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5th year, group 16

EFFECT OF MISWAK EXTRACT ON *STREPTOCOCCUS MUTANS*

Master`s Thesis

Supervisor

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Kaunas, 2017

LITHUANIAN UNIVERSITY OF HEALTH SCIENCES
MEDICAL ACADEMY
FACULTY OF ODONTOLOGY
DEPARTMENT OF DENTAL AND ORAL PATHOLOGY

EFFECT OF MISWAK EXTRACT ON *STREPTOCOCCUS MUTANS*

Master's Thesis

The thesis was done by student.....…
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Kaunas, 2017

Annex No. 7

EVALUATION TABLE OF CLINICAL–EXPERIMENTAL MASTER'S THESIS

Evaluation: .....

Reviewer: .....
(scientific degree, name and surname)

Reviewing date: .....

No.	MT parts	MT evaluation aspects	Compliance with MT requirements and evaluation		
			Yes	**Partially**	**No**
1	**Summary (0.5 point)**	Is summary informative and in compliance with the thesis content and requirements?	0.3	0.1	0
2		Are keywords in compliance with the thesis essence?	0.2	0.1	0
3	**Introduc-tion, aim and tasks (1 point)**	Are the novelty, relevance and significance of the work justified in the introduction of the thesis?	0.4	0.2	0
4		Are the problem, hypothesis, aim and tasks formed clearly and properly?	0.4	0.2	0
5		Are the aim and tasks interrelated?	0.2	0.1	0
6	**Review of literature (1.5 points)**	Is the author's familiarization with the works of other authors sufficient?	0.4	0.2	0
7		Have the most relevant researches of the scientists discussed properly and are the most important results and conclusions presented?	0.6	0.3	0
8		Is the reviewed scientific literature related enough to the topic analysed in the thesis?	0.2	0.1	0
9		Is the author's ability to analyse and systemize the scientific literature sufficient?	0.3	0.1	0
10	**Material and methods (2 points)**	IS the research methodology explained comprehensively? Is it suitable to achieve the set aim?	0.6	0.3	0
11		Are the samples and groups of respondents formed and described properly? Were the selection criteria suitable?	0.6	0.3	0
12		Are other research materials and tools (questionnaires, drugs, reagents, equipment, etc.) described properly?	0.4	0.2	0
13		Are the statistical programmes used to analyse data, the formulas and criteria used to assess the level of statistical reliability described properly?	0.4	0.2	0
14	**Results (2 points)**	Do the research results answer to the set aim and tasks comprehensively?	0.4	0.2	0
15		Does presentation of tables and pictures satisfy the requirements?	0.4	0.2	0
16		Does information repeat in the tables, picture and text?	0	0.2	0.4

17		Is the statistical significance of data indicated?		0.4	0.2	0
18		Has the statistical analysis of data been carried out properly?		0.4	0.2	0
19	**Discussion (1.5 points)**	Were the received results (their importance, drawbacks) and reliability of received results assessed properly?		0.4	0.2	0
20		Was the relation of the received results with the latest data of other researchers assessed properly?		0.4	0.2	0
21		Does author present the interpretation of results?		0.4	0.2	0
22		Do the data presented in other sections (introduction, review of literature, results) repeat?		0	0.2	0.3
23	**Conclu-sions (0.5 points)**	Do the conclusions reflect the topic, aim and tasks of the Master's thesis?		0.2	0.1	0
24		Are the conclusions based on the analysed material? Do they correspond to the research results?		0.2	0.1	0
25		Are the conclusions clear and laconic?		0.1	0.1	0
26	**References (1 point)**	Is the references list formed according to the requirements?		0.4	0.2	0
27		Are the links of the references to the text correct? Are the literature sources cited correctly and precisely?		0.2	0.1	0
28		Is the scientific level of references suitable for Master's thesis?		0.2	0.1	0
29		Do the cited sources not older than 10 years old form at least 70% of sources, and the not older than 5 years – at least 40%?		0.2	0.1	0
Additional sections, which may increase the collected number of points						
30	**Annexes**	Do the presented annexes help to understand the analysed topic?		+0.2	+0.1	0
31	**Practical recommen-dations**	Are the practical recommendations suggested and are they related to the received results?		+0.4	+0.2	0
General requirements, non-compliance with which reduce the number of points						
32	**General require-ments**	Is the thesis volume sufficient (excluding annexes)?		15-20 pages (-2 points)		<15 pages (-5 points)
33		Is the thesis volume increased artificially?	-2 points	-1 point		
34		Does the thesis structure satisfy the requirements of Master's thesis?		-1 point		-2 points
35		Is the thesis written in correct language, scientifically, logically and laconically?		-0.5 point		-1 points
36		Are there any grammatical, style or computer literacy-related mistakes?	-2 points	-1 points		
37		Is text consistent, integral, and are the volumes of its structural parts balanced?		-0.2 point		-0.5 points
38		Amount of plagiarism in the thesis.	>20% (not evaluated)			
39		Is the content (names of sections and sub-sections and enumeration of pages) in		-0.2 point		-0.5 points

		compliance with the thesis structure and aims?			
40		Are the names of the thesis parts in compliance with the text? Are the titles of sections and sub-sections distinguished logically and correctly?		-0.2 point	-0.5 points
41		Was the permit of the Bioethical Committee received (if necessary)?			-1 point
42		Are there explanations of the key terms and abbreviations (if needed)?		-0.2 point	-0.5 points
43		Is the quality of the thesis typography (quality of printing, visual aids, binding) good?		-0.2 point	-0.5 points
		***In total (maximum 10 points):**			

***Remark:** the amount of collected points may exceed 10 points.*

Reviewer's comments: _____

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Reviewer's name and surname Reviewer's signature

TABLE OF CONTENTS

Summary:

The aim: Prove that miswak has a natural antibacterial effect against the cariogenic bacteria *S.mutans.*

Hypothesis (H_a): Very high concentrations of miswak extract solution has a natural antibacterial effect against *S.mutans*.

Null Hypothesis (H_0): Very high concentrations of miswak extract solution has no natural antibacterial effect against *S.mutans*.

Introduction: Miswak has long been known to have health benefits, especially in the oral cavity as studies have proven antibacterial, antifungal, anti-periopathic and anti caries properties in aqueous extract of miswak.

Materials and methods: The experiment was divided into two parts; the first part is to confirm that the patient is positive on the test device while, the second part is to see if any given concentration would change the results into negative. Materials used include; three different miswak extract concentrations, "GC America test kit" and sterile equipment such as plastic cups, mixing spoons and sterile water.

Results: Chi square test (X^2 test), showed a lower p-value than 0.05 when comparing the different categories with each other. This indicates a strong evidence against the null hypothesis and therefore we reject the null hypothesis.

Conclusion: As the miswak extract concentration increases the antibacterial effect on *S.mutans* increases. However, the antibacterial effect of miswak extract on *S.mutans* is considered weak, as concentrations of 500 % and beyond is required to increase the possibility of an instant effect that reduces the amount of *S.mutans* under threshold.

Keywords: Miswak extract, Salvadora persica L, Streptococcus mutans, antimicrobial activity

Abbreviations: Salvadora persica L; S.perisca, Streptococcus mutans; *S.mutans*

Introduction

Tooth brushing stick, also known as miswak or Siwak, is a stick that comes from *Arak* (*Salvadorapersica*) or the tooth brush tree and is roughly the same size as a pencil with a length of 15 – 20 cm and a diameter of 1 – 1.5 cm. *Salvadorapersica* can be usually found in eastern part of India, Pakistan, Arabian countries and few African countries. Miswak is used as an alternative to the normal toothbrush with Nylon bristles seen mainly in the western world. [1]

Previously, miswak was used based on the importance it has both culturally and religiously, especially within the religion of Islam. However, recent studies have demonstrated that there is antibacterial, anti-periopathic, anti-fungal and anti-caries properties in aqueous extract of miswak. Studies have also proven oral disinfectant and anti-plaque agents present in miswak. [2]

It is estimated that more than ten different natural chemical compounds that are considered good for both oral and dental hygiene are present in miswak according to many researchers. Some of these constituents include:

- Fluorides – anti cariogenic and re-mineralizes dental enamel.
- Tannic acid – reduces candida albican counts.
- Alkaloids (salvadorine) – both stimulatory action and bactericidal effect on gingiva.
- Silica – acts as an abrasive material in order to remove any dental staining.
- Resins – amorphous product that are hard, translucent or transparent.
- Vitamin C- antioxidant, cell growth, healing and repair of tissues.
- Sulfur – has bactericidal effects.
- Volatile oils (simgrins) – has antiseptic, aroma and carminative action.
- Chlorides – reduces plaque and calculus as well as having a broad anti-bacterial spectrum.
- Sodium bicarbonate – mild abrasive, often seen in dentifrice.
- Flavenoids – antimicrobial and antifungal activities.
- Calcium – inhibits demineralization and re-mineralize enamel as well as saturation of saliva.
- Benzylisothoicyanate – It has a virucidal activity against herpes simplex virus as well as a broad spectrum bactericidal effect, inhibiting the growth and acid production of *S.mutans.* [2]

Dental plaque tops the hierarchy of initiating factors for dental caries and other periodontal diseases. Mechanical methods (toothbrush, toothpaste and chewing sticks) and chemical methods (mouthwashes) have been reported to significantly reduce the amount of dental plaque. Chlorhexidine is the gold standard as it is capable of both reducing pathogenic microorganisms such as *S.mutans* as well as reducing the amount of microbial plaque. However, chlorhexidine has some side effects which includes; Teeth discoloration, xerostomia, burning sensation and unpleasant taste alteration. Therefore, the use of it is not recommended for all patients. [3]

Herbal mouthwashes like miswak extract solutions is now increasingly becoming an alternative as it has been shown to improve periodontal health, decrease bleeding on brushing and reduce microbial plaque accumulation. In vitro studies have shown that 50 % miswak extract solution possesses antimicrobial characteristics against *S.mutans.* [3]

S.mutans is considered the primary bacterium within the dental plaque when it comes to initiation and progression of dental caries as it adhere firmly to tooth surfaces in presence of sucrose and it forms acids by fermenting dietary sugar. The chewing stick has an antibacterial effect on *S.mutans* as well as an inhibitory action on dental plaque formation. [4]

Oral flora consists of more than 700 oral microbial species that is transmitted by a circulating fluid called the saliva, which also act as a reservoir for bacterial colonization. It has been reported that the number of microorganisms living in saliva is approximately between 10^8 and 10^9 CFU/mL. Their growth and survival depends on the utilization of salivary constituents. The growth of dental plaque as well as the detachment of layers of plaque also depends on saliva. [5]

The level of certain bacterial species in saliva such as *S.mutans* can reflect their presence in plaque according to several studies which measured the correlation between salivary concentration of *S.mutans* and their proportions in plaque. Studies have also shown a correlation in relationship between *S.mutans* in saliva and increased caries initiation and progression as well as the presence of root caries. [5]

Review of Literature

There is an old proverb that reflects on the importance of the oral health within the body which states that, “if the eyes are a window to the soul, then the mouth is the doorway to the body”. Oral health is by no means the “golden gate” towards a prolonged as well as a happier life. Many present oral health products do have significant side effect or would reduce or kill oral microflora as well as pathogenic bacteria. [6]

As such, a wide range of scientific research studies have investigated the possible use of natural plant extract in both medicine and dentistry. [7] In vitro studies have proved that miswak extracts inhibited the growth of various oral aerobic and anaerobic bacteria, including *S.mutans,* as it exert antimicrobial effects on *S.mutans* due to the interaction with bacteria, preventing their attachment on the tooth surface. [8] However, it is known that bacteria acting in vitro, does not possess the same behavior as it will do in vivo or in its natural environment of biofilm. [9]

The oral cavity itself has many antimicrobial factors, such as antimicrobial peptides, lysozymes, hydrogen peroxides and lactoferrin. Antimicrobial peptides that originate from saliva, oral mucosa, gingival epithelium, gingival crevicular fluid and neutrophils are believed to have bactericidal activity against various oral bacteria, including *S.mutans*. [10] However, these natural antimicrobial factors does not eliminate or significantly reduce the amount of *S.mutans* but rather control or show signs of minimal reduction. Therefore, the oral cavity require antimicrobial aid from external sources. (Newman et al. 150 - 151)

Bacteria have the capacity to communicate with each other in a biofilm and do not exist in isolation. Growth of bacteria in microbial communities’ adherent to a surface does not act in the same way as bacteria growing suspended in a liquid environment (planktonic or unattached state). There is a significant increase in resistance of bacteria in a biofilm to antimicrobial agents. The resistance of bacteria in a biofilm to antibiotics are between 1000 to 1500 times more resistant compared with antibiotics in their planktonic state. (Newman et al. 150 - 151)

This significant increase mechanism differ from antibiotic to antibiotic, from species to species and for biofilms growing in different habitats. Factors affecting the resistance of bacteria to antibiotics include; nutritional status, pH, temperature, growth rate and prior exposure to sub effective concentrations of antimicrobial agents. A variation to these parameters will lead to a different response to antibiotics within a biofilm. Slower rate of growth of bacteria in a biofilm makes it less susceptible to many but not all antibiotics. The biofilm matrix is not a physical barrier to the diffusion of antibiotics but it has certain properties that can slow down antibiotic penetration. (Newman et al. 150 - 151)

Al-Sohaibani. S, Murungan. K (2012) conducted an in vitro and molecular docking study to evaluate the growth inhibition and antibiofilm effects of multiple extracts on cariogenic *S.mutans* isolates. Gas chromatography–mass spectrometry (GC–MS) analyses for phytochemicals and their possible mode of interaction with biofilm response regulators were revealed using LigandFit docking protocols. The extracts of S. persica all showed considerable inhibitory activity and the cariogenic *S.mutans* showed varied susceptibility when compared with controls. [12]

Various other research including epidemiological and laboratory have suggested that miswak possess a strong anti-decay effect. A study conducted in Ghana that compared miswak users with artificial tooth brush users showed that the rate of plaque formation, the development and progression of caries was less in miswak users than in those using artificial tooth brush. Some studies have also related the anti-decay effect of miswak extract to its fluoride content. [13]

Some studies such as Darmani et al found that aqueous extract of miswak was able to significantly inhibit the growth of cariogenic bacteria, while others have also proved that an increase in salivary secretion will in turn cause an increase in its buffering capacity because of the hot taste of miswak and the chewing effects of the stick. [12, 13, 14] These are few of the studies that indicate to us that miswak have some great beneficial oral health effect and confirms that there is a relationship between the antibacterial effect of miswak and *S.mutans.*

Materials and methods

This *in vivo* clinical study was conducted during the period between April 2016 and March 2017. A total of 45 adults between the age of 18 and 30 with at least two active caries agreed to participate and 98 "GC America" test devices were used. The patients were divided into three groups with different miswak concentration, each containing 15 participants. The tests do not have to be taken in the clinics but rather a clean, disinfected environment that resembles that of a clinic to avoid any contamination when it comes to sampling the results.

Figure1: Shows 20 g of miswak extract powder (on the left) and 4 ml of sterile water (on the right). Both in a sterile colourless plastic cup, prepared for a 500 % concentration test. (Sadek. M. 2017).

Upon completion of both the medical and dental questionnaire, as well as the form of consent the patient would be invited to participate in the experiment in a clean disinfected environment which was conducted in two parts.

Figure2: Shows the full content of the "Saliva Check Mutans" kit from "GC America", which was used to collect the results. (Sadek. M. 2017).

The first part:

1. The patient is asked to rinse his/her mouth for 5 minutes with 4 ml of sterile water.
2. A period of 5 minutes is given to the patient post rinsing to re-establish the natural niche environment of the oral cavity.
3. The patient is then asked to chew on the paraffin gum for 1 minute to stimulate the secretion of the saliva.
4. The stimulated saliva sample is collected in the mixing container. The volume must reach line A (see below) and any excess should be removed.
5. The bottle of reagent #1 is held vertically and 1 drop of reagent #1 is added to the saliva.
6. The opening of the mixing container is held tightly whilst tapping with the finger the mixing container 15 times over a period of 10 seconds in order to mix the saliva and to reagent #1 thoroughly and to avoid spilling of saliva.
7. 4 drops of reagent # 2 was added to the mixing container and shaken for several seconds to mix. The colour of the saliva sample changes to a light green colour (alkaline to neutral pH).
8. A graduated pipette is used to take sufficient saliva from the mixing container to fill to line 3 on the pipette and dispense into the sample window at the end of the test device.
9. The test device should be left for 15 minutes (room temperature).
10. Results should be recorded.

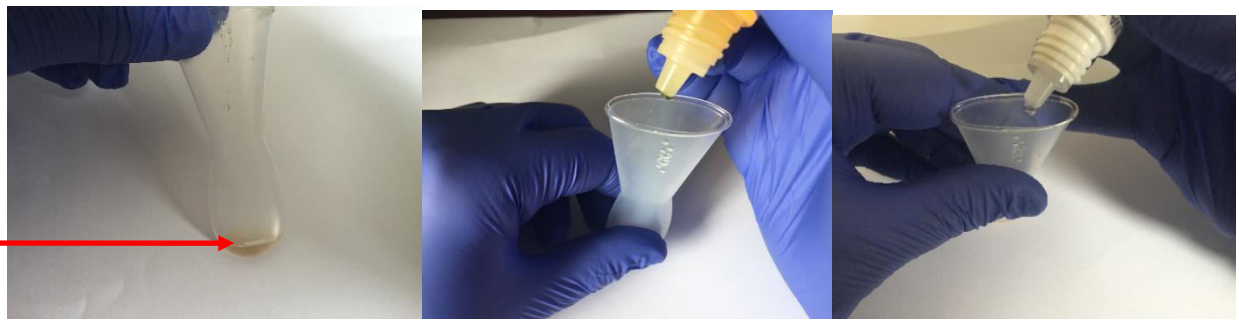


Figure3: Shows the steps described above (step, 4, 5 & 6) from left to right. (Sadek. M. 2017).

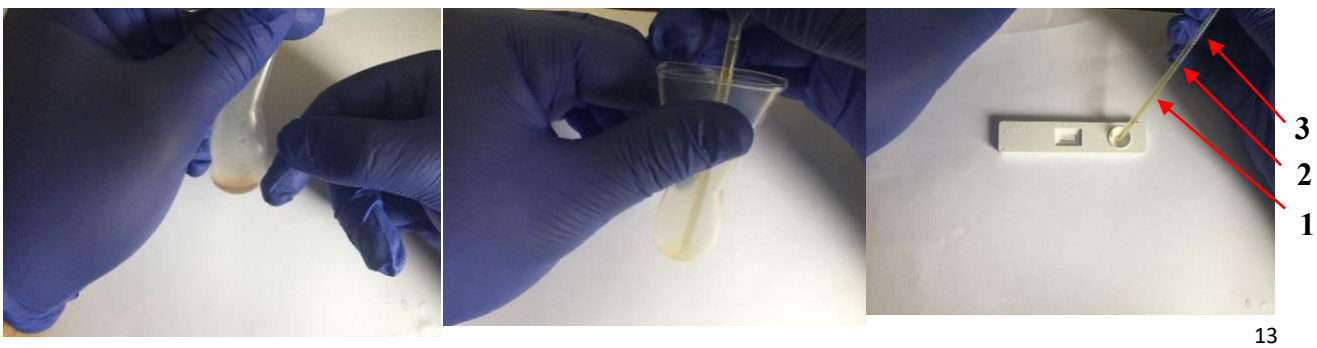


Figure4: Shows the steps described above (step, 7, 8 % 9) from left to right. (Sadek. M. 2017).

Figure₅: Shows the process within 15 minutes of taking the results (step 10). (Sadek. M. 2017).

The second part follows the same steps, except for the first, as the patients get to rinse or apply orally a specific concentration (100 %, 250 % or 500 %) of miswak extract in sterile water, prepared prior to the investigation.

Figure₆: Demonstrates the different consistencies of miswak extract. On the left, liquid state (rinsing 100%), the middle paste (applied on all teeth and tounge, 250 %) and on the right paraffin gum consistency (chewing, 500 %). (Sadek. M. 2017).

At a concentration of 100 % the miswak extract solution is still liquid and therefore the patient is asked to rinse his/her mouth. However, as the concentration of miswak extract solution increases the consistency thickens until it solidifies. Therefore, at 250 % concentration of miswak extract the consistency turns into paste. While at 500 % concentration it is more like a paraffin gum consistency.

The miswak extract used was bought from Guanjie Biotech (http://www.gybiotech.com/) on 11/04/2016 and a certificate of analysis was received upon purchase (appendix A). According the certificate of analysis, the miswak extract is pure and do not carry any contaminations or other factors that could cause conflict with the outcome of our clinical study.

Figure 7: Shows the different consistencies that has been included in the experiment. To the left (100 %) is liquid state, the middle (250 %) is paste and to the right (500 %) has a consistency of a paraffin gum. (Sadek. M 2017).

To make sure that the test is working properly, a red thick line should be observed in the control (C) window of the test device. If a thin red line appears in the test (T) window, then a positive result is indicated which means that the salivary levels of *S.mutans* are high (>5 x 10^5 CFU/mL saliva), thus a higher potential risk of future caries activity. No line can be observed after 15 minutes and indicates a low salivary level of *S.mutans* and a low potential risk of caries at this time. [15]

Note:

1. Check the results at 15 minutes after dispensing the saliva into the sample window, as the results before or after 15 minutes may be inaccurate.
2. If the line at test (T) window is very pale, it is still regarded as a positive result.
3. When the number of *S.mutans* bacteria per mL of stimulated saliva is greater than 500,000 CFU per mL (>5 x 10^5 CFU/mL) it is regarded as a positive result.
4. When the number of *S.mutans* bacteria per mL of stimulated saliva is less than 500,000 CFU per mL (<5 x 10^5 CFU/mL) it is regarded as negative result.
5. An invalid test is counted if the red line does not appear in the control (C) window.
6. Always replace the cap of the reagent bottles after its use. [15]

Reagent #1 and #2 of the "Saliva Check Mutans" kit is used to overcome difficulties in using saliva as a test sample. The two difficulties are:

1. The viscosity of the whole human saliva and in turn the ease in flow to the test device.
2. Glucans which often covers *S.mutans* bacteria may inhibit the reaction with the antibody.

Reagent #1 is NaOH solution and reagent #2 is oraganic acid solution, thus designed to remove these impediments to obtain accurate results. [15]

The mechanism of "Saliva Check Mutans" is to detect *S.mutans* in saliva using a highly specific immunochromatography process. A high level of *S.mutans* are present in saliva will react with a colloidal gold-labelled anti-*S.mutans* monoclonial antibody which is contained in the test device. These gold colloid particles attach to the surface of *S.mutans* and reacts with another anti-*S.mutans* antibody to form the red line on the T window. The C window shows any colloidal gold-labled anti-*S.mutans* monoclonial antibody that has not reacted with *S.mutans* and instead reacts with an anti-mouse immunoglobulin showing the control red line. [15]

The performance is based on four factors, namely:

1. Detectability

   - Evaluation of the detection limit is assessed by diluting the pure *S.mutans* solution ($1x10^8$ CFU/mL). Results show that the number of *S.mutans* detected is of $9.4x10^4$ CFU in the test device (300 µL of 5×10^5 CFU/mL solution). [15]

2. Sensitivity-Specificiy (correlation)

   - Evaluation on 89 saliva samples was conducted on "Saliva Check Mutans" and compared with the real-time PCR method. The results showed sensitivity (90.9% or 10/11), specificity (97.4 % or 76/78), positive predicted value (83.3% or 10/12) and negative predicted value (98.7 % or 76/77). [15]

3. Reproducibility

   - Assessment to check for intra-lot accuracy, the same positive samples and a dilution buffer solution was processed 15 times on the test devices of the same production lot with the same experimental conditions. Inter-lot accuracy was processed on three different production lots. All results were correct as expected, both intra and inter-lot accuracy. [15]

4. Interference

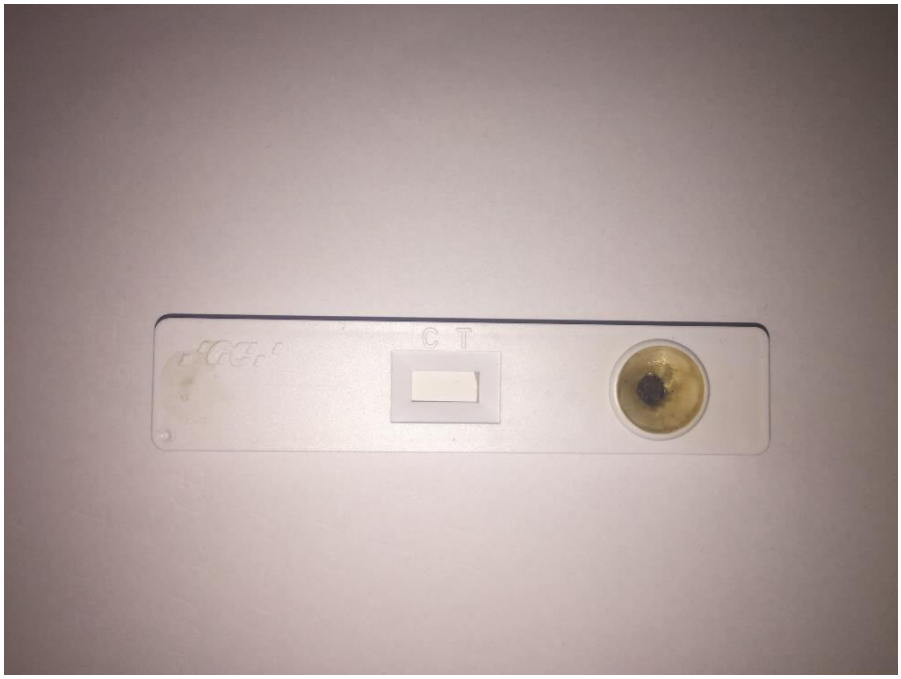
   -Testing the cross-reactivity to samples positive for the following oral bacteria was assessed and found to be negative*: Streptococci sanguis, Streptococci mitis, Streptococci salivarius, Streptococci sobrinus, Streptococcus gordonii, Streptococcus rattus, Lactobacillus casei, Lactobacillus salivarius, Lactobacillus plantarum, Lactobacillus acidophilus, Actinomyces naeslundii, Porphyromonas gingivalis, Prevotella intermedia, Tannerella Forsthensis, Eikenella corrodens, Fusobacterium nucleatum, Treponema denticola, Camplobacter rectus, Myxococcus xanthus and Escherichia colli.* [15]

Results

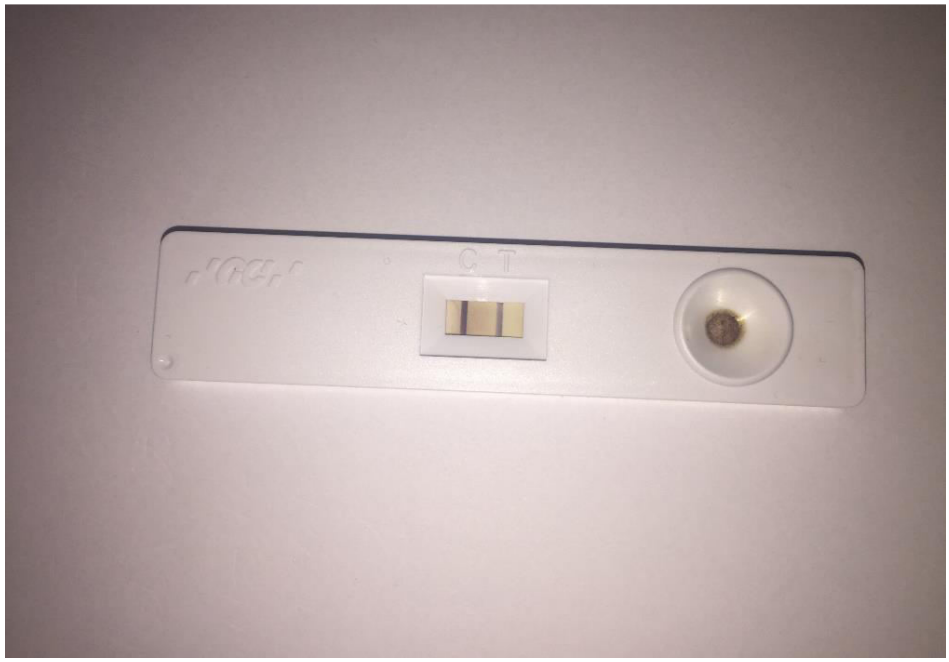
Percentage of solution	Amount (g/ml)	Amount of participants	Consistency/application	Positive results (>5 x 10^5 CFU/mL)	Negative results (<5 x 10^5 CFU/mL)
Group1: 100 %	4g/4ml	15	Liquid/rinsing	15 (100 %)	- (0 %)
Group2: 250 %	10g/4 ml	15	Paste/applying	11 (73.3 %)	4 (26.7 %)
Group3: 500 %	20g/4ml	15	Paraffin gum/chewing	2 (13.3 %)	13 (86.7 %)
Total		45		28 (62.2%)	17 (37.8 %)

Table1: shows the results obtained from the experiment.

Figure8: Shows a test device indicating a positive result after asking the participants to rinse with 4 ml of sterile water. (Sadek. M 2017).



Figure₉: Shows a test device with no C line, indicating an anomaly. (Sadek. M 2017).



Figure₁₀: Shows a test device indicating a positive result after asking the participants to rinse with 4 ml of 100 % miswak extract solution for 5 minutes. (Sadek. M 2017).

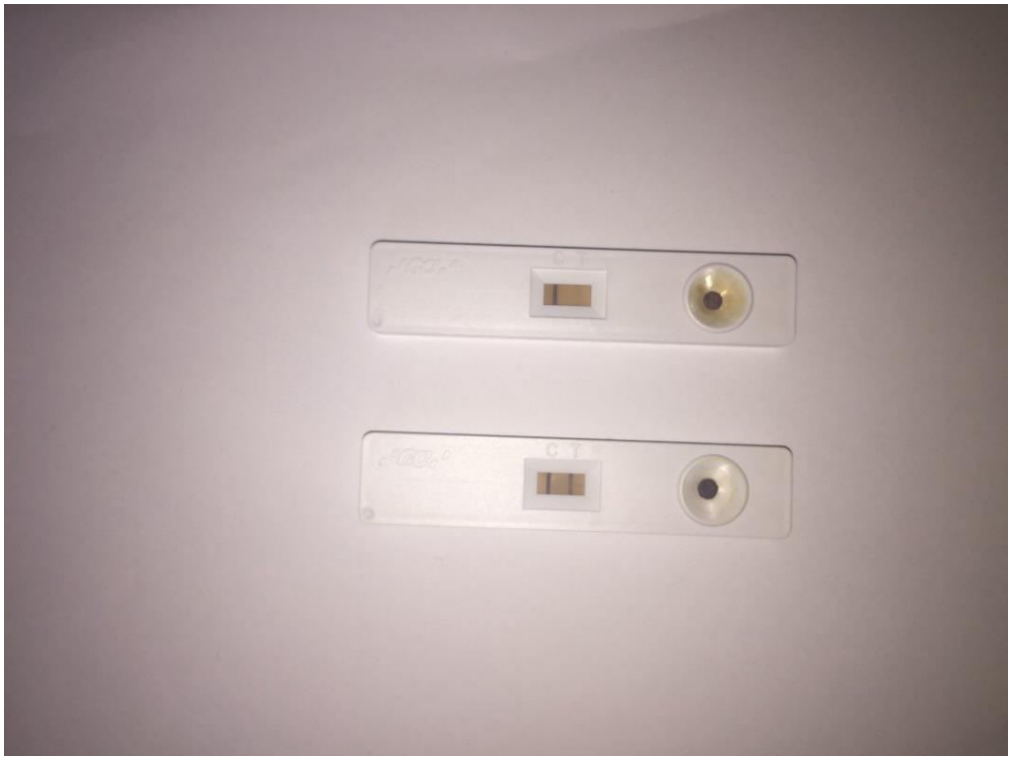
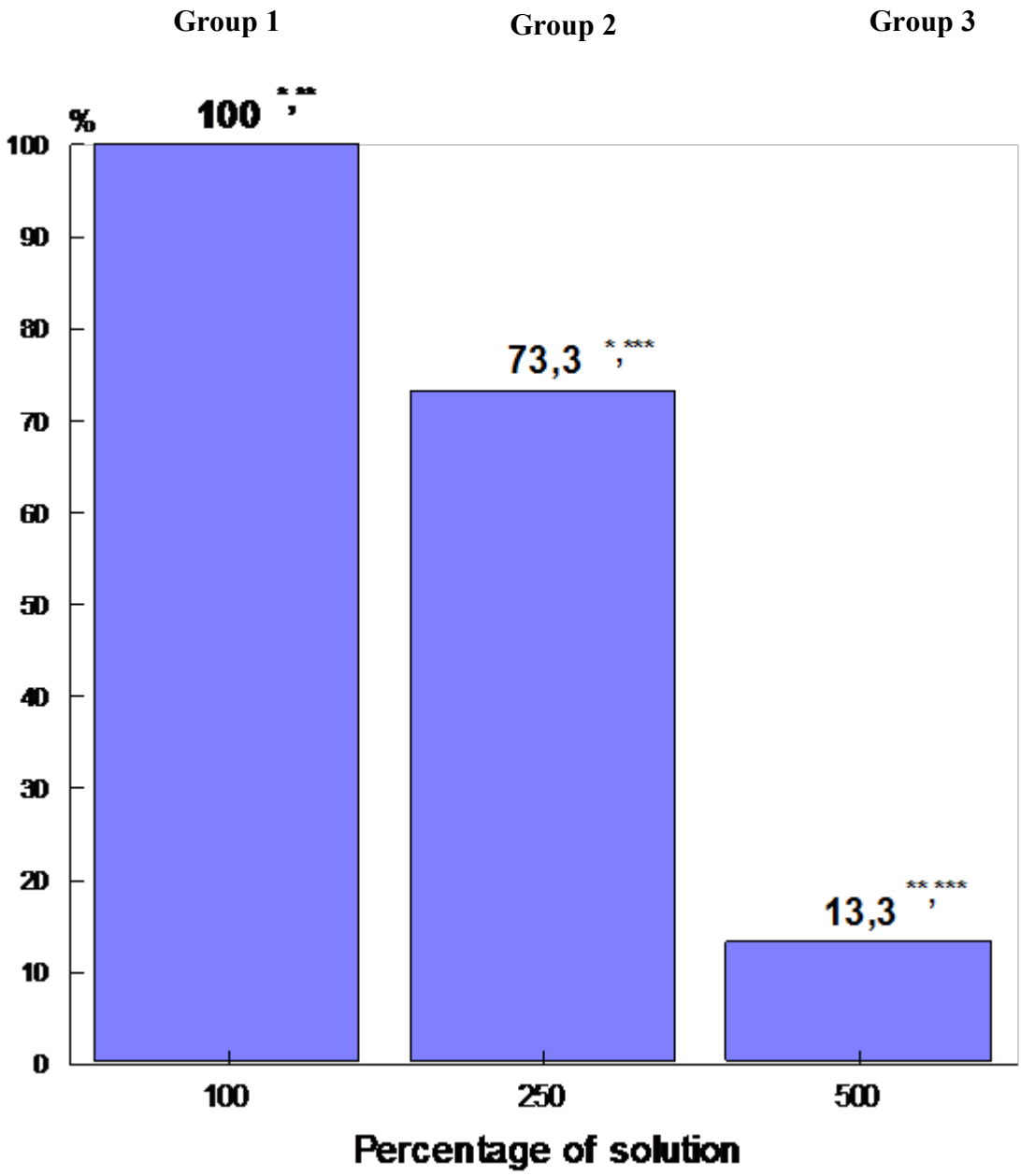


Figure11: Shows two test devices indicating a positive and a negative result after asking the participants to apply 4 ml of 250 % miswak extract paste on all the surfaces of the teeth and tongue for 5 minutes. (Sadek. M 2017).

Figure12: Shows two test devices indicating a positive and a negative result after asking the participants to chew a piece of 500 % miswak extract gum for 5 minutes. (Sadek. M 2017).



***Figure*$_{13}$**: Illustrate a bar chart that shows the percentage of positive results (>5 x 10^5 CFU/mL) obtained in all three different miswak concentrations.

Comparing groups	P-value
Groups 1 & 2	0.02
Groups 1 & 3	< 0.001
Groups 2 & 3	< 0.001

***Table*$_2$**: shows a table comparing different categorical groups of miswak extract concentrations with each other and the statistical significance obtained using the X^2 test.

Discussion

This clinical in vivo study was designed to prove that a specific tree by the name of *Salvadora persica* (tooth brush tree) which possess a purgative action and is traditionally used to treat renal stones, rheumatism and bronchial asthma, also has an antibacterial effect against *S.mutans*. [16] The research was conducted to see if at a higher concentration of miswak extract solution there will be an instant antibacterial effect on *S.mutans* which in turn would reduce the amount of this specific bacteria to under threshold limit ($<5 \times 10^5$ CFU/mL).

According to the results (table; 1) no change could be seen after 5 minutes of using a 100 % concentration of miswak extract solution as all patients had positive results post rinsing. On the contrary the 250 % concentration of miswak extract paste, did show some good signs of instant antibacterial effect as 26.7 % of the patients had negative results post oral application. However, the 500 % concentration possessed a stronger antibacterial effect on *S.mutans* as 86.7 % of the patients had negative result post chewing the miswak.

After performing the Chi square test (X^2 test) to compare the statistical significance between the different miswak concentrations. The results showed a lower p-value than 0.05 which indicates a strong evidence against the null hypothesis and therefore we reject the null hypothesis which states; "very high concentrations of miswak extract solution has no natural antibacterial effect against *S.mutans"*.

Bhat. P, Kumar. A, Sarkar. S, (2012) conducted a clinical study using participant's saliva and measuring the effect of miswak extract, tooth brush and normal saline on *S.mutans*. Thirty dental subjects aged 18-25 years were included in the study. For this study 50% of miswak extract was used. The saliva samples were analysed for the presence of *S.mutans* by serial dilution technique in Mitis salvarius agar plates. Results showed that miswak extract had a very significant detrimental effect on *S.mutans* at the tested conditions as well as a significant reduction of microbial counts as compared to toothbrush and saline in the present study. This study proved the potential beneficial effect of miswak extract in oral hygiene practice. [17]

Goyal. D, Sharma. S, Mahmood. A. (2013) examined the effect of plant phenols and aqueous extracts of certain traditionally used chewing sticks (S.persica) for prevention of dental caries on hydrolytic activity of dextransucrase on *S.mutans.* The aqueous extracts of chewing sticks produced 35-40% inhibition of dextransucrase activity at 5 mg phenol concentration. The enzyme inhibition by plant extract was maximum at pH 5-6. [4]

Rasouli. A, Rezaei. A, Mohseni. H, Yaghoobee. S, Khorsand. A, Kadkhoda. Z, Moosavi. M, Rokn. R, (2014) assessed the antimicrobial activities of methanolic extract of miswak on isolated strains from the oral fluid on 50 females university students (21.4 ± 1 year). Their un-stimulated saliva samples were obtained in sterile tubes. Strains isolated from saliva was investigated using agar disc diffusion and microdilution methods. The results showed effect of methanolic extract of S.persica was effective on growth inhibition of all strains. It was significantly more effective on gram positive bacteria than gram negative ones. [18]

Naseem S, Hashmi K, Fasih F, Sharafat S, Khanani R. (2014) made a cross-sectional study involving 100 health care workers. Oral swabs were taken and microorganisms were identified by standard bacteriological methods. Test material included four different types of miswaks. These miswaks were tested against *S.mutans* by agar diffusion method. Inhibition zone was measured after 24 hrs of incubation at 37℃. Among the miswaks used, root of the peelu tree exhibited strong antimicrobial effect against *S.mutans*. They concluded that miswak taken from the root of peely tree exhibited antimicrobial activity against *S.mutans* and could be a good oral hygiene tool in combating dental caries. [19]

Al.Dabbagh.S, Qasim.H, Al-Dersi. N, (2016) conducted a clinical study on 40 students randomly allocated into four groups. They were instructed to use miswak toothpaste, miswak mouthwash and ordinary toothpaste with water or with normal saline. Salivary samples were collected at 3-time intervals: before immediately after use and after 2 weeks of use. The effect of *S.mutans* was evaluated using caries risk test. The results showed miswak wash to have significant reduction effect on both bacteria immediately and after 2 weeks of use. Miswak paste showed a significant decrease only after 2 weeks of use. [20]

The five studies mentioned above indicate that there is an antibacterial effect between miswak extract solutions and gram positive bacteria, specifically *S.mutans* that would either reduce microbial count or inhibit growth. These findings strengthen the validity of our hypothesis, as this study have proved that miswak extract solution does provide a greater antibacterial effect at higher concentration.

Abdulbasit. I, Al-sieni. I, (2014) collected, dried and extracted miswak with either methanol or warm water and the obtained extracts were assessed for their antibacterial activity against 5 different genera of bacteira (including *S.mutans*) using agar well diffusion method. The obtained extracts exhibited considerable inhibitory effects against all the tested bacteria with various degrees of growth inhibition. It was shown that methanol extract was more effective compared to water extracts. *S.persica* showed moderate to high inhibitory activity on pathogenic bacteria with no toxicity and can be used traditionally as alternative medicine. [21]

Siddeeqh. S, Parida. A, Jose. M, Pai. V, (2016), conducted a prospective study using twigs of miswak and alcoholic and aqueous extracts were prepared. The antimicrobial properties of the extract were tested against common oral pathogens such as *S.mutans*, using agar well diffusion method and two fold broth dilution method. The tests showed no significant results with water extracts except some minimum inhibitory effect against *S.mutans.* However, a relatively significant inhibitory effect with respect to alcoholic extract of miswak. In conclusion; the alcoholic extracts have a potential beneficial effect against dental caries and periodontitis. [22]

Both studies mentioned above confirms that H_2O extracted miswak (which is used in our experiment) possess weaker antibacterial effect and could be the reason why a very high concentration was required to show an instant effect on the count of *S.mutans*. If we used alcoholic extracted miswak instead of H_2O extracted miswak, we probably would require less concentration than the ones used in our study to achieve instantly similar results. So far, there has been no other studies that prove otherwise.

Conclusion

As the miswak extract concentration increases, the effectiveness of the antibacterial activity against *S.mutans* increases. Therefore, high concentrations of H_2O extracted miswak can be used to instantly reduce *S.mutans* count or potentially lower concentration over a period of time. Miswak could be a natural substance used in dentifrices and other dental products to reduce *S.mutans* count, given its favorable effect on oral health, low cost, ready availability and simplicity of use.

It is known from studies that mothers with high caries risk can pass *S.mutans* to their infants, which in turn will predispose the infant to a high caries risk. [20] This phenomenon have a potential to be reduced or controlled by educating the society and the mothers about miswak and the benefits of it as well as recommend them to use it.

Acknowledgement

Researcher/author is extremely grateful and would like to thank the dedicated support of his supervisor (Renata Šadzevičienė). The appreciation should also be extended to the department of microbiology, Lithuanian University of Health Sciences, for providing the researcher with professional advice and suggestions.

Ethical statement

This study was approved by the head of the Bioethical Centre, Lithuanian University of Health Sciences following the guidelines of the Declaration of Helsinki and Tokyo for Humans. Reference number; 3EC-OF-73. Informed consent were obtained from all eligible participants prior to the start of the study.

Conflict of interest

There is no conflict of interest declared.

Limitations

The only restriction in this study is that the "Saliva Check Mutans" test devices from "GC America" only shows positive or negative results and does not give us a range of options like other test device such as "*Dentocult SM". This in turn does not provide us with necessary information such as; what is the exact amount of *S.mutans* count in the oral cavity of the volunteering patient pre and post investigation.

Practical recommendations

It is recommended to store the kit in a cool dark place away from high temperatures and direct sunlight. Inaccurate results may occur, if stored at 0℃ or below, or at high temperatures for a long time. Also post opening the foil bag, the test kits have to be used immediately. The shelf life is two and half years from date of manufacture. [15]

Although, this experimental research inflict no potential risk or damage to the patient throughout the process as we are using sterile water and a pure natural substance that has proven to be harmless, even if digested, there is some caution that should be taken, which include: [15]

1. Eye contact with reagent #1 or #2 should be avoided at all times. In case of eye contact, flush with copious amounts of water and seek medical attention immediately.
2. Skin contact with reagent #1 or #2 should be avoided at all times. In case of skin contact, flush with water immediately.
3. The disposal of all the components should be done according the local regulations.
4. Reagent #1 is alkaline and reagent #2 is acidic. Therefore, these liquids should be mixed together and flushed with water when disposed.
5. It clearly states, "Single use only for all components, do not reuse". [15]

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23. Sadek, Moussa. Photograph; "Figure1-13". 2017. JPG File.

Appendix A (Certificate of analysis of miswak extract powder used)

We Bring Natural Ingredient From Nature

Shaanxi Guanjie Technology Co., Ltd.

Certificate of Analysis

Product Name	Miswak Extract	**Manufacture Date**	Jan. 05, 2016
Batch No.	MI160105	**Certificate Date**	Jan. 07, 2016
Batch Quantity	100kgs	**Expiration Date**	Jan. 04, 2018
Storage Condition	Store in cool & dry place, Keep away from strong light and heat.		

Item	Specification	Result	Method
Organoleptic Data			
Appearance	Brown fine powder	Conform	GJ-QCS-1008
Odor	Characteristic	Conform	GB/T 5492-2008
Taste	Characteristic	Conform	GB/T 5492-2008
Process Data			
Method of Processing	Extraction	Conform	/
Solvent(s) Used	Water	Conform	/
Drying Method	Spray drying	Conform	/
Extract rate	10:1	Conform	/
Physical Characteristics			
Particle Size (80 mesh)	100.0%pass 80mesh	Conform	GB/T 5507-2008
Loss on Drying	<5.0%	3.66%	GB/T 14769-1993
Ash Content	<5.0%	2.21%	AOAC 942.05, 18th
Solvent Residue	None	Conform	GJ-QCS-1007
Heavy Metals			
Total Heavy Metals	<10 ppm	Conform	USP <231>, method II
As	<1.0 ppm	Conform	AOAC 986.15, 18th
Pb	<1.0 ppm	Conform	AOAC 986.15, 18th
Hg	<0.1 ppm	Conform	AOAC 971.21, 18th
Cd	<0.1 ppm	Conform	AOAC 986.15, 18th
Pesticide Residue			
666	<0.2ppm	Conform	GB/T5009.19-1996
DDT	<0.2ppm	Conform	GB/T5009.19-1996
Microbiology			
Total Plate Count	<1,000cfu/g	87cfu/g	AOAC 990.12, 18th
Yeast & Mold	< 100cfu/g	11cfu/g	FDA (BAM) Chapter 18, 8th Ed.
E. Coli	Negative/1g	Negative/1g	AOAC 997.11, 18th
Salmonella	Negative/10g	Negative/10g	FDA (BAM) Chapter 5, 8th Ed.
S. aureus	Negative/1g	Negative/1g	AOAC 997.11, 18th

Conclusion: Complies with specification

Tested by: Liu gang **Date:** 2016.01.07

Approved by: Susan Sun, Ph.D **Date:** 2016.01.07

Tel: 0086-29-88253272 FAX:0086-29-68740290 Http://en.gybiotech.com/
Add: 6th floor, 2nd building, Xijing NO.3, XiJing industrial park , DianZi western street, Xi'an, Shaanxi, China

Appendix B (Ethical paper)

LIETUVOS SVEIKATOS MOKSLŲ UNIVERSITETAS

BIOETIKOS CENTRAS

Kodas 302536989, Tilžės g. 18, LT-47181, Kaunas, tel.: (8 37) 327233, www.lsmuni.lt., el.p.: sochumkatedra@lsmuni.lt

Medicinos akademijos (MA) 2017-03-14 Nr. BEC-OF-75
Studijų programa – ODONTOLOGIJA
V k. stud. Moussa Sadek

DĖL PRITARIMO TYRIMUI

LSMU Bioetikos centras, įvertinęs (MA) studijų programos – ODONTOLOGIJA V k. stud. Moussa Sadek (mokslinio darbo vadovas: doc. Renata Šadzevičienė, LSMUL KK Dantų ir burnos ligų klinika) mokslinio-tiriamojo darbo temos: „Miswak ekstrakto baktericidinis poveikis Streptoccocus mutans“ tiriamojo darbo anotaciją, tiriamojo asmens informavimo formą, tiriamojo asmens sutikimo formą ir anketą, kurie leidžia spręsti, jog planuojamame tyrime neturėtų būti pažeistos tiriamojo teisės, todėl šiam tyrimui pritariama.

Bioetikos centro vadovas dr. Eimantas Peičius

Appendix C (Consent form concerning master thesis experimental study)

Experimental Purpose & Procedure

The purpose of this experiment is to prove that miswak has a natural antibacterial effect against the cariogenic bacteria *Streptococcus mutans*. The experiment consist of two parts during which the patient will be involved, first part:

1. The patient is asked to rinse his/her mouth for 5 minutes with sterile water.
2. 5 minutes is given to the patient for the environment of the oral cavity to re-establish its niche.
3. The patient is then asked to chew on the paraffin gum for 1 minute to stimulate the secretion of the saliva.
4. The stimulated saliva sample is then collected into a mixing container.

Second part:

1. The patient is asked to rinse his/her mouth for 5 minutes with a specific concentration of miswak extract in sterile water.

2. 5 minutes is given to the patient for the environment of the oral cavity to re-establish its niche.

3. The patient is then asked to chew on the paraffin gum for 1 minute to stimulate the secretion of the saliva.

4. The stimulated saliva sample is then collected into a mixing container.

Note: The patient will be contacted few days after the experiment to rule out any side effect caused by the mouthwash. Furthermore, none of the tasks is a test of your personal oral hygiene discipline nor to test any of your knowledge and/or intelligence.

Confidentiality

The only data that will be recorded will be the positive or the negative results shown on the test device. All data will be coded so that your anonymity will be protected in any research papers and presentations that result from this work.

Finding out about the results

If interested, you can find out the result of the study by contacting the researcher (Moussa Sadek), after date: 01/05/2017. His phone number: 0037068918474 and/or on his email address: M93@live.se.

Record of consent

Your signature below indicated that you have understood the information about this (effect of miswak extract on *Streptococcus mutans*) in-vivo study and consent to your participation. The participation is voluntary and you may refuse participate or withdraw from the study at any time with no penalty. This does not waive your legal rights. If you have further questions related to this research, please contact the researcher.

…… ……

Participants signature Date

…… …….

Researchers signature Date

Appendix D (Medical and dental health questionnaire)

The following information is required to enable us to evaluate and assess the eligibility of your participation in this study (Effect of miswak extract on *Streptococcus mutans*) conducted by Moussa Sadek. Failure in filling this form is classed as ineligible and therefore would not be allowed to participate in this study.

Medical condition

Do you think there is a health obstacle which prevents you from participating in this study?

…….

Would you like to tell us something about your health problems?

…….

Are you being treated for any medical condition at the present or in the past year?

…….

Are you taking any medications, non-prescription drugs or herbal supplements of any kind?

……

Do you have any allergies?

…….

Are you pregnant?

…….

Dental condition

Last dental visit?

……..

What was done at the visit?

…….

Do you have any oral health problems you would like to inform us about?

……

Patient certification and consent

The undersigned, certify that all the above medical and dental information is correct to the best of my knowledge and that I have not omitted any pertinent information. I agree to participate in the experiment and will follow any instruction being told.

……. …….

Participants signature Date

……. …….

Researcher signature Date

CHAPTER V
ANNEXES

Annex No. 1

INDIVIDUAL DEVELOPMENT PLAN FOR THE MASTER'S THESIS

Graduate student _____,

of the year _____, and the group _____ of the integrated study programme of Odontology

Duration of studies from _____till_____

Supervisor _____

MT title: _____

_____

MT annotation:

_____
_____
_____
_____
_____

Aim of the work:

_____
_____

Tasks of the work

_____
_____
_____
_____

Schedule of the works

No.	Description of MT task	Performance deadline	Done/not done (supervisor's evaluation and signature)

Graduate student's name, surname and signature _____

Supervisor's name, surname and signature _____