1992). There is also an immediate antibacterial effect of Miswak on saliva with immediate significant reduction of *S. mutans* and non significant reduction in *Lactobacilli* when comparing Miswak with toothbrush (Almas and Al-Zeid, 2004).

SALIVA BUFFERING CAPACITY AND ANTIMICROBIAL COMPOSITION

Saliva is an essential component required for maintenance of the ecologic balance in the oral cavity. Saliva does not only lubricate oral tissues, making swallowing and speaking possible, but also protects oral tissues, teeth, and mucosal surfaces in many ways. These largely protective functions are best observed in xerostomic patients, when severe reduction of salivary output occurs (Edgar and O'Mullane, 1996). Saliva is important in maintaining a neutral pH level in plaque and in the oral cavity while constantly bathing the teeth and the oral mucosa. Stimulated saliva is a powerful buffer that plays a critical role in limiting pH changes after acid production by plaque bacteria. Increased salivary flow results in increased pH and buffering capacity because of the increased rate of clearance of oral sugar and plaque acid due to higher salivary film velocity, and the increase in bicarbonate, which is the most important

buffering system in the saliva at high flow rates. However, in unstimulated saliva, the level of bicarbonate ions is too low to be an effective buffer (Dawes and Kubieniec, 2004). The phosphate system has an important role at low salivary rates, where its concentrations are very high. However, phosphate concentrations become very low with salivary stimulation (Edgar and O'Mullane, 1996). Other factors such as urea content of saliva are important as many plaque organisms possess urease activity, converting urea to ammonia; thus, raising plaque pH (Edgar and O'Mullane, 1996; Dawes and Kubieniec, 2004).

The antimicrobial functions of saliva are probably one of the most important functions of saliva. Human mixed whole saliva contains two principal defensive peroxidase systems in the oral cavity, salivary peroxidase (hSPO) secreted from the acini of human parotid and submandibular glands, and myeloperoxidase (hMPO) from polymorphonuclear leukocytes coming into saliva from the gingival crevicular fluid. The major function of hSPO and hMPO is to catalyse the oxidation of thiocyanate (SCN^-) in the presence of hydrogen peroxide (H_2O_2), resulting in end products with wide antimicrobial potential (Ihalin et al., 2006; Ashby, 2008). Human salivary peroxidase systems belong to the group of innate defence factors. The antibacterial spectrum of salivary peroxidase systems covers both Gram positive and negative oral and non-oral bacteria (Tenovou, 1998). In addition to being an antibacterial, the peroxidase systems have antiviral as well as antifungal activities (Lenander-Lumikari, 1992; Mikola et al., 1995). Salivary peroxidase systems have various antibacterial mechanisms, some which are bacteriostatic inhibiting bacterial growth, and some of which are more bacteriocidal, killing the microorganism. Gram negative bacteria are more susceptible than Gram positive (Marshall and Reiter, 1980; Ihalin et al., 2001), and anaerobically-grown bacteria are more susceptible than aerobically-grown bacteria (Carlsson et al., 1983). As human saliva contains a variety of innate defence mechanisms, which act in the concerted manner and may exhibit synergistic effects (Lenander-Lumikari, 1992), the role of a single defence factor is difficult to define (Ihalin et al., 2006).

PLAQUE pH MEASUREMENTS

The oral environment is in continuous state of flux, and changes in a large number of factors can be envisioned to alter the response of plaque to fermentable carbohydrates. There are many host factors influencing plaque response to fermentable carbohydrates such as buffering capacity of the saliva, plaque, and ions released from enamel; calcium and phosphate concentration of saliva; flow rate and viscosity of saliva; presence and age of the plaque at caries prone sites in the dentition; components of the plaque matrix affecting diffusion; anatomy of the dentition; microstructure of enamel; fluoride content of enamel and plaque; pattern of mastication, sucking, rinsing, and swallowing; and the frequency of food ingestion (Schachtele and Jensen, 1982).

There are three different methods used to assess plaque pH: the sampling, the microtouch, and the telemetric. The advantages and limitations of each method are discussed in different studies.

The Sampling method

The Sampling method involves repeated removal of small samples of plaque from a number of teeth at intervals after food ingestion, dispersion of the sample, and in vitro measurement of pH. This method does not require sophisticated equipment, and is efficiently used on a large number of subjects. However, the main disadvantage is that plaque sampling must be critically timed unless bacterial metabolism is blocked after plaque removal.

The Telemetric method

This is the most sensitive method for measuring changes in plaque pH. It involves the placement of glass microelectrodes or ion-sensitive field effect transistors within the dentition. Plaque is allowed to accumulate on the microelectrodes, and the pH changes are subsequently transmitted by radio or wire. The main advantages of this method are continuous monitoring of the pH of undisturbed plaque and the ability to study older plaque (i.e. weeks to months). However, technically, it is more demanding than other methods as it requires sophisticated equipment, patient selection is limited by the need for appropriate space in the dentition, and the microbial content of plaque on sensing surface might not be normal.

The Microtouch method

This method uses glass or antimony microelectrodes, which are placed onto plaque in situ where direct readings can be obtained. The main disadvantage is that some electrodes are fragile and lack stability. However, this method had the requirements needed for our study in that it does not require any sophisticated equipment and could be efficiently used on a large number of subjects with numerous individual accessible sites that can be monitored with few seconds between them (Jensen et al., 1982; Schachtele and Jensen, 1982; Yamada et al., 1982; Harper et al., 1985; Harper et al., 1986).

MECHANICAL AND CHEMICAL PLAQUE CONTROL

Plaque formation starts almost immediately after a tooth surface has been cleaned. Saliva is seldom in direct contact with the tooth surface. A thin layer of 10-100µm thick acellular layer of absorbed salivary proteins and other macromolecules, called the acquired pellicle, separates saliva from the tooth surface. This thin layer forms the base for subsequent adhesion of microorganisms, and is then termed dental plaque (Gibbons, 1984; Fejerskov and Kidd, 2003). For descriptive purposes plaque development is divided into three phases: phase I, phase II, and phase III (Fine, 1995; Lindhe et al., 2003). Today, there is strong evidence that both dental caries and periodontal diseases are associated with oral microorganisms, in other words, dental plaque (Løe et al., 1965; Lindhe and Axelsson, 1973). The concept of anti-plaque agents can be used in one of the different phases of plaque development: to interfere with the adhesion of oral bacteria to surfaces and prevent biofilm formation; to interfere with co-aggregation mechanisms or to affect bacterial vitality, which thereby prevents future growth of colonies; or, to remove or disrupt existing biofilms (Baehni and Takeuchi, 2003).

In periodontal disease treatments, some cases progress even after mechanical removal of plaque, thus, conventional periodontal treatments are insufficient for preventing progression of the disease (Hirschfeld and Wasserman, 1978; Isidor and Karring, 1986; Fine, 1995). In such cases, additional chemical plaque control could be of importance (Löue et al., 1976; Gordon et al., 1985; Grossman et al., 1986; DePaola et al., 1989; Svaton et al., 1990). Three of the most commonly used antimicrobial agents are chlorhexidine, essential oil (EO), and triclosan (Fine, 1995). At low concentrations, chlorhexidine acts as a bacteriostatic and damages the bacterial cell membrane: at high concentrations chlorhexidine causes precipitation and coagulation of the bacterial cytoplasm leading to bacterial death (Grossman et al., 1986; Jones, 1997; Baehni and Takeuchi, 2003). EO mouthwashes are also important antiplaque agents (Ouhayoun, 2003). Essential oil acts on microorganisms by disrupting their cell wall and inhibiting enzymatic activity (Fine et al., 1985; Gordon et al., 1985; DePaola et al., 1989). The advantage of EO mouthwashes over chlorhexidine is the ability to penetrate plaque biofilm (Ouhayoun, 2003). Triclosan affects microorganisms in a similar manner to EO (Fine, 1995).

The majority of the bacteria in the biofilm on teeth may be considered commensal bacteria. Commensalism is the situation when one population or individual (the bacteria) benefit from the relationship while the other (the host) neither benefits nor is harmed. In periodontal diseases, the biofilm life-style is advantageous to some bacteria; and if they are not removed by hygiene measures, they may expand and cause disease. These bacteria have thus become opportunistic pathogens. In the oral cavity, bacteria such as *Porphyromonas gingivalis* and *Streptococcus mutans* are opportunistic pathogens. This is contrary to pathogenic bacteria that never constitute part of the commensal community of a host and always cause disease to the particular host. One such example of a pathogenic bacterium is *Salmonella enteritidis*, which may invade the human intestine. The border between commensals and opportunists (also called conditional pathogens) is sometimes vague, essentially when a group of bacteria expand in a bacterial community out of the host control, there is always a risk the bacteria may cause disease to the host (Samaranayake, 2006).

In this thesis the intestinal pathogen *Salmonella Typhimurium*, the opportunistic pathogens *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Haemophilus influenzae* as well as the commensals *Escherichia coli* (two different strains), *Enterococcus faecalis*, *Enterococcus faecium*, *Lactobacillus fermentum*, and the oral opportunistic pathogens *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Streptococcus mutans* and *Lactobacillus acidophilus* were used. Dental caries is a multifactorial disease in which, among others, mutans streptococci (*S. mutans* and *S. sobrinus*) and *lactobacilli*, separately or together, play an important role (Featherstone, 2000; Selwitz et al., 2007). Increased levels of mutans streptococci are strongly associated with caries initiation, and lactobacilli are strongly associated with deep carious lesions requiring restoration (Beighton et al., 1996; Ajdic et al., 2002; Fejerskov and Kidd, 2003). The World Workshop in Periodontics (report., 1996) reported there is sufficient evidence to incriminate *P. gingivalis*, *Tannerella forsythia* (previously *T. forsythensis*), and *A. actinomycetemcomitans* (previously *Actinobacillus actinomycetemcomitans*) as causative factors for periodontitis. Studies show that strains of *A. actinomycetemcomitans* and *P. gingivalis* are capable of

invading epithelial cells derived from human epithelial periodontal pockets or gingival sulci (Fives-Taylor et al., 1995; Dierickx et al., 2002; Andrian et al., 2006). *A. actinomycetemcomitans* also produces leukotoxin that lyses polymorphonuclear leukocytes and monocytes, which enables it to evade the innate defence line of the periodontal pocket (Johansson et al., 2000). *P. gingivalis* is characterised by production of an unusually extensive array of proteases, collectively designated the gingipains. These gingipains play major roles in *P. gingivalis* colonisation, host tissue invasion, destruction of collagen and fibrinogen, and hydrolysis of haemoglobin (Brien-Simpson et al., 2003).

This research was directed towards the field of natural antibacterial substances. Plants have innate protective mechanisms to protect against microbial attacks. Many toothbrush plants have been used in traditional medicine to treat diseases other than oral diseases. Chewing sticks or Miswak as a traditional cleaning toothbrush might offer simultaneous mechanical and chemical plaque control.

AIMS

Overall aim

The main aim of this thesis was to test the hypothesis that Miswak offers a unique combination of mechanical and chemical supragingival plaque control.

Specific aims

Clinical part

- To document the changes in plaque pH when an acidic challenge was followed by rinsing with water Miswak extract.
- To evaluate the effect of Miswak rinse on parotid gland secretion rate.
- To evaluate the effect of active and inactive Miswak on dental plaque, subgingival microbiota, and gingival inflammation in patients with gingivitis.

Laboratory part

- To test in vitro the antibacterial effect of *Salvadora persica* Miswak pieces, without extraction, on bacteria implicated in the etiology of periodontitis and caries.
- To find an extract of Miswak that could show the strong antibacterial activity achieved by Miswak pieces.
- To test these extracts for an antimicrobial effect similar to the stick on a range of Gram negative and Gram positive bacteria.
- To identify, characterise, and isolate the active compound or compounds found in Miswak.

MISWAK USED IN ALL FOUR STUDIES

The Miswak used in all four studies was roots of the *Salvadora persica* shrub. Miswak was bought fresh from the local market in Makkah city, Saudi Arabia. Identification of the plant as *Salvadora persica* (Arak) was performed by a professional Arak seller and by Meshari Al-Otaibi (one of the authors of Paper IV). The Miswak used in Papers I, II, and III was sent on a 5-day journey from Makkah to the Karolinska Institute, Sweden, where it was vacuum packed and stored at -80°C until it was used. The Miswak in Paper IV was used fresh with no storage, as the study was performed in Makkah.

CLINICAL PART (PAPERS I & IV)

The two clinical papers included participants who used Miswak in two different ways: a rinse of a Miswak water extract prepared immediately before use (Paper I), and fresh or deactivated Miswak used as a toothbrush (Paper IV). The clinical effects indicated Miswak is viable for further investigations and presents opportunity for new research fields.

MATERIALS AND METHODS

PAPER I

Miswak water extract preparation

Miswak was used as a water extract mouth rinse. Fresh Miswak was cut into small pieces and ground with a grinding machine into a wet powder. The fresh powder was mixed with distilled water, 5g of Miswak to 20 ml of distilled water, to give a 20% (w/w) concentration. The mixture was allowed to soak for one hour at 4°C and then centrifuged at 2500 rpm (1400 gravity) for 10 min. The remaining pellet was centrifuged for another 5 min to remove the remaining liquid. The supernatants were passed through filter paper (class1 No.00M) and the extract was used immediately after filtration.

Laboratory experiment testing the buffering capacity of Miswak water extract

Thirty ml of 20% (w/w) freshly prepared Miswak extract was titrated with 0.02% hydrochloric acid (HCl), pH 2.30. The pH of the Miswak extract was measured, and then every five minutes, 0.5 ml of Hydrochloric acid (HCl) was added to the Miswak extract while the mixture was continuously stirred, and then pH was measured.

Participants

Ten volunteers (4 men and 6 women) aged 30 to 45 years (mean 35.2 years) participated in the study. The mean Decayed, Missing, and Filled Teeth (DMFT) (13.6), mean stimulated whole saliva flow rate (1.66 ml/min), mean saliva buffer capacity (4.28 pH units) were registered. The buffering capacity was measured

according to Ericsson (1959). Participants had good general and good oral health, and had ≥ 20 natural teeth, and did not wear orthodontic appliances or removable partial dentures. None of the participants were on any medications. All participants gave their informed consent. This study was approved by the Regional Research Ethics Committee at Karolinska Institute, Stockholm, Sweden (Registration No. 2005/76-31).

Study design

The participants were instructed to refrain from tooth brushing, from all other oral hygiene procedures, and from chewing gum for three days before start of the study. They were instructed not to eat or drink anything but water in the last three hours before each test session. Each participant attended three sessions; one for testing the Miswak oral rinse, one for water rinse (placebo), and a third session with no activities after the sucrose rinse (control) [Figure 4]. The three test sessions were in a randomised order. Plaque pH was measured by the microtouch method (Lingström et al., 1993a) [Figure 3]: an iridium touch microelectrode* (0.1mm in diameter) was inserted into the interproximal plaque, under the contact area in the right and left maxillary premolar regions. For each test session, plaque pH was measured at baseline (0 min). The subject then rinsed for 2 min with 15 ml of 5% (w/w) aqueous sucrose rinse. Nine min after the sucrose rinse, the subjects were given either a) a rinse with 15ml of 20% Miswak extract, or b) a rinse with 15ml of water, or c) no rinse. The rinses were performed for 2 min. Plaque pH was measured at set time points from the start to the end of the experiment: 0, 2, 5, 8, 13, 15, 30, 45, 60 min [Figure 4].



Figure 3: Photograph of the microtouch instrument, to the right, an enlarged picture of iridium touch microelectrode with a diameter of 0.1mm.

* Beetrode; WPI Instruments, Sarasota, FL, USA.

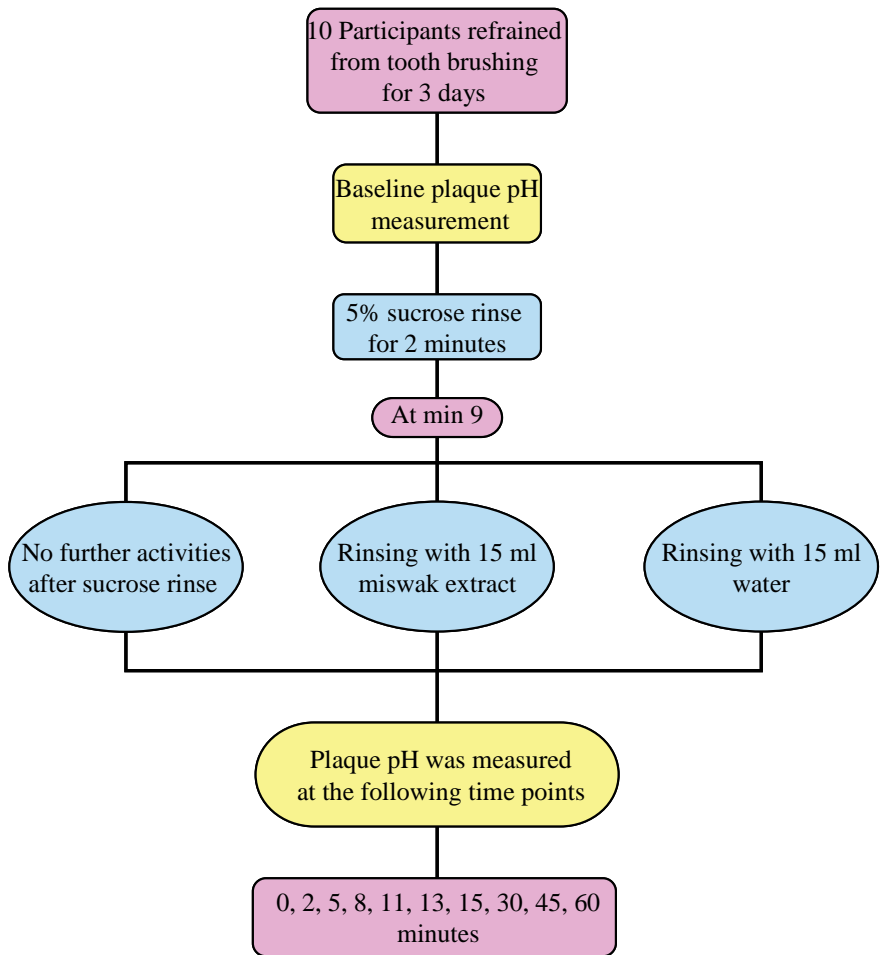


Figure 4: The general plan of the study.

In a separate session, the effect of Miswak rinse on parotid gland secretion rate was tested. Unstimulated parotid saliva was collected for 7 min with a modified Carlson-Crittenden device [Figure 5] (Shannon et al., 1962). Then parotid saliva was collected during stimulation with Miswak extract rinse for 2 min, and during the 5 min directly after the rinse (in total for 7 min).

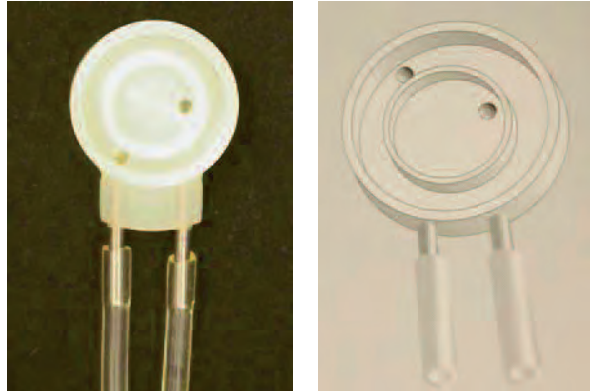


Figure 5: Modified Carlson-Crittenden device.

Statistical analyses

Two-way repeated-measures ANOVA revealed a significant method×time interaction. Thus, comparisons between methods were performed at each time point. Paired t-tests were used to compare plaque pH readings between right and left sides, and to compare stimulated and unstimulated parotid saliva flow rates. The data were analysed using the STATISTICA (7.0) program . $P < 0.05$ was considered statistically significant.

PAPER IV

Miswak

Active Miswak was used fresh form the market. Inactive Miswak was obtained by boiling the fresh Miswak sticks in water for two hours. Deactivation was confirmed by in vitro antibacterial testing on *H. influenzae* (ATCC 49247). *H. influenzae* was grown and tested with active and inactive Miswak [Figure 6]. Each subject was provided with six Miswak sticks 20 cm in length and 7mm in width and they were instructed to keep the sticks in the refrigerator until use.

* StatSof, Inc. Tulsa, Oklahoma, USA.

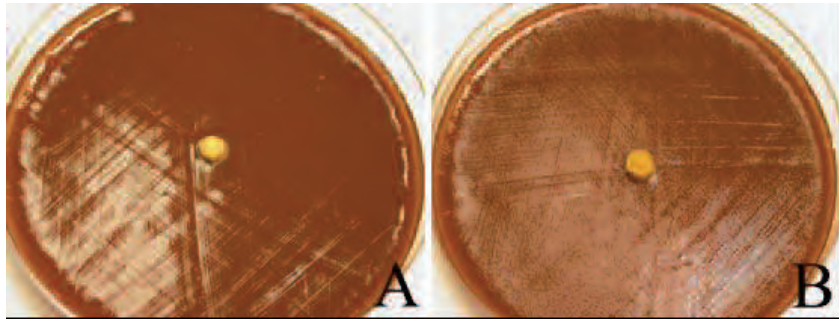


Figure 6: Bacterial growth with active and inactive Miswak. A) Total bacterial inhibition with active Miswak. B) Complete bacterial growth with inactive Miswak.

Participants

Sixty eight (21 males and 47 females) regular dental patients at the Security Forces Polyclinics, Dental Department, Makkah City, Saudi Arabia, were invited to participate in the study. To be included, the patients had to be ≥ 18 years of age and have at least 24 teeth. Exclusion criteria were systemic disease, long term medication, antibiotics during the last 6 months, and pregnancy. Participants with gingival pockets >5 mm and/or orthodontic appliances were also excluded. All participants were familiar with the use of Miswak and gave their informed consent. The participants were asked to refrain from Miswak use for two weeks prior to the study start. The study was approved by the General Medical Affairs Administration at Makkah City and the regional Research Ethics Committee in Stockholm (Registration No. 2009/1077–31/4).

Study design

This study was a double-blind, randomised clinical trial. The participants were randomised by assigning an even or odd number by the random binary outcome of the toss of a dice. Participants with odd numbers were assigned to active Miswak (test group) and participants with even numbers were assigned to the inactive Miswak (control group).

One week before the start of the study, the participants received an intraoral examination, scaling, and professional tooth cleaning. The volunteers were asked to continue their usual oral hygiene during the following week.

One week later the participants underwent baseline clinical examination and samples of subgingival microflora were collected. The participants then received either active or inactive Miswak sticks according to their assigned group. They were instructed to use the respective Miswak five times a day over the subsequent three weeks, and not to use a toothbrush, inter dental cleaners, chewing gum or any other Miswak apart from the one given, during the time of the study. After three weeks, a follow-up examination and sample collection were performed in the same manner as at baseline. Neither the participants nor the dentist knew to which group the participant belonged. The dental assistant controlled randomisation and grouping.

Clinical Examination

The clinical parameters measured were: a) modified gingival index (GI) (Løe and Silness, 1963; Løe, 1967), and b) plaque index (PI) according to Turesky modified Quigley-Hein plaque index (Quigley and Hein, 1962; Turesky et al., 1970). Both gingival inflammation and plaque were registered at four sites per tooth (buccal, mesial, distal, and lingual) for all teeth, except the third molar. Plaque was stained with erythrosine before scoring. Approximal plaque (AP) were registered and expressed separately.

Subgingival microflora

Sterile paper points were used to collect subgingival plaque samples from four sites in each subject. Supragingival plaque was removed by a sterile cotton roll. Samples were collected from the distobuccal aspects of all second molars (in case of missing second molar, the first molar was used). The four samples were pooled in one Eppendorf tube and kept at -20°C until subsequent analysis. All samples were sent to the microbiology laboratory at the University of Bern, Switzerland, for analysis by checkerboard DNA-DNA hybridisation technique (Socransky et al., 1994; Socransky et al., 2004; Katsoulis et al., 2005). The assay included a panel of 74 bacterial species (Baumgartner et al., 2009).

Statistical analyses

The sample size calculation was based on a previous cross over study comparing Miswak and toothbrush in 15 participants, in which a significant difference in PI and GI between Miswak and toothbrush was found (Al-Otaibi et al., 2003). Based on this study, the power calculation revealed a sample size of 25 in each group would have 80% power to detect a difference between groups in plaque index means of 0.19, assuming that the common standard deviation is 0.23 using a two group t-test with a 0.05 two-sided significance level. The data were analysed using the Statistica (8.0) program*: $p \leq 0.05$ was considered statistically significant. The results from the plaque registrations were normally distributed (tested with Kolmogorow–Smirnow test), and were expressed as mean and standard deviation. Differences were tested using Student's t-test. The gingival index results were not normally distributed and were presented as median and quartile range and the differences were tested using Mann Whitney U-test and Wilcoxon Signed rank test.

* StatSof, Inc. Tulsa, Oklahoma, USA.

RESULTS

PAPER I

The pH of the 20% (w/w) freshly prepared aqueous Miswak extract ranged from 4.50 to 4.60. Titration of this extract with 0.02% HCl (pH 2.3) provided no evidence of a buffering capacity of the Miswak extract. The Miswak extract did not keep the pH of the mixture at any time point, as demonstrated in Figure 7.

All subjects had the classical plaque pH drop after 5% sucrose rinse. The maximum pH drop was recorded after 8 min, with a mean minimum pH of 4.5 (95% CI 4.22 to 4.79): the pH curve generated from one of the subjects is presented in Figure 8. Rinsing with Miswak extract or water at 9 min raised the plaque pH immediately. However, Miswak rinse maintained the elevated plaque pH level until the end of the session, whereas, water rinsing did not. The difference in plaque pH between water and Miswak sessions was statistically significant at 30 min ($p < 0.001$), 45 and 60 mins ($p < 0.05$) [Figure 9 and Table 1].

The difference in plaque pH between readings at baseline and each time point reading was calculated for both Miswak and water. This change was most pronounced at 30 min. The mean (95% CI) change in the plaque pH was -0.5 (-0.8 to -0.2) and -0.9 (-1.2 to -0.5) for Miswak and water, respectively; this difference was statistically significant ($p < 0.001$) [Figure 10].

The measurements also revealed that plaque pH values of the right side were lower than those of the left side. The mean (95% CI) of the minimum readings of right and left sides were 4.75 (4.47: 5.03) and 4.88 (4.62: 5.14) respectively, and was statistically significant ($p < 0.05$) [Figure 11].

Miswak extract stimulated parotid saliva flow rate significantly ($p < 0.01$). The mean unstimulated flow rate was 0.06 ± 0.03 (SD) ml/min and the stimulated rate was 0.2 ± 0.06 ml/min. Stimulation was high during the 2 min rinse, then, it decreased progressively during the subsequent period (0-5 min after stimulation).

Table 1: Mean (95% CI) and statistical significance of sucrose, Miswak, and water sessions at different time points.

Time (min)	Plaque pH			P-values for comparisons		
	Sucrose	Miswak	Water	Sucrose vs Miswak	Miswak vs water	Sucrose vs water
13	5.47 (5.22 to 5.72)	6.03 (5.65 to 6.40)	5.63 (5.25 to 5.99)	< 0.001	< 0.01	> 0.05
15	5.80 (5.46 to 6.14)	6.31 (5.91 to 6.70)	5.91 (5.43 to 6.39)	< 0.01	> 0.05	> 0.05
30	5.74 (5.36 to 6.13)	6.11 (5.72 to 6.49)	5.57 (5.21 to 5.94)	< 0.05	< 0.001	> 0.05
45	6.12 (5.57 to 6.68)	6.22 (5.88 to 6.57)	5.92 (5.59 to 6.24)	> 0.05	< 0.05	> 0.05
60	6.34 (5.85 to 6.81)	6.44 (5.97 to 6.85)	6.08 (5.67 to 6.48)	> 0.05	< 0.05	< 0.05

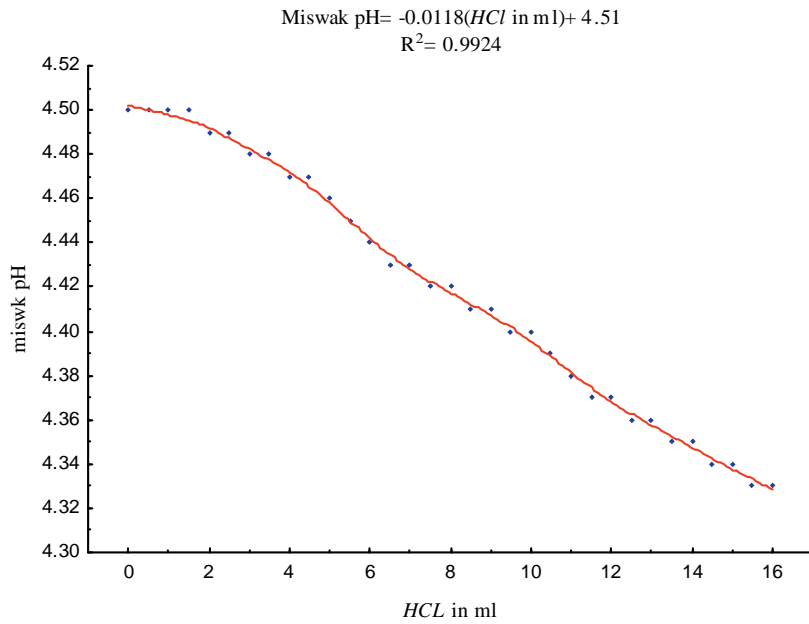


Figure 7: The buffering capacity of Miswak water extract. The linear relation between the added volume of acid and the resulting measured pH of the Miswak extract showing no buffering capacity of the extract.

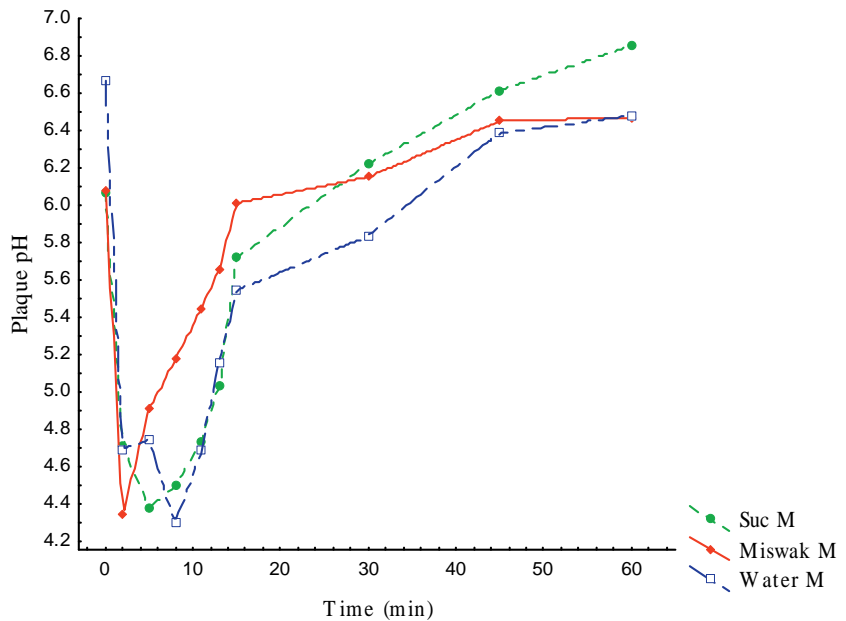


Figure 8: A classic Stephan pH curves in the three sessions for one subject.

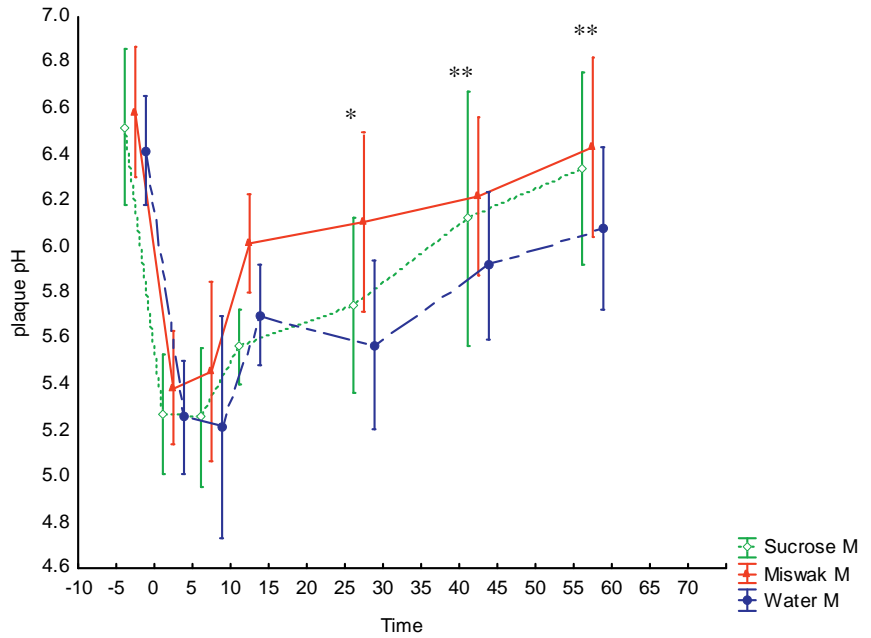


Figure 9: Means (95% CI) of plaque pH measurements during the Miswak, water, and no rinse sessions. Statistically significant differences between the three test sessions are marked in the figure (* $p < 0.001$; ** $p < 0.05$).

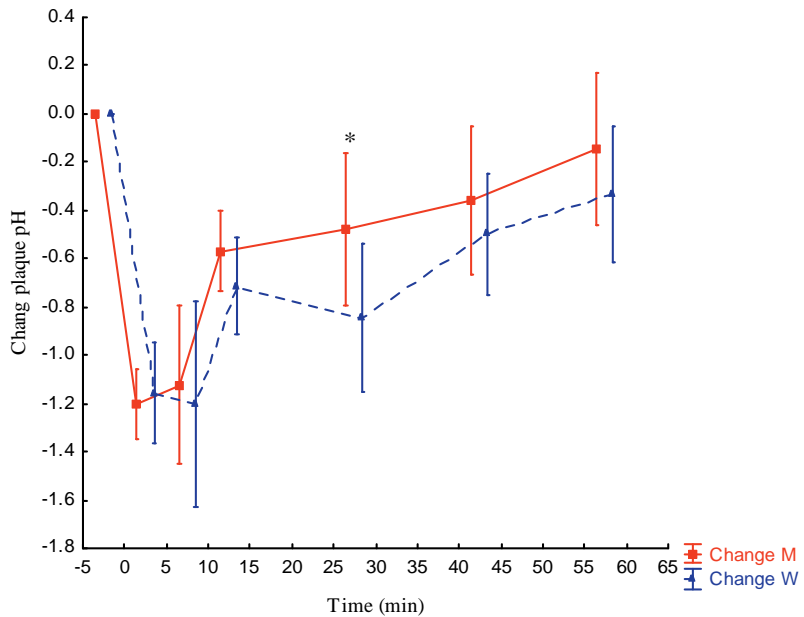


Figure 10: Change in the plaque pH for the Miswak and water. Each point on the graph represents the mean change and 95% CI for the 10 readings for water and Miswak sessions.

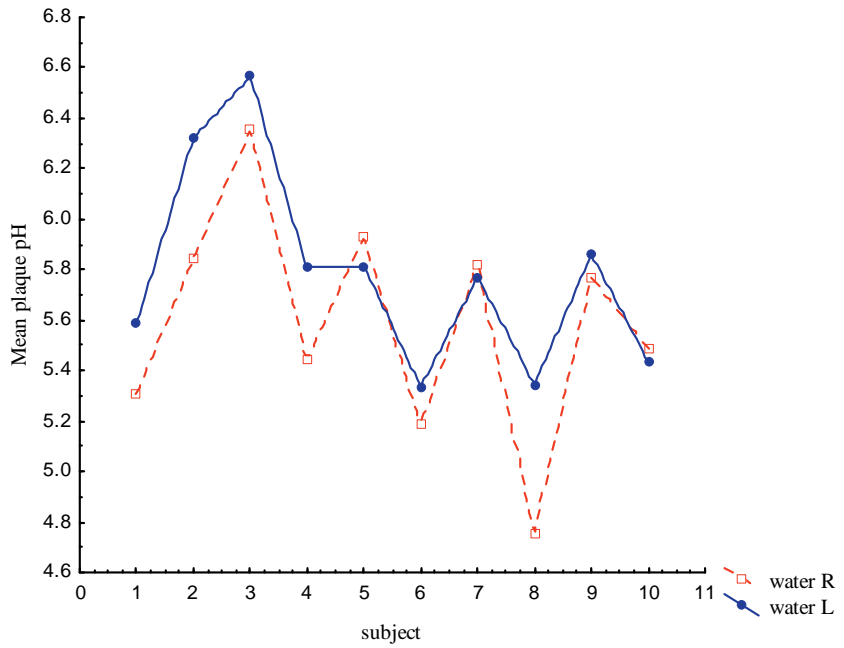


Figure 11: The mean readings between right and left sides of water sessions. Each point represents the plaque pH reading for each subject, showing right and left sides separately.

PAPER IV

Study Population

Fifty-eight of the original 68 participants completed the study. Of the 10 dropouts, two men and three women were from the test group and five women from the control group [Table 2].

Table 2: Number of subjects, gender, and mean age (range).

Group	Subjects	Gender (Male- Female)	Mean age (range)
Test	30	16M – 14F	32.8 (18-54)
Control	28	3M – 25F	27.7 (18- 49)

M = male; F = female.

Plaque Index

In the test group, supragingival plaque decreased significantly ($p=0.007$) during the three-week study, however, the decrease in the control group did not reach significance ($p=0.46$). While the PI of the test and control groups was similar at baseline ($p=0.18$), the difference at follow-up was significant ($p=0.016$). Both the test and control groups showed intergroup significant decrease in AP ($p=0.024$ and $p=0.003$ respectively). However; there was no significant difference in the change of PI and AP between the two groups [Table 3].

Table 3: Mean and standard deviation of plaque index and approximal plaque at baseline and follow-up.

Parameter	Time point	Test group	Control group	<i>p</i> -value (between the two groups)
Plaque Index (PI)	Baseline	2.27 ± 0.57	2.54 ± 0.89	0.18
	Follow-up	2.00 ± 0.53	2.44 ± 0.78	0.016
	<i>p</i> -value (between time points)	0.007	0.46	NA
Approximal plaque (AP)	Baseline	2.62 ± 0.65	2.79 ± 0.89	0.39
	Follow-up	2.30 ± 0.55	2.29 ± 0.84	0.95
	<i>p</i> -value (between time points)	0.024	0.003	NA
Change in PI	NA	-0.27 ± 0.51	-0.10 ± 0.71	0.31
Change in AP	NA	-0.32 ± 0.72	-0.51 ± 0.83	0.36

NA = not applicable.

Gingival Index

In the test group, the GI decreased significantly by the end the three-week test period ($p=0.02$), while no such reduction occurred in the control group ($p=0.15$). There was no significant intergroup decrease in approximal GI in either group ($p=0.46$ for test group and $p=1.00$ for control group). The change in GI and approximal GI did not differ significantly between the two groups [Table 4].

Microbial analysis

The microbial analyses disclosed no significant changes between baseline and follow-up samples in either group (data not shown).

Table 4: Median and quartile range of gingival index and approximal gingival index at baseline and follow-up.

Parameter	Time point	Test Group	Control group	<i>p</i> -value (between the two groups)
Gingival Index (GI)	Baseline	1.07 (0.22)	1.00 (0.13)	0.10
	Follow-up	1.04 (0.22)	1.00 (0.18)	0.11
	<i>p</i> -value (between time points)	0.02	0.15	NA
Approximal GI	Baseline	1.09 (0.16)	1.00 (0.29)	0.008
	Follow-up	1.08 (0.23)	1.00 (0.19)	0.007
	<i>p</i> -value (between time points)	0.46	1.00	NA
Change in GI	NA	- 0.03 (0.16)	- 0.02 (0.15)	0.50
Change in Approximal GI	NA	- 0.02 (0.15)	0.00 (0.36)	0.98

NA = not applicable

DISCUSSION

The first study was a small clinical trial aiming to test a conventional crude Miswak extract, a water extract used in several studies to test Miswak from different aspects. Rinsing with Miswak extract had a neutralising effect on plaque pH after a previous sucrose exposure. The data indicated rinsing with Miswak extract raised the plaque pH for a more prolonged time as compared to water rinsing.

The pH of Miswak extract prepared in this study ranged from 4.50 to 4.60: this pH value was consistent with previous studies (Almas et al., 1997; Almas, 1999). Although, Almas (2001) reports the pH of Miswak extract was 5.7, the difference in the pH of Miswak extract may be explained by differences in the methods of extract preparation. In our study, the extract was prepared from fresh Miswak, whereas in Almas' study, Miswak sticks were dried for two days before preparation of the extract. The time Miswak is kept before preparation also affects its pH: the pH of fresh Miswak extract is 4.9 and the pH of one month-old Miswak is 5.5 (Almas et al., 1997).

The mechanism behind the elevated plaque pH with Miswak rinse could be due to a buffering capacity of the Miswak extract, salivary stimulation due to Miswak taste, and/or antibacterial activity against acid-producing bacteria. The primary laboratory tests proved Miswak extract was acidic, and did not indicate any buffering ability when titrated with 0.02% HCl. This suggested the rise in plaque pH was due to other reasons [Figure 7]. Miswak stimulated salivary secretions as it has a relatively strong taste. It is well known that salivary stimulation increases salivary flow rate washing out and diluting acids (Edgar and O'Mullane, 1996). Detailed in vitro studies indicate (flow-dependant) salivary film velocity is a more important factor in pH recovery of thinner plaque, whereas, bicarbonate availability is more important for thicker plaque layers (Macpherson et al., 1991). The effect of Miswak was most pronounced after 30 min and remained up to 60 min suggesting an additional mechanism with increased salivary flow. *S. Persica* has an antibacterial effect on different types of bacteria, including *mutans streptococci* (Al-Lafi and Ababneh, 1995; Almas et al., 1997; Almas, 1999; Almas and Al-Bagieh, 1999). However, information on the biologically active compounds contributing to the reported antibacterial effects of *S. Persica* Miswak are relatively limited (Wu et al., 2001). The growth and acid production of *mutans streptococci* are inhibited by different types of substances such as Xylitol (Kakuta et al., 2003; Miyasawa et al., 2003), and it is possible that Miswak may contain substances that inhibit growth and acid production of *mutans streptococci*.

Earlier studies show that the consumption of food containing fermentable carbohydrates causes rapid decrease in plaque pH, and recovery of low pH values is rather slow. However, this recovery is detected differently, depending on the plaque pH measuring method (Harper et al., 1985; Lingström et al., 1993a; Lingström et al., 1993b; Imfeld et al., 1995). The micro-touch electrode method is effective and reliable for assessing changes in plaque pH, and for identifying and ranking acidogenic foods (Scheie et al., 1992; Lingström et al., 1993b). Plaque pH dropped after the 5% (w/w) sucrose rinse, indicated good acid production by the subjects even though they

collected plaque for only three days before the start of the study. In the water session [Figure 9], the plaque pH did not go back close to base pH value, even at the last two time points (45 min and 60 min); whereas, at the end of the sucrose session, the plaque pH reached a level close to baseline pH. It is possible that rinsing with water diluted the buffering effect of saliva, whereas in the sucrose session the buffering effect of saliva was maintained, as subjects did not have a second rinse after the sucrose rinse (Edgar and O'Mullane, 1996).

The difference between right and left plaque pH readings was statistically significant in the water session ($p < 0.05$); however, the difference was not statistically significant in the Miswak and sucrose sessions. Earlier studies show that plaque pH is lower in the upper jaw than in the lower and there are differences in plaque pH between different sites in the mouth (Kleinberg and Jenkins, 1964; Lingström et al., 1993a). However a difference between the right and left side has not been reported.

The difference found in this study could be explained by differences in the amount and quality of plaque present on each side, although visual inspection did not indicate such differences. Studies on the distribution of plaque in relation to recession and dentine hypersensitivity indicate that in the upper arch, buccal plaque is more predominant on all right-sided teeth than on left contra lateral teeth; corresponding to a predominantly right-handed toothbrushing population. This means that right-handed subjects brush their upper left side better than their upper right side (Addy et al., 1987a; Addy et al., 1987b; Rees, 2000; Addy and Hunter, 2003). Professional tooth cleaning before the start of the study could have resulted in no difference between right and left readings; although, more than three days "of no brushing" may be needed to allow for sufficient plaque accumulation for acid production to be detected. However, none of the plaque pH studies has included professional teeth cleaning before plaque pH measurement.

As the crude Miswak extract was tested in the first study (Paper I), it was necessary to test Miswak as it is used in real life. It was also important to compare Miswak with something that has a similar mechanical plaque removing technique, the idea of deactivating Miswak and using it as a control came into action. In this thesis, the effect of Miswak on plaque and gingival indexes was tested. Clinical studies comparing adult habitual Miswak users and habitual toothbrush users reveal better periodontal status in Miswak users (Gazi et al., 1990; Darout et al., 2000a). Moreover, it has been proven that the chewing stick is as or more effective than the toothbrush in reducing plaque and gingivitis (Olsson, 1978; Danielsen et al., 1989; Gazi et al., 1990; Al-Otaibi et al., 2003). The findings from the present study supported previous findings about Miswak.

In order to evaluate a possible chemical effect of the Miswak, approximal plaque (AP) was analysed separately and both fresh and deactivated Miswak reduced AP significantly, which is also emphasised in the results of another study comparing the effectiveness of Miswak and toothbrush on proximal plaque control (Al-Otaibi et al., 2003). However, in the present study, intergroup comparison disclosed greater reduction of AP in the control group than in the test group. This suggested a limited chemical effect of fresh Miswak in areas difficult to reach, as is mentioned by Gazi et al (1990). This difference might be attributable to the fact that deactivated Miswaks used as a control lacked their natural moisture and content. Thus, the participants in the

control group may have needed to be more assiduous in mechanical cleaning to attain the level of cleanliness they were accustomed to achieving with routine Miswak use. In order to isolate the chemical effects of Miswak from the mechanical effects, it would probably be necessary to use Miswak extract of some kind rather than the sticks *per se*.

Darout et al. (2003) use the checkerboard hybridisation technique to compare subgingival plaque samples of regular miswak and toothbrush users, and report higher *A. actinomycetemcomitans* in the plaque of regular miswak users. In a clinical, controlled, cross-over study, Al-Otaibi et al. (2004), use the same technique to compare subgingival plaque samples of Miswak and toothbrush groups and report significantly lower *A. actinomycetemcomitans* in the Miswak group. However, the present study disclosed no differences in subgingival microbiota for any of the bacterial species tested. One explanation might be that all the participants of the present study were regular Miswak users and this had already influenced their subgingival microbiota. Consequently, the two-week wash-out period might have been too short for eliminating the effect of previous Miswak use. A different result might have been achieved if the participants selected for the study had never used Miswak before.

The participants of the present study had gingivitis with no signs of periodontitis. Theilade et al. (1966) describe the sequential development of gingival plaque, from a simple monolayer of Gram-positive coccoid bacteria colonising the enamel surface and the marginal gingiva to a complex microbial plaque dominated by Gram-negative anaerobic cocci, filaments, and spirochetes. However, the present studies indicate that Miswak had higher antibacterial effect on Gram-negative bacteria. Moreover, Miswak essential oil extract tested on seven Gram-positive and seven Gram-negative bacterial species (Paper III), indicated that Miswak extract was more effective against Gram-negative than Gram-positive species. Thus, a corresponding study on patients with periodontitis, with predominant Gram-negative microflora, might have disclosed clinically relevant differences in the subgingival microflora.

The active and deactivated Miswak groups had more female than male participants. Testing for an influence of the differences in gender on the data did not reveal any significant effect on the study results. Moreover, the number of dropouts did not have any effect on the results as it was equal in both groups. The reasons for not continuing participation are unrelated to the study.

LABORATORY PART (PAPERS II & III)

Miswak water extract had an effect on the plaque pH, and brushing with fresh Miswak reduced dental plaque and gingival inflammation (Papers I & IV). This raised the question of how did Miswak really affect oral bacteria and whether laboratory testing of Miswak as it is used clinically, with out extraction, would have any effect on oral pathogens. As Miswak displayed a stronger antibacterial activity against the oral pathogens (Paper II) than previous reports on Miswak extracts, the need to have an extract that represented the same strong antibacterial activity as was achieved with fresh Miswak was raised. Extraction of Miswak by steam distillation and by Medium Pressure Liquid Chromatography produced an extract with the strong antibacterial activity required. This lead to the necessity of chemically characterising the components of the Miswak sticks and to defining if one or more of these components was the active component (Paper III).

MATERIALS AND METHODS

PAPER II

Miswak pieces preparation

Standardised Miswak pieces were prepared with sticks 0.5 mm wide. The sticks were cut into pieces weighing 0.14, 0.07, 0.03, or 0.015 g. The larger pieces (0.14g and 0.07g) were tested against all bacteria used in this study, while the smaller pieces (0.03g and 0.015g) were only tested against *P. gingivalis*. The outer cork layer of the Miswak [Figure 12] was removed just before weighing and testing.

Bacterial strains and cultivation

The bacterial strains selected for antimicrobial testing of the Miswak were: *S. mutans* (CCUG 27624; Ing-Britt), *L. acidophilus* (NCTC 1723), *A. actinomycetemcomitans* (HK 1519), a highly leukotoxic strain*, *P. gingivalis* (ATCC 33277), and *H. influenzae* (ATCC 49247).

S. mutans and *L. acidophilus* were grown for two days under anaerobic conditions on Colombia base agar[†] supplemented with 0.01% tryptophan[‡] and citrated horse blood (5%). *A. actinomycetemcomitans* was grown for two days on the same medium, but incubated in 95% air and 5% carbon dioxide. *P. gingivalis* was grown anaerobically for four days on Colombia base agar supplemented with hemin (0.05 mg/ml), vitamin K (0.01 mg/ml), and citrated horse blood (5%). *H. influenzae* was grown for one day in 95% air and 5% carbon dioxide on haematin-agar[§] supplemented with haemoglobin (1%) and isovitalex (1%).

* Dr M. Kiliens strain collection, Aarhus, Denmark.

† Acumedia, Baltimore, MD, USA.

‡ Merck, WWR International AB, Stockholm, Sweden.

§ GC-agar, Acumedia, Baltimore, MD, USA.

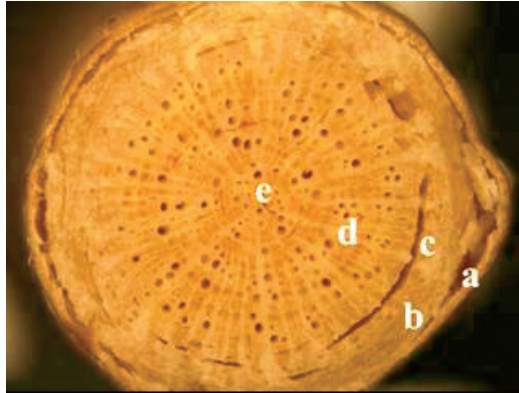


Figure 12: A cross section of the Miswak showing the different layers of the root: (a) Cork, (b) Cortex, (c) Phloem, (d) Xylem, (e) Pith.

Preparation of bacterial suspensions

The suspension of *S. mutans* and *L. acidophilus* was prepared in PBS (Phosphate Buffer Solution). *A. actinomycetemcomitans* and *H. influenzae* were suspended in HTM broth (Haemophilus Test Medium, according to CLSI document M7-A7, 2006). *P. gingivalis* was suspended in PYG (Peptone Yeast Glucose media, prepared anaerobically). The turbidity of all suspensions was standardised for each bacterial strain using a spectrophotometer*. The suspensions were adjusted to give $\approx 3 \times 10^8$ CFU/ml. Each bacterial suspension was then swabbed over the surface of the special agar plate.

Antibacterial testing

Two types of tests were conducted

In the first test, a standardised round hole, 5 mm in diameter, was punched into the middle of each inoculated agar plate, and a 0.14 g Miswak piece was placed in the hole. This test was repeated ten times for each strain.

The second test was designed to determine the presence of volatile, air-borne antibacterial compounds. A 0.14 g Miswak piece was suspended by a thread 3 mm above each inoculated agar plate. This test was repeated five times for each bacterial strain. The inoculated plates were incubated as described above and growth inhibition was evaluated. In both tests, two plate sizes, 8 and 14 cm, were used, depending on the strength of the antibacterial effect of the Miswak piece on the bacterial strain. In addition, in the second test lower weights of Miswak pieces were tested in duplicate.

Statistical analyses

The data were not normally distributed and non-parametrical statistical tests were used. The Mann-Whitney U-test was used to compare the inhibitory effects of 0.14 g Miswak

* Ultraspec® 3000, Cambridge, England.

samples embedded in inoculated agar plates and samples suspended above the plates. It was also used to compare the inhibitory effects of 0.14 g and 0.07 g Miswak samples suspended above the inoculated agar plates. The Kruskal-Wallis test was used to compare the inhibitory effects on *P. gingivalis* of four different weights of suspended Miswak. The data were analysed using the STATISTICA (7.0) program*. P<0.05 was considered statistically significant.

PAPER III

Laboratory work was in four parts: Miswak extracts preparations, Miswak extracts chemical analysis, antibacterial testing of the Miswak extracts, and transmission electron microscopy.

I. Miswak extracts preparations

- 1) Miswak oil prepared by steam distillation.
- 2) Combined extraction-fractionation of fresh Miswak using Medium Pressure Liquid Chromatography (MPLC).

II. Chemical analyses of Miswak extracts

- 1) Gas Chromatography-Mass Spectrometry (GC-MS) analysis of Miswak oil and Miswak fractions obtained from MPLC.
- 2) Analysis of volatiles from fresh Miswak using Solid phase microextraction (SPME).

III. Antibacterial testing of the Miswak extracts

- 1) Growth conditions and bacterial strains selected for antimicrobial testing of the Miswak extracts.
- 2) Testing Miswak oil.
- 3) Testing Miswak pooled fractions.

IV. Transmission electron microscopy.

I. Miswak extracts preparations

1) Miswak oil preparation by steam distillation

Many organic compounds tend to decompose at high sustained temperatures. The test for deactivation of Miswak indicated Miswak loses its antibacterial activity on heating; thus, separation by normal distillation was not an option. Therefore, steam distillation was chosen for obtaining the Miswak oil. The addition of water or steam depresses the boiling points of the compounds, allowing the compounds to evaporate at lower temperatures, preferably below the temperatures at which deterioration of the material becomes detectable. Miswak sticks (1.5 Kg) were cut into pieces of 1- 2 cm in length. The pieces were then smashed with a stone mill, place into a 5 litre round-bottom flask [Figure 13], then, 700 ml of distilled water was added and the mixture was heated. The flask was fitted with a thermometer and a condenser. The temperature was maintained at 80°C. The steam from the heated flask was condensed in the condenser with cold tap water [Figure 14]. The result was Miswak oil mixed with water. The miswak oil was separated from the water by extracting the oil/water mixture with hexane[†]. The hexane was dried with magnesium sulphate (Mg₂SO₄), and then evaporated under pressure

* StatSof, Inc. Tulsa, Oklahoma, USA.

† Merck[®], Val-De-Reuil Cedex, France.

using the Rotavapor®* [Figure 16, C]. This procedure resulted in 12 grams of pure Miswak oil.



Figure 13: A) Miswak roots cut into small pieces. B) Miswak root smashed with a stone mill. C) Miswak root pieces in round bottomed flask.

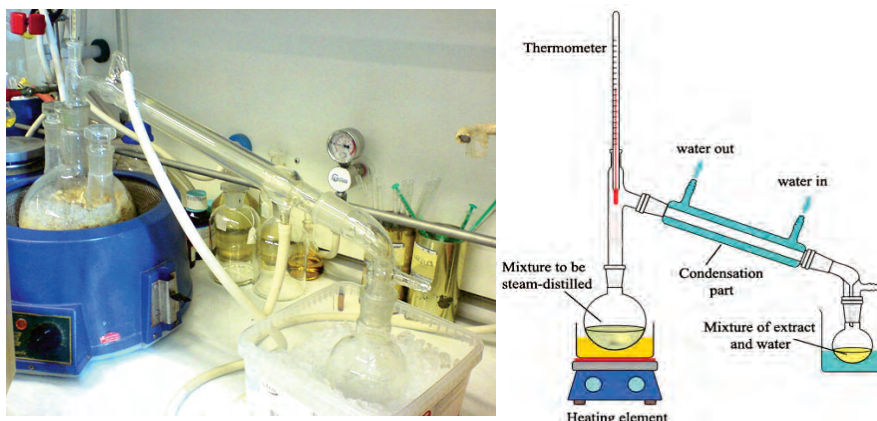


Figure 14: Steam distillation set-up.

2) Combined extraction-fractionation of fresh Miswak using Medium Pressure Liquid Chromatography (MPLC)

The preparative liquid chromatography technique used in this study was the same as described by Liblikas et al. (2005). Fresh Miswak was cut into 1-2 cm pieces and

* Rotavapor® R-210/ R215, BÜCHI Labortechnik AG, Flawil, Switzerland.

ground with silica gel* in a 1:1 mixture (50.4 g Silica + 50.4 g Miswak) by means of a household meat grinder. The material was passed through the grinder four times to produce a free flowing powder [Figure 15]. The Miswak silica powder (100.8g) was dry packed in a 30 mm internal diameter glass column followed by fresh silica gel (18.4 g). The length of the loading zone was 19 cm and that of the fresh silica was 5.6 cm. The column was sealed with pistons that could be adjusted to the resulting bed length [Figure 16, A].



Figure 15: A) Miswak pieces, B) Fresh silica, C) Ground Miswak with silica.

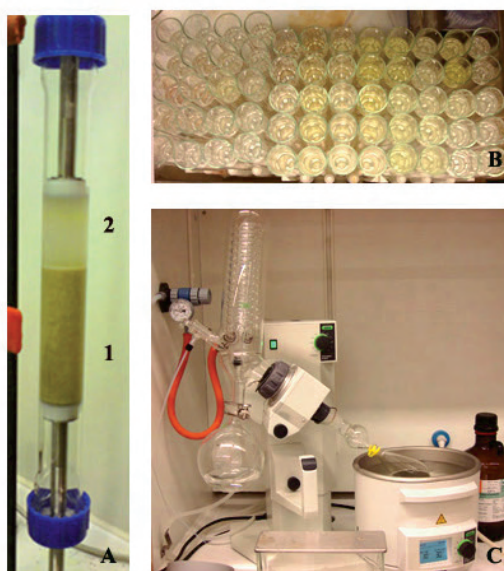


Figure 16: A) The column with Miswak and silica: 1. Silica & sample (19 cm), 2. Pure silica (5.6 cm), where separation takes place, B) fractions eluted from the column in A, C) The Rotavapor[®] used to reduce the amount of solvents.

The combined extraction and chromatography was performed with the setup depicted in Figure 17. Two consecutive gradients were used. The first gradient was attained with 100 ml of 100% hexane in the mixing chamber followed by consecutively adding 100 ml of mixtures of hexane and ethyl acetate (EtOAc) (1.25, 2.5, 5, 10, 20, 40, and 80), ending with 300ml of 100% EtOAc to the solvent reservoir. This was followed by

* Merck 60, 230-400 mesh (0.040- 0.63 mm).

consecutively adding 100 ml of mixtures of methanol (MeOH) in EtOAc (1.25, 5, 10, 20, 40, and 80), ending with 300ml of 100% MeOH. The eluent was collected in 25 ml test tubes [Figure 16, B].

All fractions were checked with thin layer chromatography (TLC) performed on silica gel plates (Merck 60, pre-coated aluminum foil) and eluted with: 10% EtOAc in hexane for fractions 1-25; 20% EtOAc in hexane for fractions 20-41; or, 30% MeOH in ethyl acetate for fractions 40-71. The plates were then sprayed with a solution containing 3 g vanillin dissolved in ethanol (EtOH) 99% with 0.5% sulphuric acid, and developed by heating the TLC plates [Figure 19]. The MPLC fractions were pooled guided by the TLC results; fractions containing compounds with similar Retardation factor (Rf) values. This Resulted in eight pooled fractions [Figure 18, Table 5]. The solvents in the eight pooled fractions were evaporated using Rotavapor[®]. The pooled fractions for the antibacterial tests were recollected by dissolving a defined amount in dimethyl sulfoxide (DMSO). For GC-MS analyses, pooled fractions were recollected in Dichloromethane (DMC), except sample seven that was recollected in 50% DMC/EtOH.

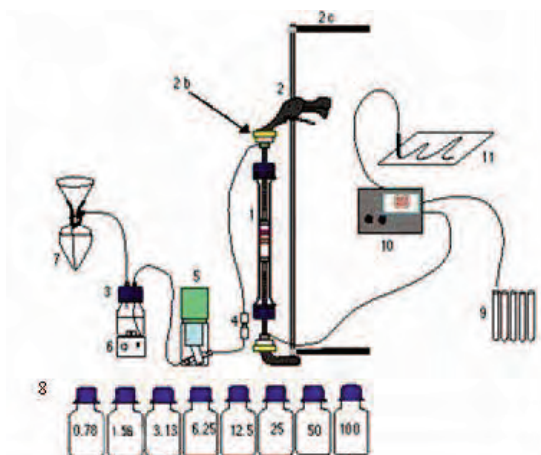


Figure 17: Describing the setup for chromatography. 1. Column; 2. Quick Grip Vise; 2 b. Column holder; 2 c. Stainless steel mounting device; 3. Airtight mixing chamber; 4. Luerlock connectors; 5. Pump; 6. Magnetic stirrer; 7. Solvent reservoir; 8. Stock solutions for gradient eluent; 9. Fraction collector; 10. UV-detector; 11. Chart recorder. (Baeckström)

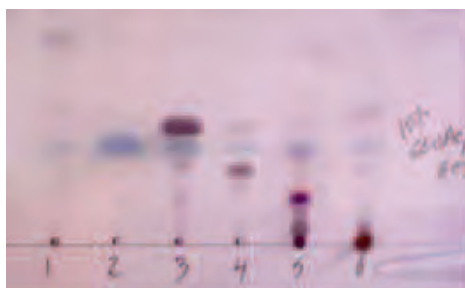


Figure 18: TLC for pooled MPLC fractions (1-6), resulting in fractions eluted in 10% ethyl acetate in hexane.

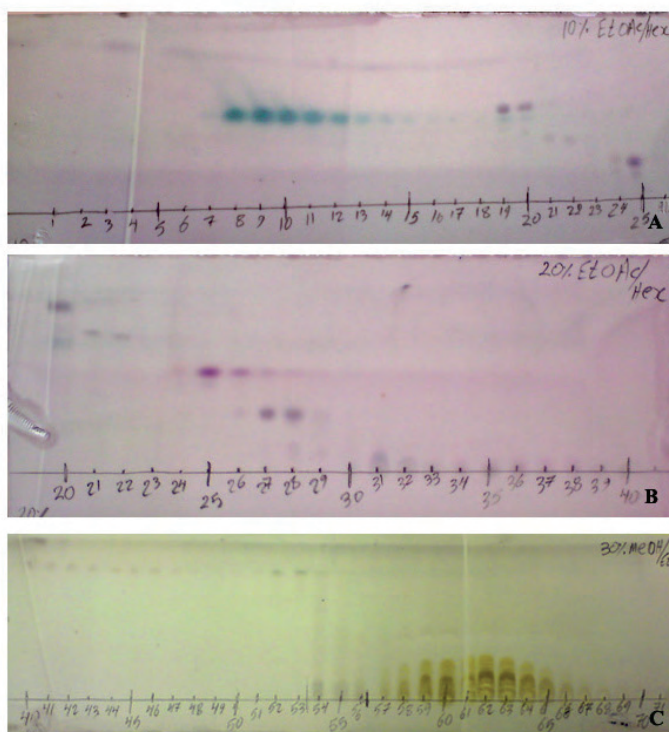


Figure 19: A) MPLC fractions 1-25 eluted with 10% ethyl acetate in hexane, B) Fractions 20-41 eluted with 20% ethyl acetate in hexane, C) Fractions 40-71 eluted with 30% methanol in ethyl acetate.

Table 5: The pooled MPLC fractions gave 8 pooled fractions, their Retardation factor and amount in grams.

Pooled Fractions	Rf	Tube number	Fraction weight	Pooled Fractions	Rf	Tube number	Fraction weight
1	1.0	1-7	0.014 g	5	0.2	24-30	0.044 g
2	0.4	8-18	0.234 g	6	0.1	31-53	0.08 g
3	0.5 & 0.4	19-20	0.029 g	7	> 0.1	54-56	0.117 g
4	0.4 & 0.3	21-23	0.039 g	8	> 0.1	57-70	2.721 g

Rf = Retardation factor

II. Qualitative and Quantitative Chemical analyses for Miswak extracts

1) GC-MS analysis of Miswak oil (from steam distillation) and Miswak pooled fractions from MPLC

Gas chromatography-mass spectrometry (GC-MS) was performed using a Varian 3400* GC connected to a Finnigan SSQ 7000 quadropole mass spectrometer†. The GC was equipped with a split/splitless injector (splitless mode 30 sec), and a DB-wax capillary column‡ (30 m, 0.25 mm id, and 25 µm film thickness). Injector temperature was isothermally set at 230°C. The carrier gas (He) was of high purity and delivered at a constant pressure of 10 bars. The temperature programme was 40°C for 1 minute, followed by an increase in temperature of 4°C/min up to 235°C: the temperature was then maintained at 235°C for 10 minutes. The transfer line temperature was kept at 240°C. The filament off time was 4 min and the MS ion source temperature was 150°C. Mass spectra were obtained at 70 eV with a mass range of 30 to 600 m/z.

All MPLC pooled fractions and oil samples for GC-MS analysis were prepared in hexane by dissolving 10 mg of each completely dried pooled fraction in 1 ml of hexane; a 100 µl ethyl acetate were added to increase solubility. The pooled fractions were further diluted to 2mg/ml by addition of hexane, of which 1 µl was injected for the analysis: pooled fractions 7 and 8 were not run on GC-MS for technical reasons. For analysis of the volatiles from the cut Miswak sticks, the SPME fiber was desorbed in the injector for 4 minutes. The constituents of the pooled fractions were identified by comparison with the Finnigan of the national institute of standards and technology library (NIST) and confirmed by analysing the reference compounds using the same chromatographic parameters.

For quantitative determination of the main constituent (benzyl isothiocyanate) in oil distillate and pooled MPLC-fractions, five different concentrations of a synthetic reference of benzyl isothiocyanate (BITC)§ were injected into the GC-MS under the

* VARIAN 3400 Chromatography system, Walnut Creek, California, USA.

† SSQ 7000 Finnigan Mat, Thermo Scientific system Inc, California, USA.

‡ J & W Scientific, Folsom, California, USA.

§ >98% GC-purity, Lancaster Synthesis Inc., Windham, NH, USA.

same conditions as for the pooled fractions and oil dilutions. A calibration curve was obtained with $Y = 4.7495E7 + 1.7387E9*x$ and $R^2 = 0.9953$ [Figure 20].

2) Analysis of volatiles from fresh Miswak using Solid phase microextraction (SPME):

A piece of Miswak stick (20 mm) from the roots of *Salvadora persica* was placed in a 5 ml beaker. The volatiles from the cut root were collected for 15 minutes on a solid-phase microextraction (SPME) fiber coated with the polymer polydimethylsiloxane/divinylbenzene*. The volatiles were analysed by GC-MS, see above.

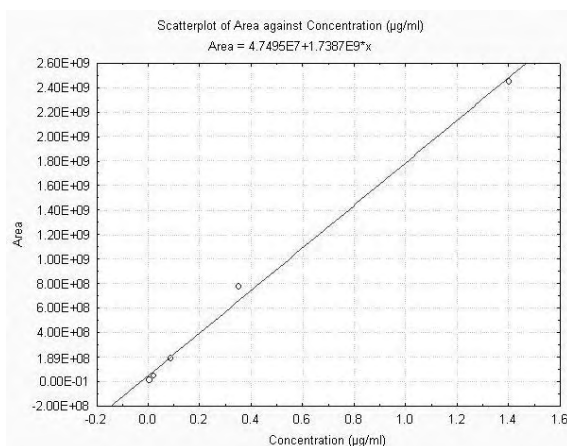


Figure 20: The calibration curve of synthetic Benzyl isothiocyanate generated from five different concentrations. The curve formula is: $y = 4.7495E7 + 1.7387E9*x$; $p = 0.0001$; $R^2 = 0.9953$.

III. Antibacterial testing of the Miswak extracts

1) Growth conditions and bacterial strains selected for antimicrobial testing of the Miswak extracts

Gram-negative bacteria: *Aggregatibacter actinomycetemcomitans* HK 1519[†], *Haemophilus influenzae* (ATCC 49247), *Escherichia coli* strain MC4100, *Escherichia coli* strain D21 (9), *Salmonella enterica* serovar *Typhimurium* (ATCC 14028), *Pseudomonas aeruginosa* strain PA01, and Gram-negative bacteroidetes *Porphyromonas gingivalis* (ATCC 33277).

Gram-positive bacteria: *Streptococcus mutans* (CCUG 27624), *Lactobacillus acidophilus* (NCTC 1723), *Streptococcus pyogenes* clinical isolate[‡], *Staphylococcus*

* Supelco, Sigma-Aldrich, Sweden.

[†] Dr M. Kiliams, Aarhus, Denmark.

[‡] Dr Martin Lindberg, Uppsala, Sweden.

aureus strain 8325-4^{*}, *Lactobacillus fermentum* (DSM 20052), *Enterococcus faecalis* (ATCC 29212), and *Enterococcus faecium*, clinical isolate[†]

Growth conditions: *A. actinomycetemcomitans* was propagated for two days on Colombia base agar[‡] supplemented with 0.01% tryptophan[§], and citrated horse blood (5%) in 5% CO₂ gas-pack generated atmosphere^{**}. *P. gingivalis* was propagated for six days on Colombia base agar supplemented with hemin (0.05 mg/ml), vitamin K (0.01 mg/ml), and citrated horse blood (5%) in anaerobic atmosphere generated with gas-packs^{††}. For the antibacterial assays, colonies of *A. actinomycetemcomitans* and *P. gingivalis* were suspended in Peptone Yeast Glucose medium (PYG) to an optical density of 0.5 at $\lambda=590$ nm. *S. mutans* was grown on BHI^{‡‡} (Brain Heart Infusion) agar plates for two days in 5% CO₂ atmosphere generated with gas-packs. For antibacterial assay, colonies were suspended in BHI broth (Oxoid) to the same optical density as above. *H. influenzae* was propagated over-night on GC-agar^{§§} supplemented with haemoglobin (1%) and isovitalex (1%) in 5% CO₂ atmosphere generated with gas-packs. For the antibacterial assay, bacteria colonies were suspended in 5% CO₂ preconditioned BHI medium with 5% Fildes Enrichment (BBL[™]) to the same density as above. *L. acidophilus* and *L. fermentum* were propagated for two days on Lactobacilli MRS agar plates^{***} in 5% CO₂. For antibacterial assay, colonies were suspended in *Lactobacilli* MRS Broth to the same optical density as above. *S. pyogenes*, *S. aureus*, *E. faecalis*, and *E. faecium* were all propagated over night on TSB agar plates^{†††} in normal atmosphere, supplemented with 0.5% yeast extract (BBL[™]). The bacteria were suspended in TSB with 0.5% yeast extract as above for the antibacterial assay. *E. coli* MC4100, *E. coli* D21, *P. aeruginosa*, and *S. typhimurium* were propagated over night in normal atmosphere on Luria Agar Base, Miller (Difco[™]). For the antibacterial assay, the bacteria were grown in Luria Broth (Difco[™]) to the same optical density as above.

2) Testing Miswak oil

Dose-response analysis of S. persica essential oil and pure synthetic BITC

Miswak oil was tested for antibacterial activity in six dilutions and one pure oil sample. Bacteria were incubated for 90 minutes in respective growth medium at 37 °C in the presence of different concentrations of essential oil. Dilutions of oil was prepared in

^{*} Dr S Arvidsson, Karolinska Institutet, Stockholm, Sweden.

[†] Inger Kühn, Karolinska Institutet, Stockholm, Sweden.

[‡] Acumedia, Baltimore, MD, USA.

[§] Merck, WWR International, Sweden.

^{**} BBL[™], CampyPak[™], Becton Dickinson, Sweden.

^{††} GasPak[™], Becton Dickinson, Sweden.

^{‡‡} Oxoid, Malmö, Sweden.

^{§§} Acumedia, Baltimore, MD, USA.

^{***} Difco[™], Becton Dickinson, Sweden.

^{†††} Tryptic Soy Broth and Bacteriological agar No 1, Bacto[™], Becton Dickinson, Sweden.

DMSO* and 5 µl of each dilution or undiluted oil was added to 500 µl bacteria suspension to obtain final dilutions of 1%, 0.1%, 0.05%, 0.02%, 0.01%, 0.005%, and 0.001% oil. These oil dilutions corresponded to 28 µmole, 2.8 µmole, 1.4 µmole, 0.56 µmole, 0.28 µmole, 0.14 µmole and 0.028 µmole pure BITC respectively. Only 5 µl of DMSO was added to the controls. After incubation, the bacteria were plated on respective agar-medium plates for determination of live bacteria by counting the number colony forming units (CFU). Each experiment was in duplicate and every experiment was repeated three times.

Pure synthetic Benzyl isothiocyanate (BITC) with a dilution of 1%, 0.1%, 0.05%, 0.02%, 0.01%, 0.005%, and 0.001% was used for the dose response test. The test was performed on *E. coli* MC 4100 and *A. actinomycetemcomitans* to compare its effect to corresponding dilutions of *S. persica* oil on the same bacterial strains.

Killing kinetics of the essential oil

To test the kinetics of the Miswak oil, one of the dilutions was used on two Gram negative bacteria. The number of viable *A. actinomycetemcomitans* and *E. coli* MC 4100 was assessed after co-incubation with essential oil at a final dilution of 0.1% and 0.02%. Aliquots were removed at 2, 5, 10, 20, 40, and 90 minutes, and plated on respective agar-medium plates for determination of colony forming units. The experiment was in duplicate and repeated three times.

Bactericidal effect of essential oil with increasing ratio of bacteria to oil

In this test the number of bacteria in the suspension tested was increased against fixed amounts and dilutions of Miswak oil. The tests were with 0.1% essential oil on *A. actinomycetemcomitans* and 0.05% essential oil on *E. coli* MC4100. Bacteria were each incubated for 90 minutes in growth medium at 37 °C with the respective oil dilution. Five different densities with 10 times increasing steps of bacteria were assessed: 10^4 , 10^5 , 10^6 , and 10^7 bacteria/ml. After incubation, the bacteria were spread on agar-medium plates for determination of live bacteria by number of colony forming units (CFU). Each experiment was in duplicate and every experiment was repeated three times.

3) Testing the eight pooled fractions from MPLC

Miswak fractionation gave 8 pooled fractions that were tested on two Gram negative bacteria *E. coli* MC4100 and *A. actinomycetemcomitans* and two gram positive bacteria *L. acidophilus* (NCTC 1723) and *S. pyogenes* clinical isolate. Five µl of each pooled fraction were added to 500 µl of bacterial suspension. Bacterial suspensions were incubated for 90 minutes in respective growth medium at 37 °C. To test synergistic effect, all 8 pooled fractions were added together and five µl were tested on each bacteria. After incubation, the bacteria were plated on the respective agar-medium plates for determination of live bacteria by counting the number of colony forming

* D2650, Sigma-Aldrich, Stockholm, Sweden.

units (CFU). Each experiment was in triplicate and every experiment was repeated two times.

IV. Transmission electron microscopy

In order to understand the nature of the fast killing exerted by the essential oil on Gram negative bacteria, electron microscopy analysis was performed. The susceptible bacterium *A. actinomycetemcomitans* was chosen to test the essential oil and to compare that with BITC and ampicillin antibiotic. For control of essential oil and BITC one sample was tested by adding 1% DMSO, and for the control of ampicillin 1% water was tested. *A. actinomycetemcomitans* was incubated for 2, 20, or 40 minutes in 37 °C with 0.1 % of essential oil (corresponding to 2.8 μmole BITC). To determine if there was a similar effect of BITC, the same test was run on 2.8μmole of synthetic BITC. For comparison 1mg/ml of ampicillin antibiotic* was tested. At each time point an aliquot of bacteria suspension was removed and centrifuged for 10 min. The supernatant was discarded and bacteria were suspended over night in fixation buffer containing 2% glutaraldehyde in 0.1M sodiumcacodylate buffer with 0.1M sucrose and 3mM CaCl₂, pH 7.4 at 4°C. After fixation the bacteria were centrifuged to a pellet. The pellet was rinsed in 0.1 M phosphate buffer (pH 7.4) followed by postfixation in 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4), at 4°C for 2 h, dehydrated in ethanol followed by acetone and embedded in LX-112 †. Sections were contrasted with uranyl acetate followed by lead citrate and then examined in a Leo 906 transmission electron microscope‡ at 80 kV(Laane et al., 2009). Digital images were taken using a Morada digital camera§.

* Sigma-Aldrich.

† Ladd, Burlington, Vermont, USA.

‡ Zeiss, Oberkochen, Germany.

§ Soft Imaging System, GmbH, Münster, Germany.

RESULTS

PAPER II

The Miswak had powerful inhibitory effects on the growth of *A. actinomycetemcomitans*, *P. gingivalis*, and *H. influenzae*, less effect on *S. mutans*, and much less effect on *L. acidophilus*. The 0.14 g suspended Miswak exhibited significantly greater inhibition on *A. actinomycetemcomitans* and *H. influenzae* than the Miswak embedded in agar ($P < 0.01$, and $P < 0.001$ respectively). The 0.14 g suspended Miswak was less effective than the corresponding embedded Miswak on *S. mutans* ($P < 0.001$), and had no inhibitory effect on *L. acidophilus* [Table 6].

Table 6: Growth inhibition (cm) of different bacteria with various Miswak weights.

Bacterial strain	0.14-g Miswak in Agar (median [range])	P-value Embedded & Suspended Miswak	Suspended Miswak		
			0.14g (median [range])	P-value	0.07g (median [range])
<i>A. actinomycetemcomitans</i>	10.9 (10.0-14.0)	0.008	13.0 (12.0-14.0)	0.021	8.0 (7.8-8.2)
<i>P. gingivalis</i>	14.0 (11.0-14.0)	0.370	14.0 (14.0-14.0)	0.008	5.0 (5.0-5.0)
<i>S. mutans</i>	3.2 (2.6-4.6)	0.001*	0.46 (0.28)	0.021*	No inhibition
<i>L. acidophilus</i>	1.4 (1.2-1.6)	0.007	No inhibition	NA	Not tested
<i>H. influenzae</i>	9.3 (5.1-11.5)	0.001	12.0 (11.5-13.0)	0.008	8.8 (7.2-9.0)

NA = not applicable. * Inhibition was less when Miswak was suspended.

The 0.07g suspended Miswak pieces exhibited significantly less inhibition on *A. actinomycetemcomitans* and *H. influenzae* than the 0.14 g suspended piece ($P < 0.05$), and no observable effect on *S. mutans*. The strain most sensitive to the Miswak piece was *P. gingivalis* and least sensitive was *L. acidophilus*. The overall differences among inhibition zones around *P. gingivalis* associated with Miswak pieces of different weights were significant ($P = 0.011$).

Figure 21 shows the dose response of different suspended Miswak weights (0.14g, 0.07g, 0.03g, and 0.015g) on *P. gingivalis*. Examples of inhibition zones associated with different methods of testing the Miswak are presented in Figure 22, which also shows the testing of different Miswak weights and full growth of bacteria in the absence of Miswak.

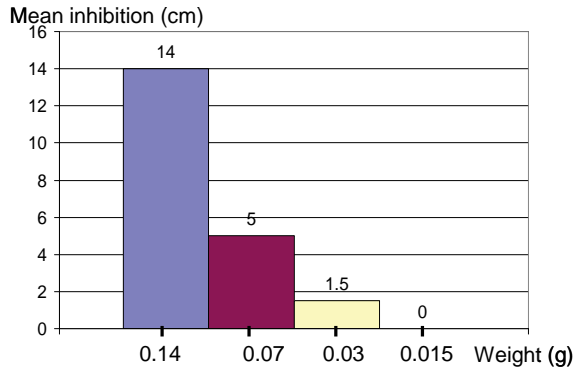


Figure 21: Correlation of *P. gingivalis* growth inhibition to Miswak of various weights suspended with a thread 3mm above the agar plate.

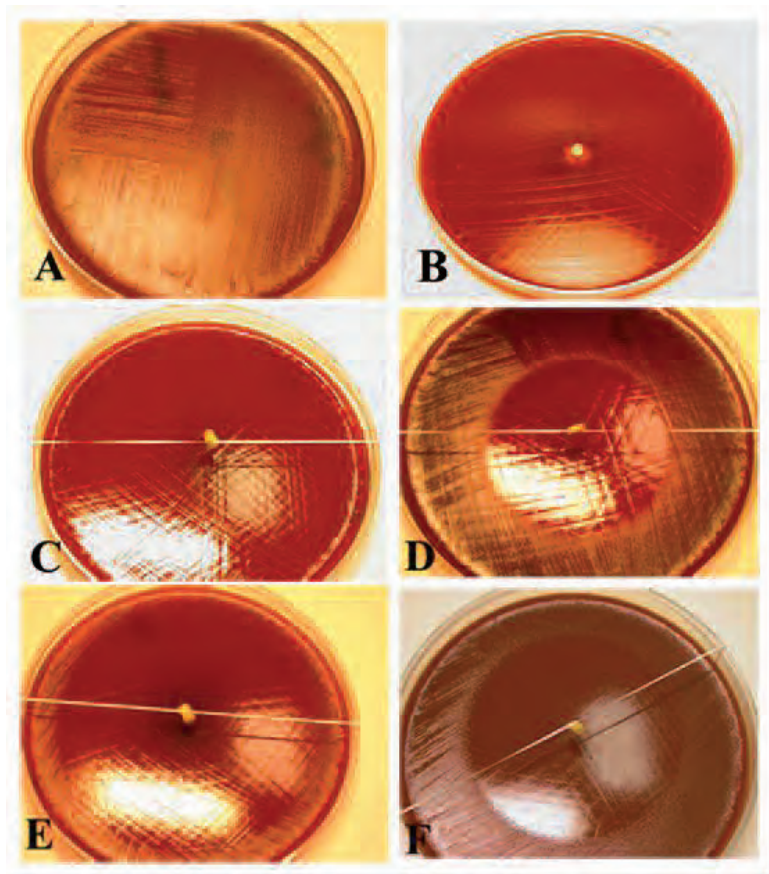


Figure 22: Miswak-induced growth inhibition, all with 14 cm plates. (A) *P. gingivalis* growth in the absence of Miswak. (B) *P. gingivalis* inhibition with 0.14g Miswak piece embedded in agar. (C) *P. gingivalis* inhibition with 0.14g suspended Miswak. (D) *P. gingivalis* inhibition with 0.07g suspended Miswak. (E) *A. actinomycetemcomitans* inhibition with 0.14g suspended Miswak. (F) *H. influenzae* with 0.07g suspended Miswak.

PAPER III

The steam distillation produced 12 g of volatile essential oil, and that MPLC gave 8 pooled fractions. The next step was analysis of both oil and samples using GC-MS to characterise and quantify the components of these two extracts.

Chemical analyses of Miswak extracts

GC-MS and SPME analyses of Miswak extracts

The GC-MS analysis for identification of Miswak oil optioned by steam distillation gave: 69.6% Benzyl isothiocyanate (BITC) and 29.5% Benzyl nitrile (BN, benzyl cyanide), with retention times of 35.6 and 31.2 minutes respectively [Figure 24].

The antibacterial testing of Miswak eight pooled fractions obtained by MPLC revealed that the pooled fractions two, three, and five were the most effective against bacteria. Identification analysis of these fractions with GC-MS revealed that fraction two was 100% BITC with a retention time of 35.6 min, fraction three was 99.2% BITC with retention time of 35.6 min; and fraction five was 66.1% BITC, and 29.4% Benzyl nitrile (Benzyl cyanide), with retention times of 35.6 and 31.2 minutes respectively.

The GC of all three active pooled fractions is presented in Figure 25. The MS for the compound presented in this GC: compound A (Benzyl isothiocyanate) and compound B (Benzyl nitrile) are presented in Figure 24 (II) and (III) respectively.

The SPME analysis of 15 minutes collection of volatiles from Miswak stick gave 98% benzyl isothiocyanate with retention time of 28.9 min [Figure 23]. The MS of BITC is shown in Figure 24 (II).

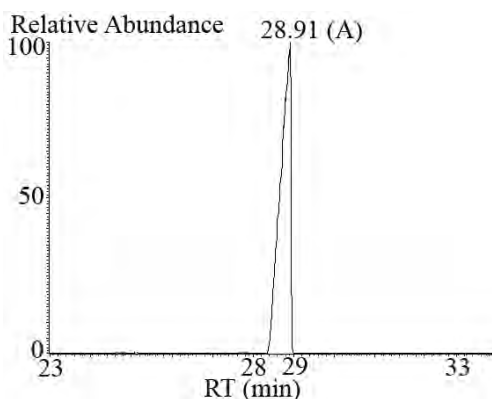


Figure 23: GC-MS chromatogram of Miswak volatiles showing BITC compound (A) as the main component.

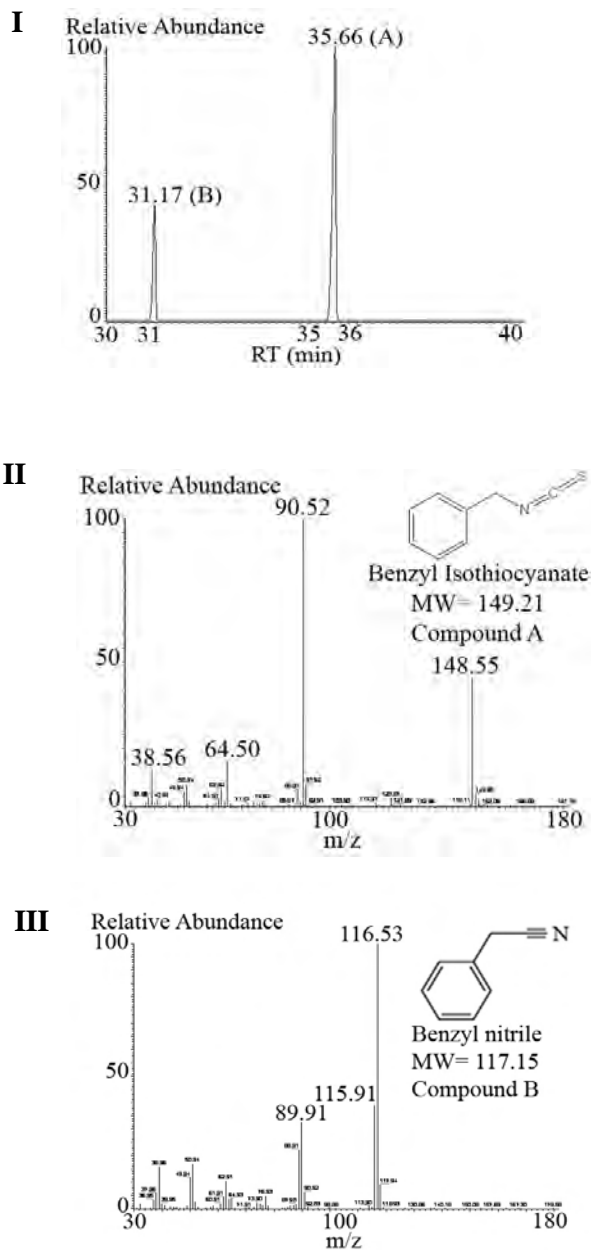


Figure 24: Gas chromatography-mass spectrometry (GC-MS) analysis of *S. persica* essential oil. *I*) Shows GC-MS of *S. persica* essential oil with two main compounds, *II*) MS and chemical structure of compound (A) Benzyl isothiocyanate. *III*) MS and chemical structure of compound (B) Benzyl nitrile.

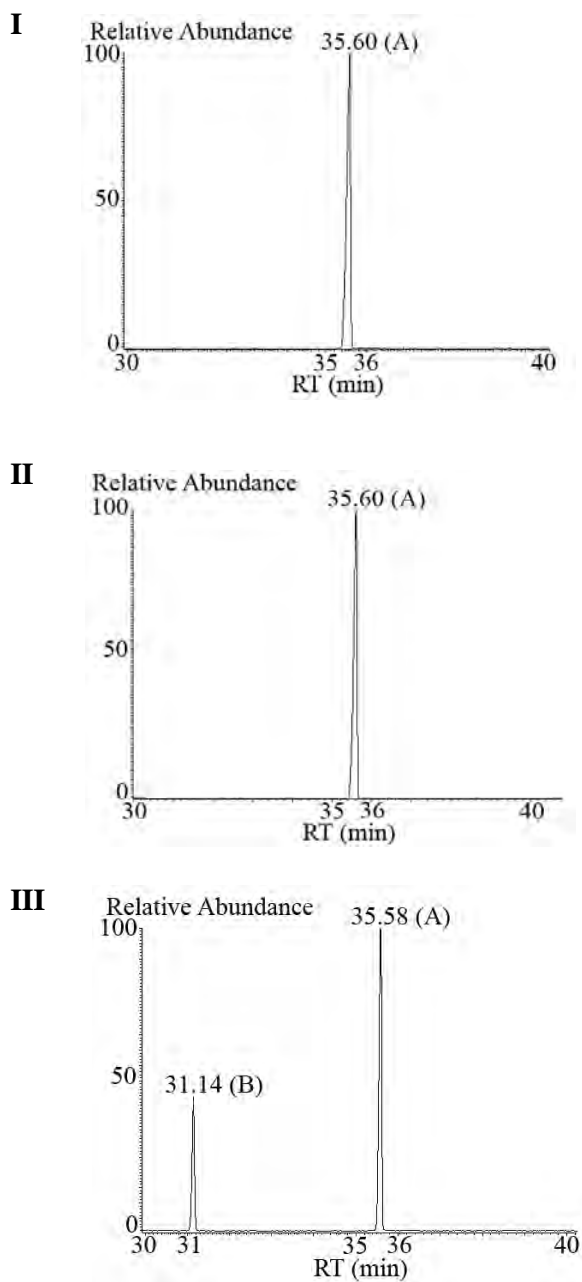


Figure 25: Gas chromatography (GC) of active antibacterial pooled fractions from MPLC. *I)* GC-MS for pooled fraction no. two with only one compound (BITC). *II)* GC-MS for pooled fraction no. three with only one compound (BITC). *III)* GC-MS for pooled fraction no. five with two compounds (BITC and BN).

The major constituent of Miswak essential oil, the active fractions, and the volatiles of fresh Miswak was Benzyl isothiocyanate. Using the formula from the calibration curve of the synthetic reference of BITC, the quantity (the % age) of BITC present in Miswak essential oil and each pooled fraction was calculated [Table 7]. The amount of BITC added to the bacteria cultures, considering that BITC constitutes 73.8% of the oil [Table 3], was 28µmole (1% dilution), 2.8 µmole (0.1%), 1.4µmole (0.05%), 0.56 µmole (0.02%), 0.28 µmole (0.01%), 0.14µmole (0.005%), and 0.028 µmole (0.001%) for the different oil dilutions used. Synthetic BITC, used at the same concentration as it occurs in the oil, was almost equally efficient against the oil-susceptible bacteria *A. actinomycetemcomitans* and *E. coli* [Figure 26].

Table 7: Percentage of Benzyl isothiocyanate in essential oil and 1-6 pooled fractions from MPLC

Pooled fraction	Benzyl isothiocyanate	Pooled fraction	Benzyl isothiocyanate
1	6.6%	5	22.9%
2	89.4%	6	6.1%
3	72.2%	Essential Oil	73.8%
4	5.2%		

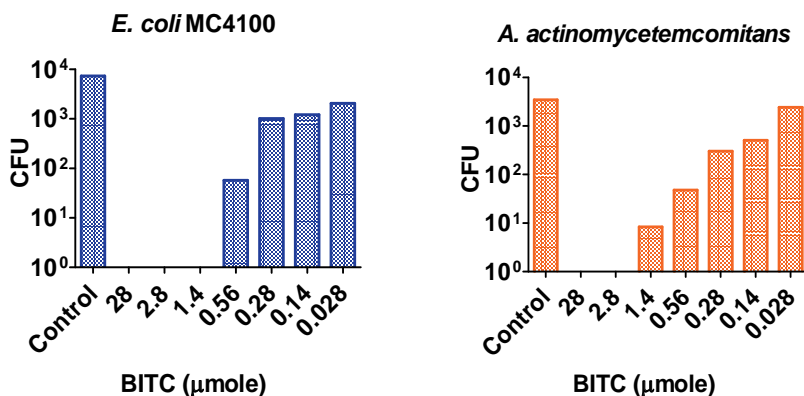


Figure 26: Dose-response analysis of antibacterial activity of synthetic BITC. The different amount of BITC applied corresponds to the BITC content in the different oil dilutions of Figure 27.

Antibacterial testing of Miswak extracts

Dose response to S. persica Miswak essential oil and pure BITC

Miswak oil with different dilutions (1%, 0.1%, 0.05%, 0.02%, 0.01%, 0.005%, 0.001% oil) had a strong antibacterial activity. The activity varied with different dilutions and different bacterial strains.

In general, Gram negative bacteria were more sensitive to all oil dilutions than Gram positive. The most sensitive Gram negative bacteria were *P. gingivalis*, and the most resistant were *P. aeruginosa*. Miswak oil with 0.05% was very effective against most Gram negative bacteria tested in this study. *P. gingivalis* and *S. typhimurium* had no growth up to 0.02% oil dilution [Figure 27, A].

All Gram positive bacteria tested in this study were to some extent resistant to the oil. It can be seen from the pattern of growth of all Gram positive bacteria that they were affected, but not to the extent of the dramatic growth inhibition seen for Gram negative bacteria. Only *S. aureus* displayed no bacterial growth at 1% oil dilution; however, with the rest of dilutions tested, *S. aureus* had close to normal growth [Figure 27, B]. Pure BITC had the same antibacterial power as the *S. persica* essential oil extract. It can be observed that the killing pattern of BITC on *E. coli* MC 4100 and *A. actinomycetemcomitans* was comparable to that of the essential oil [Figure 26].

Killing kinetics of the essential oil

In this section bacterial growths with two different dilutions tested at different time points are presented.

A. actinomycetemcomitans: 0.02% Miswak oil reduced the viable bacterial number from 1×10^4 to 1×10 by the end of the session, whereas, 0.1% Miswak oil reduced the viable bacterial count from 1×10^4 to almost zero viable bacteria in 10 minutes, ending with a completely sterile culture.

E-coli MC4100: 0.02% Miswak oil reduced the viable bacterial count from 1×10^4 to almost zero in two minutes but ended with 1×10 , whereas, 0.1% Miswak oil reduced the bacterial growth from 1×10^4 to completely sterile culture in 5 minutes [Figure 28].

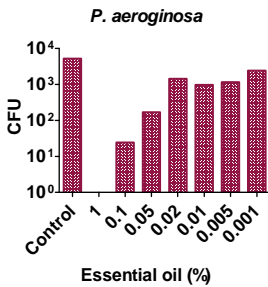
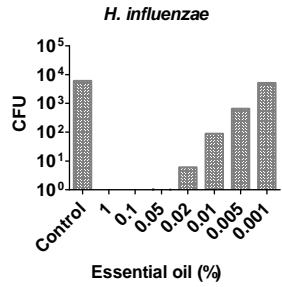
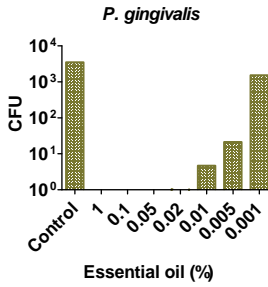
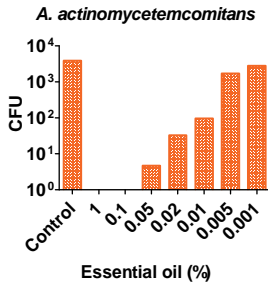
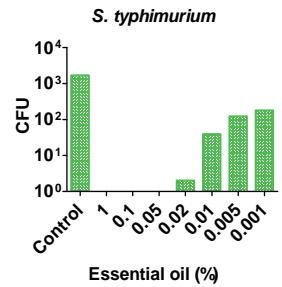
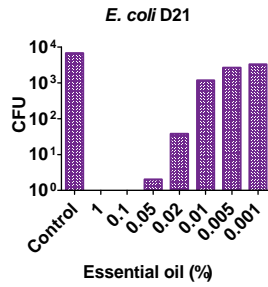
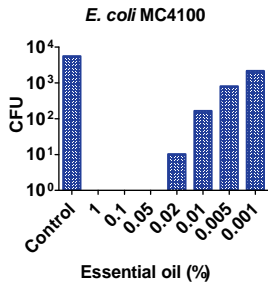


Figure 27, A.

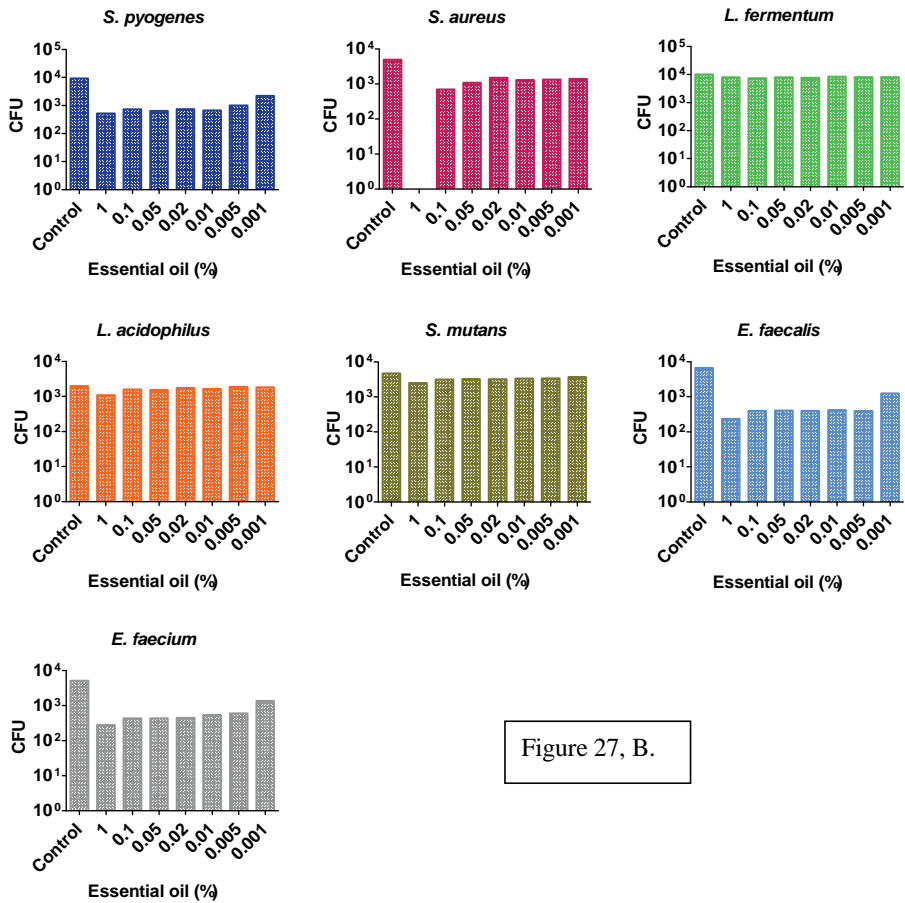


Figure 27, B.

Figure 27: Dose-response analysis of antibacterial activity of Miswak essential oil.

The numbers of surviving bacteria were determined by counting colony forming units (CFU) after 90 min co-incubation with essential oil. The essential oil concentration is the percentage of final assay volume. Control is with DMSO only. The different concentrations correspond to 28 μmole, 2.8 μmole, 1.4 μmole, 0.56 μmole, 0.28 μmole, 0.14 μmole and 0.028 μmole pure BITC. A) Gram-negative bacteria, B) Gram-positive bacteria.

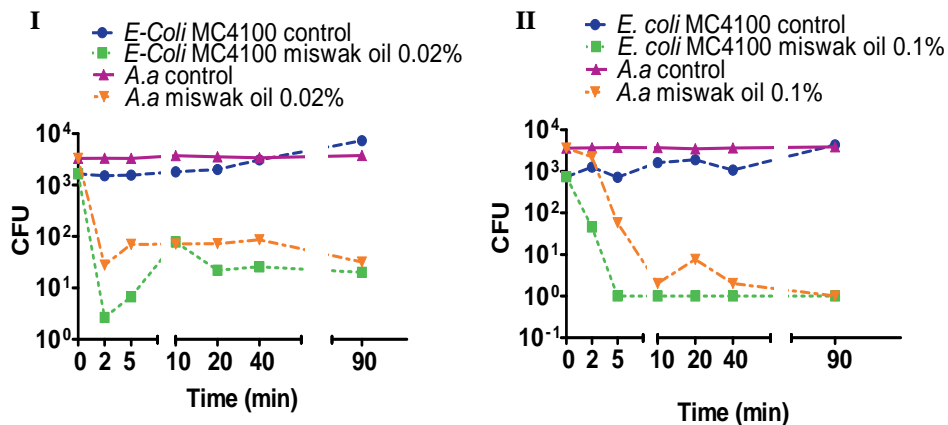


Figure 28: Assessment of surviving CFU of Gram-negative bacteria *E. coli* MC 4100 and *A. actinomycetemcomitans* HK 1519 in response to two *S. persica* oil dilutions at the times indicated. *I*) Kinetics of bacterial killing with 0.02% oil dilution (= 1.4 μ mole pure BITC). *II*) Kinetics of bacterial killing with 0.1 % oil dilution (= 2.8 μ mole pure BITC). Control was with DMSO only.

Bactericidal effect of essential oil with increasing ratio bacteria to oil

The results showed that the number of the surviving CFU decreased dramatically compared to the control with no oil. It also showed that the CFU did not change proportionally with every ten times increase in bacteria numbers [Table 8].

Table 8: Number of colony forming units (CFU) with increasing number of bacteria and constant essential oil concentration with 0.1% oil for *A. actinomycetemcomitans* and 0.05% oil for *E. coli* strain MC4100.

Bacteria	Time point (min)	CFU			
<i>A. actinomycetemcomitans</i>	0	9×10^4	9×10^5	9×10^6	9×10^7
	90	6	4	136	7264
<i>E. coli</i> MC 4100	0	6×10^5	6×10^6	6×10^7	6×10^8
	90	1	4	30	590

The pooled fractionation from MPLC

The test of pooled fractions on two Gram negative and two Gram positive bacteria indicated that the pooled fractions were strong bactericidal against *E-coli* MC4100 and *A. actinomycetemcomitans*, but were not that strong against *S. pyogenes*, and *L. acidophilus*. Fractions 2, 3, and 5 were the most effective against *E-coli*. Fractions 2 and 3 were the most effective against *A. actinomycetemcomitans*.

Fractions from 1 to 6 killed 85% of *S. pyogenes*, while *L. acidophilus* was not much affected by the any of the oil fractions. Fractions 7 and 8 had no major impact, compared to the control. In fact, *S. pyogenes* growth was enhanced in comparison to the control. All 8 pooled fractions were remixed together to test for synergistic effect. The mixed fractions indicated no increased antibacterial capacity on any of the tested bacteria [Figure 29].

The antibacterial repertoire of the three pooled fractions 2, 3, and 5 was similar to *S. persica* essential oil with respect to killing activity against Gram-positive and Gram-negative bacteria. This emphasised the fact that BITC is the major antibacterial component of the *S. persica* root [Figure 30].

Transmission electron microscopy

The electron micrographs of bacteria treated with oil exhibited dramatic effects on the cell membrane. In the presence of the *S. persica* essential oil, the bacterial membrane exhibited protrusions already at 2 minutes incubation time. These protrusions increased in size and prevalence with increasing incubation time. The commercially available pure BITC induced similar protrusions on the bacterial membrane. The reference antibiotic ampicillin, which targets the cell-wall synthesis, did not exert the same cell membrane protrusion effect on the bacteria [Figure 31].

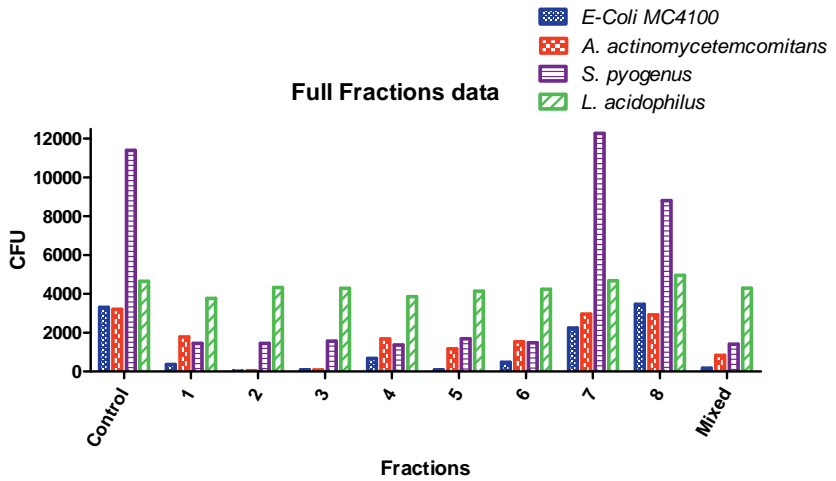


Figure 29: Antibacterial activity pooled MPLC fractions of *S. persica* root: Two Gram-negative (*E. coli* MC 4100 and *A. actinomycetemcomitans*) and two Gram-positive bacteria (*S. pyogenes* and *L. acidophilus*) were used for bactericidal activity detection. Controls were with DMSO only. Pooled fractions 1-8, combined.

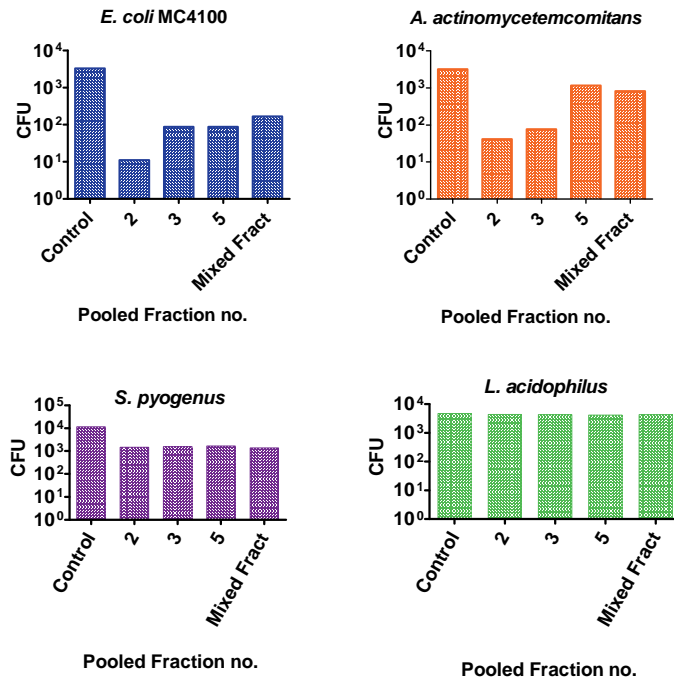


Figure 30: Active pooled fractions of *S. persica* root. Controls were with DMSO only. Pooled fractions 1-8, combined.

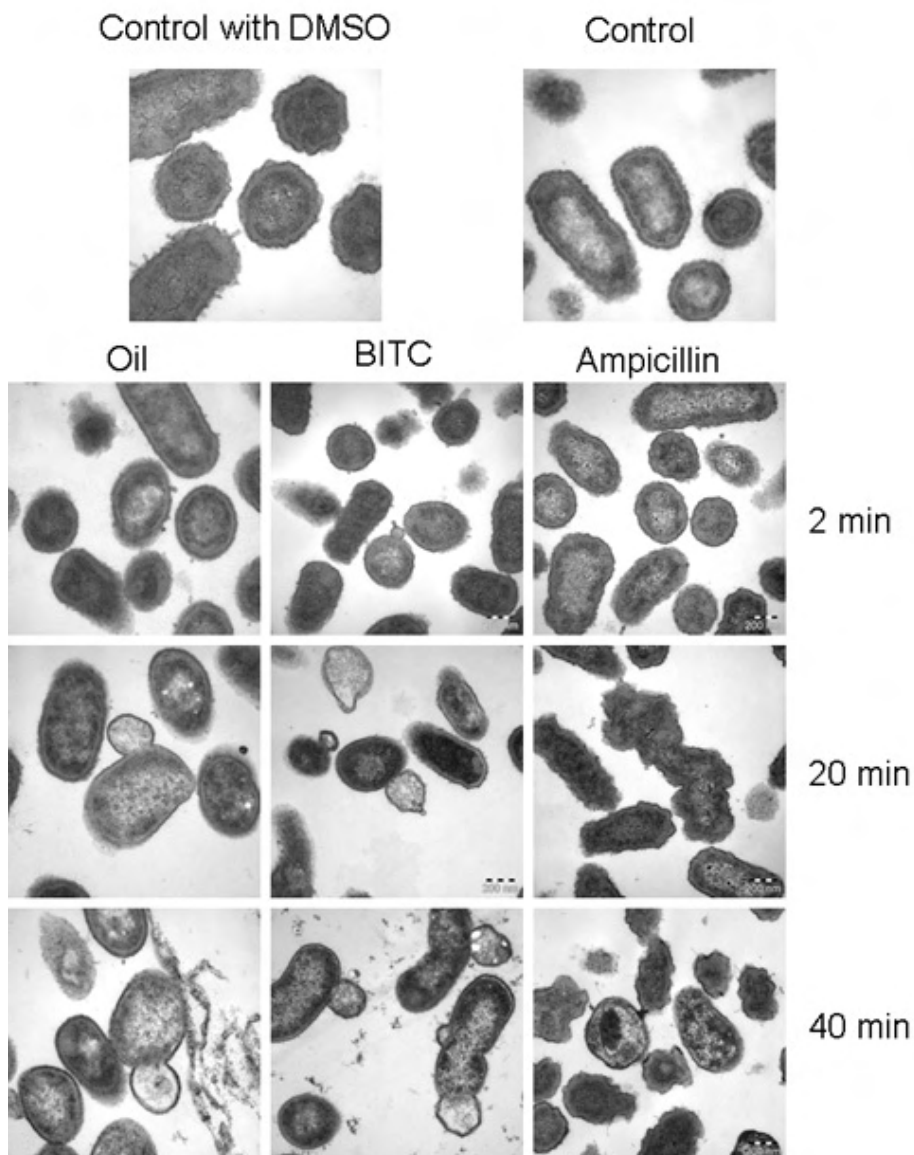


Figure 31: Transmission electron micrographs of *A. actinomycetemcomitans* treated with *S. persica* essential oil, synthetic BITC or Ampicillin. Samples were withdrawn at time points indicated. Concentration of essential oil was 0.1% corresponding to 2.8 μ mole BITC, synthetic BITC was 2.8 μ mole and ampicillin was 1 mg/ml. Control for essential oil and BITC was with 1% DMSO. For ampicillin, the control was with 1% water.

DISCUSSION

Our findings supported the hypothesis that Miswak stick pieces, without extraction, have a strong antibacterial effect against most bacterial species tested. Corresponding or greater antibacterial effects were associated with Miswak suspended 3 mm above the inoculated agar plates, suggesting the presence of volatile active antibacterial compounds.

The inhibition zones associated with the Miswak pieces clearly demonstrated much stronger inhibitory effects than the aqueous Miswak extract. For example, for *S. mutans*, the Miswak pieces caused unexpectedly large inhibition zones of 3.4 cm (2.6-4.6); whereas, our preliminary tests of aqueous Miswak extract yielded an inhibition zone against *S. mutans* of only 0.2 cm. This result was in accordance with earlier studies in which 50% Miswak aqueous extract achieved an inhibition zone of 0.2-0.3 cm (Al-Lafi and Ababneh, 1995; Almas et al., 1997; Almas, 1999; Almas and Al-Bagieh, 1999). The weak antibacterial effect observed with aqueous Miswak extract suggested that the active compounds were not extracted or were deactivated during preparation of the crude aqueous extract. Using this kind of extract may probably not reflect the real antibacterial activity of Miswak.

The antibacterial effect of Miswak pieces on *A. actinomycetemcomitans*, *P. gingivalis*, *H. influenzae* and *L. acidophilus* cannot be compared with the crude extract, as there are no published studies on the effect of Miswak extract on these bacterial strains. It was also difficult to compare the antibacterial effect of Miswak pieces with that of known antibacterial substances as the exact content and amount of Miswak pieces is unknown. Thus, the need to evaluate Miswak antibacterial effect by standard methods of evaluating antibacterial substances prompted an alternative extraction method for obtaining an active Miswak extract.

Pharmacological studies indicated that with steam distillation it is possible to obtain essential volatile oil from the roots, stems, and leaves of *Salvadora Persica* (Bader et al., 2002; Alali and Al-Lafi, 2003; Alali et al., 2004). However, no studies were conducted to test the antibacterial activity of the root oil on oral bacteria. From GC-MS analyses, the root oil comprises mainly of Benzyl isothiocyanate (BIT) (70%), limonene (9.4%), α -pinene (8.7%) and flavonoids (2.55%) (Abdel-Wahab et al., 1990; Bader et al., 2002). Some of these compounds are known to have antibacterial activity. Studies on BITC and flavonoids separately proved that they have antibacterial, antifungal, and antiviral activities (Al-Bagieh and Weinberg, 1988; Abdel-Wahab et al., 1990; Al-Bagieh, 1992; Al-Bagieh, 1998; Cushnie and Lamb, 2005).

The Miswak exhibited stronger antibacterial activity against Gram negative bacteria than Gram positive bacteria, as evidenced by the pronounced differences in inhibition zones associated with the Gram negative species *A. actinomycetemcomitans*, *P. gingivalis*, *H. influenzae* and the Gram positive species *S. mutans* and *L. acidophilus*. Studies on the effects of BITC and flavonoids on Gram negative and Gram positive

bacteria present contradictory results (Cushnie and Lamb, 2005). This may be due to the different assays used to test antibacterial effects and to variations within each assay. Well standardised studies are needed to identify which components of the oil extract have an antibacterial effect against Gram negative and Gram positive species.

Comparison of the effect of suspended and embedded Miswak pieces disclosed that the suspended Miswak pieces had similar or stronger effects on Gram negative bacteria, whereas, the opposite was true for Gram positive bacteria where the effect of the suspended Miswak was substantially reduced. Most probably, the compounds that affect Gram negative bacteria are more volatile than those affecting Gram positive bacteria. Studying the properties of different Miswak compounds and their reactions with surrounding environments is important in order to determine the best method available for testing its antibacterial activity. The effect of suspended 0.14 g Miswak on *P. gingivalis* was so strong that all of the 14 cm agar plates had complete growth inhibition. However, our laboratory facilities did not allow anaerobic incubation of larger agar plates. In order to verify that the strong inhibitory effect of 0.14 g pieces was a real effect of the Miswak and not an artefact, a dose response experiment was conducted. This experiment revealed a linear correlation between Miswak weight and inhibitory effect ($R^2 = 0.99$).

H. influenzae is a well characterised upper respiratory tract commensal in humans and associated animals. It is a major etiological agent of upper respiratory tract infections and acute exacerbations of chronic bronchitis (Samaranayake, 2006). Initially, *H. influenzae* served as a control for the incubation environment of *A. actinomycetemcomitans*. However, when the Miswak presented a strong antibacterial effect against *H. influenzae*, it was necessary to include these results in the study.

The diverging reports on the chemical nature and antimicrobial capacity of Miswak sticks prepared from the roots of *S. persica*, and the strong and air borne antimicrobial activity achieved in this work, prompted systematic chemical characterisation and antibacterial testing to identify the antimicrobial components present and investigate their properties for possible use as future therapeutic antimicrobials.

The volatiles from cut Miswak chewing-sticks from the root of *S. persica* are highly antimicrobial, these volatiles consisted of >98% (determined by GC-MS) of benzyl isothiocyanate (BITC). The steam-distilled essential oil generated from *S. persica* roots contained 78% BITC and this essential oil exhibited high antimicrobial activity, mainly against Gram-negative proteobacteria. With MPLC, the contents of *S. persica* milled roots were fractionated. The fractions that exhibited the highest antimicrobial activity had BITC as a dominating component and displayed the same repertoire of bacterial killing as with the oil. Through three different methods of preparation, BITC was identified as the main antimicrobial factor in the root of the shrub *S. persica*. Although there are naturally other, more polar, components in the roots that could be obtained through water or alcohol extraction, and which may contribute to the bactericidal activity, these components are far less potent in antimicrobial capacity and have low activity against Gram-negative bacteria (Al-Bagieh et al., 1994; Darout et al., 2000b; Almas et al., 2005; Hammad and Sallal, 2005; Darmani et al., 2006).

Upon plant tissue damage, BITC is released as an effector molecule of the plant defence system through hydrolysis of benzyl-glucosinolate by the enzyme myrosinase, (Rask et al., 2000). The plant enzyme myrosinase and its substrates glucosinolates are physically separated in plants, and therefore, plant tissues damage is a prerequisite for releasing isothiocyanates (Al-Bagieh et al., 1994; Holst and Williamson, 2004): Cruciferous vegetables of the genera *Brassica* and *Sinapis* are rich in benzyl-glucosinolates. Hence, BITC is released into the oral cavity and gastrointestinal tract upon consumption of uncooked cruciferous vegetables. The same mechanism appears active in the release of BITC when a Miswak stick is chewed on prior to mechanical cleansing of the teeth. As boiled Miswak sticks lose antibacterial activity, most likely due to inactivation of the myrosinase, this assumption is confirmed.

It has previously been shown that BITC exerts antimicrobial activity; however, the reported repertoire of susceptible bacteria and killing efficiency differs, probably due to variations in the methods used for antimicrobial testing (Aires et al., 2009; Beevi et al., 2009). Earlier, zone-inhibition assay was used, but the medium-containing agar appeared to inhibit the diffusion of the substances released from pieces of Miswak sticks. To avoid this inhibitory effect the essential oil or dilutions thereof to bacterial suspensions were used and BITC-containing essential oil exhibited dose-dependant antimicrobial activity against a number of medically important Gram-negative bacteria. The Gram-positive bacteria, all belonging to the firmicutes phyla, were more resistant or totally unaffected by the oil. Of special interest were the Gram-negative oral pathogens *A. actinomycetemcomitans* and *P. gingivalis*, which exhibited high sensitivity to the essential oil. This suggested the use of Miswak chewing-sticks for cleaning teeth may be protective against oral pathogens strongly associated with the pathogenesis of periodontal disease and tooth loss.

The kinetics of bacterial killing mediated by the essential oil was rapid. Within minutes the bacteria load was diminished by 1000 times. The short exposure required for bactericidal effect supported the assumption Miswak chewing-sticks may be efficient for improving oral health. There are few studies evaluating the *in vivo* effect of the practice of Miswak sticks on oral health and conclusions vary with study design (Darout et al., 2000a; Al-Otaibi et al., 2004). Larger studies with DNA-based analysis of the microbiota would be necessary in order to evaluate the benefit of Miswak chewing sticks in oral hygiene.

The rapid killing mediated by *S. persica* root essential oil suggested BITC-containing oil might target the bacterial membrane. Electron micrographs of the Gram-negative bacteria *A. actinomycetemcomitans* displayed protrusions of the bacteria cell membrane. Similar membrane protrusions were found with synthetic BITC at the same concentration as in the essential oil. This membrane effect resembled those reported for antimicrobial peptide treated bacteria (Lehrer et al., 1989) and the rapid antimicrobial effect (Boman, 2003). Antimicrobial peptides bind to and destabilise bacterial membranes and during this process the peptides are essentially consumed (Shai, 2002). This mechanism of killing confers a stoichiometric relation between required numbers of bound peptide molecules in order to kill a certain number of bacteria (Steiner et al., 1988). Our results on BITC bactericidal effects with increasing number of bacteria

suggested the essential oil targeted a vital enzymatic process rather than exerting its effect through binding to the bacterial membrane.

It has been demonstrated that the electrophilic end group of BITC can interact directly with glutathion-reductase and thioredoxin-reductase enzymes that are essential for maintaining the redox-potential in the cell (Hu et al., 2007). Interestingly, in a recent publication Daniels et al (2010) suggest Gram-positive bacteria of the firmicutes phyla exclude cysteins in their secreted and cytoplasmic proteins at a gene level, as a means of coping with a highly oxidative environment. This was in contrast to Gram-negative proteobacteria, in which cystein-containing secreted proteins mature in the oxidative periplasm assisted by glutathion-like oxidoreductases for disulfide bond formation (Nakamoto and Bardwell, 2004). One possible mechanism for BITC bacterial killing could be that BITC, which is rather lipophilic but also has electrophilic properties, may pass the cell membrane and bind to the bacterial glutathion-like oxidoreductases, such as the Dsb family (Nakamoto and Bardwell, 2004), thus, interfering with the bacteria cell redox-potential. The repertoire of bacteria killed by BITC, Gram-negative proteobacteria and the anaerobic Gram-negative *P. gingivalis* of the bacteroidetes phyla, would concur with this speculation

Naturally occurring isothiocyanates released during consumption of cruciferous vegetables have demonstrated cancer-preventive activity in animal models, and in epidemiological studies, high dietary intake of isothiocyanates is associated with a reduced risk for lung, colorectal, breast, and prostate cancer in humans (Fenwick et al., 1983; Conaway et al., 2002; Wu et al., 2004). There are numerous reports on the chemoprotective activity of synthetic BITC (Miyoshi et al., 2004; Zhang and Gordon, 2004; Warin et al., 2009) and some also address the possible adverse effects of BITC (Kassie et al., 1999). Interestingly, genotoxic effects of BITC in terms of DNA damage are demonstrated *in vitro* on liver- and gastric cell lines at low doses (5µg/ml) after one hour of exposure. This is contrary to the *in vivo* results in which doses of 220 mg BITC/kg body weight are required to cause a shift in the DNA migration pattern of rat gastric and colonic mucosa cells. The discrepancy between *in vivo* and *in vitro* data suggests that BITC is detoxified in the living organism (Munday and Munday, 2004). Indeed it has been demonstrated that the chemopreventive activity of BITC is partially through activation of detoxification systems (Shapiro et al., 1998). Together these data concerning the effects of BITC on eukaryotic organisms suggests the possible antimicrobial therapeutic interval of BITC is viable for further investigation due to the rather high *in vivo* tolerance and the documented positive health effects when BITC is ingested via the consumption of vegetables.

MAIN FINDINGS

- Rinsing with Miswak extract resulted in protracted elevation of plaque pH (>6.0). The difference in plaque pH between Miswak extract and water rinse was statistically significant at 30 minutes ($p < 0.01$).
- Miswak water extract stimulated parotid saliva flow rate significantly ($p < 0.01$).
- Toothbrushing with fresh Miswak significantly reduced dental plaque and gingival inflammation while the deactivated Miswak did not.
- Both fresh and deactivated Miswak reduced approximal plaque significantly, but there was no significant change in the approximal gingival index.
- Testing the antibacterial effect of fresh Miswak pieces embedded in inoculated agar plates indicated Miswak had very strong antibacterial activity.
- The inhibitory effect was most pronounced on *P. gingivalis*, *A. actinomycetemcomitans*, and *H. influenzae*, less on *Streptococcus mutans* and least on *Lactobacillus acidophilus*.
- Suspending Miswak pieces on top of inoculated agar plates had a comparable effect to the embedded Miswak pieces.
- A dose response effect was demonstrated when different Miswak weights were tested on *P. gingivalis*.
- Miswak essential oil had a strong antibacterial activity against seven Gram negative bacterial strains.
- Benzyl isothiocyanate is the main antimicrobial component of Miswak from *Salvadora persica* roots. Synthetic Benzyl isothiocyanate had comparable antibacterial activity to Miswak essential oil.
- Electron microscopy revealed bacterial cell membrane exhibited protrusions upon treatment with Miswak essential oil and synthetic Benzyl isothiocyanate. The bacterial cell membrane presented different changes when treated with ampicillin.

CONCLUSIONS

Miswak extract from *Salvadora persica* raised plaque pH after acidic challenge; this elevation was maintained until the end of the testing session. Previous studies report an immediate reduction of *S. mutans* count and a significant increase in calcium and chloride concentrations. These findings together with the protracted elevation of plaque pH determined in this thesis suggested Miswak might have a positive roll in the reduction and prevention of dental caries. Fresh Miswak had a significant effect on dental plaque and gingival inflammation, suggesting that Miswak might play a potential role in periodontal disease prevention. However, fresh Miswak did not have superior properties to deactivated Miswak on interproximal embrasures, which are difficult to access, suggesting a limited chemical effect on the microflora in our study population. Therefore, Miswak can be used as a dental hygiene method for maintaining good oral hygiene; however, additional interproximal cleaning aides are needed in combination with Miswak use.

Miswak prepared from the roots of *Salvadora persica* had strong antibacterial activity when tested without extraction. The experiment with pieces of Miswak suspended over the agar plate revealed the antibacterial component was volatile. Miswak oil, extracted with steam distillation, had the same strong antibacterial effect as the root *per se*. Gas chromatography and fractioning identified Benzyl isothiocyanate as a major antibacterial effector molecule of *Salvadora persica* root. Benzyl isothiocyanate is released from the root upon tissue damage and when Miswak sticks made from *Salvadora persica* root are used for oral hygiene, an antibacterial effect is obtained fresh and instantaneously. The difference in bacterial cell wall change when treated with Benzyl isothiocyanate and ampicillin suggested Benzyl isothiocyanate killing mechanism of action is different to that of ampicillin.

FUTURE PERSPECTIVES

Further studies are warranted for exploring and identifying the underlying mechanisms of action of Miswak on bacteria. The strong and rapid antimicrobial effect against Gram-negative gammaproteobacteria, a class of bacteria that include medically important pathogens such as *Salmonella enterica*, *Porphyromonas gingivalis* and *Haemophilus influenzae*, advocates in favour of investigating the potential of Benzyl isothiocyanate as an antimicrobial substance for therapeutic use. The volatile character of Benzyl isothiocyanate might be an avenue for exploring applications where confined body spaces require antibacterial treatment. Studying the effect of brushing with Miswak on populations with periodontitis, having a predominantly Gram-negative flora, would be necessary for testing the effectiveness of Miswak in the treatment and prevention of periodontal disease. To test a chemical antibacterial effect of Miswak without mechanical interference would require the use of an extract rather than the sticks *per se*. Laboratory and clinical investigations of antiviral and antifungal activities together with the effect on periodontal bacteria need to be performed. A possible anti-inflammatory effect of a Miswak extract should also be explored. The cytotoxic

activities of fresh Miswak and Miswak oil need to be evaluated before the development of oral applications becomes a future reality.

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