Tufts University

School of Dental Medicine

Benefits of Thymoquinone, a *Nigella Sativa* Extract in Preventing Dental Caries Initiation and Improving Gingival Health

Thesis submitted in partial fulfillment of the requirement for the degree of Master of Science

Hanadi A. Al-Wafi, B.D.S.

[Department of Public Health and Community Service]

October 2013

Thesis Committee

Principal Investigator:

Driss Zoukhri, Ph.D.

Associate Professor, Department of Diagnosis and Health Promotion, Tufts University School of Dental Medicine.

Committee Members:

Paul C. Stark, M.S., Sc.D.

Professor, Department of Public Health and Community Service, Director of Advanced and Graduate Education, Tufts University School of Dental Medicine.

Cheen Y. Loo, D.M.D., M.P.H., Ph.D.

Associate Professor, Director of the Postdoctoral Program in Pediatric Dentistry, Tufts University School of Dental Medicine.

Soad Shaker Ali, Ph.D.

Professor of histology and cytology, Faculty of medicine, Anatomy Department, King Abdulaziz University in KSA.

Yong Hur, D.M.D., M.S.

Assistant Professor, Department of Periodontology, Tufts University School of Dental Medicine.

Ahmad Shaker Ali, Ph.D.

Associate Professor, Department of Pharmacology, Faculty of Medicine, King Abdulaziz University, Jeddah, KSA.

ABSTRACT

Background: Thymoquinone (TQ) has a variety of pharmacologic properties, including antibacterial, anti-inflammatory, and anti-oxidative activities. Because of these properties, TQ could play an important role in preventing caries initiation and gingivitis. The aim of this study is to evaluate the potential preventive effect of TQ on caries initiation through inhibition of plaque formation in a rat experimental model.

Materials and methods: Sixteen, germ-free, 21-day old Fischer rats were divided into four experimental groups, four per group: G1 – negative control fed standard normal diet and received no treatment; G2 – positive control fed caries inducing diet without treatment; G3 – TQ (10 mg/kg body weight) in oral gel; G4 – TQ (10 mg/kg body weight) in drinking water. In order to induce experimental caries and gingivitis, pups in G2, G3 and G4 were fed a sucrose rich diet and challenged with *S. mutans*. The following parameters were measured: caries score (CS), plaque index (PI), bleeding on probing (BOP), and subgingival bacterial count (BC). Caries fissures and plaque scores were assessed using caries and plaque indicator solutions. Mandibular specimens were examined histologically to determine the degree of inflammation. **Result:** Rats treated with TQ in drinking water or as an oral gel showed lower, statistically significant differences in all the measured parameters: CS ($p=0.02$), PI ($P=0.01$), BOP ($P=0.01$), and BC ($p=0.02$) when compared to both control groups. Furthermore, compared to both the negative and positive control groups, mandibular specimens from the TQ treated groups showed no signs of inflammation.

Conclusion: The present study showed that administration of TQ either as an oral gel or mixed with the drinking water diminished caries and plaque formation in a rat gingivitis model.

DEDICATION

To my great supportive family: my mom Samirah, my dad Abdullah, my husband Tariq and my two eyes Lara and Lillian.

ACKNOWLEDGMENTS

I have had the good fortune of being a student at Tufts University School of Dental Medicine and being able to know and work with a great team.

I would like to thank my husband, Tariq Madkhali, for the unconditional love, help and support he has given me throughout this entire journey which keeps me going on and for giving me the opportunity to be here to fulfill my dream. To my parents, Samirah and Abdullah, my role models, for their continuous support to pursue my goals in all my studies and for always believing in me. To my brothers Abdulrahim, Abdulrhman and my sisters Hala and Heba: Thank you for always being there for me and for your moral support.

Dr. Driss Zoukhri has always been my model. I had the honor of being one of his students for the last two years. Throughout this project I have been blessed with his excellence in science, patience, advice, kindness, encouragement, talent and knowledge. I would not have been able to do this project without his tireless support, leadership and advice. I would also like to express my gratitude to Dr. Paul Stark for his warm reception and assistance with the statistical analyses. Dr. Soad Ali for her invaluable guidance in research, generous support, continuous help, encouragement throughout my project and for help with the histological study and great advice. Dr. Ahmad Ali for his help in preparing the thymoquinone oral gel in his lab and for his cooperation during the study execution. Drs. Loo, Hur and Horsono for providing me with the appropriate references, helpful discussions and advice throughout this study. Ms. Claire Kublin for her greatly appreciated help and effort with animal handling and working in the laboratory. Dr.layla Abuljadayel for her great help. My neighbors, colleagues and friends for your help. The Saudi Arabian Cultural Mission (SACM) for financial support.

Table of Contents

List of Tables

List of Figures

Appendices

Benefits of Thymoquinone, a *Nigella Sativa* Extract, in Preventing Dental Caries Initiation and Improving Gingival Health

I. Introduction

1. Dental Caries

Dental caries is a multifactorial disease, which is characterized by local destruction of the tooth. Caries, starting as small-demineralized areas, are caused by bacterial fermentation of dietary carbohydrates to lactic acid, within the dental plaque.[1] It is a progressive, irreversible microbial disease affecting the calcified tissues of the teeth characterized by demineralization of the inorganic portion and destruction of the organic portion of the tooth (Figure1). Almost all people are affected by dental caries, only the severity differs.[2]

Dental caries have several etiologies. The five most important risk factors for dental caries are: (1) dental plaque, (2) cariogenic diet, (3) susceptibility of dental tissues to decalcification, (4) length of exposure to the pathogenic factor on teeth and (5) saliva and its characteristics and composition.[3] The primary etiologic agent of coronal caries and root caries are the *M. streptococci*, particularly *Streptococcus mutans* and *Streptococcus sobrinus.*[4] These two microorganisms are considered major pathogens in the initiation and progression of dental caries.[5] Secondarily implicated is the *Lactobacillus* species and perhaps non- *M.streptococci* in coronal caries, particularly the acid-tolerant strains: *Streptococcus sanguis*, *Streptococcus gordonii*, and *Streptococcus oralis*. The ability of *S. mutans* to produce extracellular polysaccharides, mainly glucans, has been recognized as a critical factor in the pathogenesis of dental caries.[4] Glucans, synthesized from dietary sucrose by glucosyltransferases, are essential for *S. mutans* and other oral microorganisms to adhere and accumulate on the tooth surface, leading to the formation of cariogenic biofilm communities.[5] Adherence to the tooth surface by

S. mutans is an important step in initiation of dental caries and is considered to be the predominant one. Caries go through several stages in their development: phosphate metabolism disorders in dental tissue; calcium metabolism disorder; abnormal glycemia; declining ascorbic acid level and activation of the insular apparatus followed by its exhaustion. Within the multifactorial context, sugars cause dental caries.[6] Furthermore, teeth morphology, enamel development defects or lack of topical fluoride and whether the teeth are permanent or deciduous will affect the caries attack on the enamel. The ability of saliva to constantly deliver fluoride to the tooth surface makes salivary fluoride an important player in caries protection largely by promoting remineralization and reducing demineralization.[7] It has been demonstrated, both experimentally and clinically, that caries-inducing factors are particularly dangerous in childhood when carbohydrate metabolism is disturbed, especially due to exposure to refined sugars.[8]

2. Prevalence of Caries

Dental caries is one of the most prevalent diseases in humans worldwide. When different stages of the disease are taken into account, from the initial to the clinically manifest lesion, very few individuals are unaffected. In most industrialized countries, 60%–90% of school-aged children are affected. The prevalence among adults is even higher and in most countries the disease affects nearly 100% of the population.[9] Worldwide, most children and an estimated 90% of adults have experienced caries, with the disease being most prevalent in Latin America, the Middle East, and South Asia.[10] In the United States, dental caries is the most common contagious childhood disease from age 5 to age 17 and is considered five times more prevalent than asthma

and seven times more than hay fever.[11] Over 50% of 5-9 year-old children have at least one caries or restoration, and that proportion increases to 78% among 17 year olds.[12] In China the overall weighted prevalence of caries is 84% for children.[13] One study involving 5-year-old children at the tertiary hospitals in Saudi Arabia showed that 91.9% of medically compromised and 84 % of healthy children had evidence of caries.[14] The severity and the incidence of caries in primary and permanent teeth has become a major problem, which deserves more attention as the percentage in the developing countries increases with the degree of urbanization.

3. Dental Plaque

Dental plaque was the first biofilm defined as the diverse community of microorganisms found on the tooth surface. It is also defined as a layer of matrix enclosed bacteria attached to a surface.[15] It is the most complex biofilm, as it is home to at least some three hundred species of organisms able to communicate with each other via signal molecules within the biofilm. This dental biofilm is comprised of mushroom–shaped micro colonies of bacteria protruding from the tooth surface separated by water channels.[16] The metabolic processes of the plaque bacteria cause a drop in the pH value in the plaque; as a result, the underlying enamel may begin to dissolve and, eventually, caries may develop. In addition, a build-up of plaque may lead to inflamed gums, known as gingivitis. If left untreated, gingivitis may progress to periodontitis.

There are three steps involved in dental plaque formation. The first step is when salivary molecules are adsorbed to the enamel as soon as the tooth has been cleaned. The second step is bacterial interaction with this acquired pellicle via several specific cell to surface interactions.[17] During the third step, other bacterial species such as *S. mutans* adhere to the primary colonizers

by cell-to-cell interactions. Subsequent bacterial growth on the tooth surface leads to formation of dental plaque biofilm.[18]

Dental plaque plays an etiological role for major diseases to the oral cavity including dental caries, periodontitis, and gingivitis.[19] Development of dental plaque usually leads to an increased level of caries inducing bacteria in the oral cavity, e.g. *S. mutans* and *Lactobacillus spp.*[20]

Control of dental plaque is generally carried out through mechanical and or chemical methods. Although the mechanical methods (toothbrush and dental floss) are considered efficient, they are not sufficient in certain cases such as compromised patient.[21] Preventive effect of plaque-control on gingivitis and periodontitis is clinically accepted but there is a question as to whether or not the development of caries can be avoided or reduced by improved oral hygiene.[22] These questions regarding the effectiveness of improved oral hygiene offered us with several reasons to initiate the research into additional methods of plaque control test to test its effect on gingivitis and dental caries.

Furthermore, certain patients may not be willing or able to perform adequate mechanical plaque removal on a regular basis. Such patients could benefit from anti-plaque agents as adjuncts to mechanical removal. Topical antimicrobials in dental products have four general mechanisms of action; in fact, they can decrease the rate of new plaque accumulation, decrease or remove existing plaque, suppress the growth of pathogenic micro flora, or inhibit the production of virulence factors.[23]

4. Dental caries prevention

Over the past decades, the prevention of oral diseases has become an essential component of dentistry and dental research.[24] Despite the great improvements in the field of prophylaxis, caries remains a challenging disease that highlights the need for reasonable and worldwide accessible preventive methods.[25] Public health strategies have been focused on water fluoridation, topical fluoride application, and the use of fluoride rinses.[12] Despite the indisputable role of fluoride in the prevention of caries, it has not eliminated dental caries and many communities are not exposed to optimal quantities of fluoride.[26] School oral health educational programs emphasize proper tooth brushing with fluoride dentifrice and flossing, proper diet and regular dental office visits.[12] Since dental plaque is implicated in the etiology of dental caries, gingivitis, and periodontitis, the removal of plaque is thought to play a key role in the prevention of these diseases.[27] Furthermore, since an unbalanced carbohydrates diet with high levels of sucrose results in an increased production of acid in the dental plaque [28], a subsequent reduction in the levels of caries-associated bacterial species in the dental place has been show to prevent caries.[29]

Good dental hygiene has been shown to lead to significant decreases in dental plaque index scores. In addition, other materials that could supplement or increase the efficacy of good dental hygiene are being explored; such materials can be placed between the teeth or in the pits and fissures of the teeth. For example, the two materials, glass ionomer and resin-based fissure sealants, appear to be equally suitable as fissure sealant materials.[30]

5. Gingivitis

Gingivitis is an inflammation of the gingiva resulting from gingival bacteria and

bacterial byproducts.[31] It is well established that not all gingivitis will progress to periodontitis, and while it has not been characterized as a public oral health problem, gingivitis is the most prevalent periodontal disease in childhood and adolescence.[32] Studies examining the prevalence of gingivitis in children have shown different results, but generally the prevalence of gingivitis has been >80 percent.[33] Gingival disease is further characterized into plaque-induced and no plaque-induced categories.[34] The most common form of gingivitis is in response to bacterial biofilm is the plaque-induced gingivitis (Figure 2).[27] Plaque-induced gingivitis is characterized by the presence of gingival inflammation without detectable loss of bone or clinical attachment and is common in children.[35] As biofilm accumulates, gingivitis develops over a period of several days in the presence of periodontal bacteria.[27] This accumulation results in an inflammatory reaction, with clinical signs of redness, edema, gingival bleeding, and sometimes pain.[32] Gingivitis may be a nonspecific bacterial infection dependent on the level of plaque present.[36]

It is well established that supragingival plaque is the cause of gingivitis and plays a primary role in the initiation of periodontitis[37], which has been linked to various systemic conditions[38] (e.g., cardiovascular diseases, ischemic stroke, diabetes mellitus), as well as tooth loss. Therefore, the prevention of gingivitis by daily and effective supragingival plaque control is necessary to arrest its progression into periodontitis.[39]

The clinical assessment of the gingival inflammatory response to plaque accumulation relies on several parameters.[40] One of them is the visually assessment which includes the extent and severity of changes in the physical status of the gingiva, such as changes in color, in surface anatomy (contour), and in bleeding tendency. Diagnosis of periodontal disease relies on the

clinical evaluation including bleeding on probing, clinical attachment level, probing depth and radiographic examination.[41] Several indices have been proposed for the clinical evaluation of gingival inflammation, including the papilla, marginal, attached (PMA) index,[42] the papillary bleeding index (PBI)[43], and the gingival index (GI).[44] The visually assessed signs of inflammation correspond to histopathological tissue changes.[45, 46] The various indices have been shown to each have its own limitations. Methods and indices used to quantify the accumulated dental bacterial plaque like the plaque index [47], have been necessary in the assessment of the gingival response to the plaque. Use of these indices has helped demonstrate the relationship between the extent of plaque deposits and the severity of gingivitis.[48, 49]

Good oral hygiene is crucial to maintaining good oral health so Along with receiving professional care, such as regular dental prophylaxis, patients can choose from various oral hygiene products aimed at controlling plaque-induced gingivitis. Studies have demonstrated that good oral hygiene practices, including tooth brushing and flossing, using proper mouth rinses, and receiving periodic dental prophylaxis, can maintain gingival health.[50] However, while it may be possible, under controlled conditions, to remove most plaque with a variety of mechanical oral hygiene aids, many patients lack the motivation or skill to attain and maintain a plaque-free state for significant periods of time.

6. Nigella Sativa

Nigella sativa is an annual herbaceous plant belonging to *Ranunculaceae*. It is known under many names such as black cumin (English), black-caraway seeds (U.S.A.), *habba-tu sawda* (Arabic), *kalonji* (Urdu and Hindi), *krishnajirika* (Sanskrit), *kalajira* (Bangali) and

shonaiz (Persian).[51] Nigella sativa and its oil have been used for over thousands of years both as food additives and as natural remedies for many ailments, including asthma, hypertension, diabetes, cough, bronchitis, headache, eczema, dizziness, and influenza.[52] The seeds or its oil are used as a carminative, diuretic, lactagogue and vermifuge.[53]

Several active components have been isolated from *Nigella sativa*, including thymoquinone, thymohydroquinone, dithymoquinone, thymol, carvacrol, nigellimine-N-oxide, nigellicine, nigellidine, and alphahederin (Figure 3).[54] The antibacterial effect of the phenolic fraction of Nigella sativa oil was first reported in 1965.[55] Crude extracts of *Nigella sativa* were reported to have a promising effect on multi-drug–resistant organisms, including gram-positive and gram-negative bacteria.[56] With regards to the safety of *Nigella sativa*, the seeds' powder did not produce any toxic effects at very high doses (28 g/kg) when given orally to rabbits. Its oil was also very safe when given orally to rats (LD50 of 28.8 mg/kg).[57] Administration of either the seed extract or its oil has been shown not to induce significant adverse effects on liver or kidney functions.[52]

Thymoquinone (2-Isopropyl-5-methylbenzo- 1,4-quinone) is the bioactive constituent of the volatile oil of Nigella sativa. It has been studied for its activities in both in vitro and in vivo models since the first extraction in 1960s. Thymoquinone has a potent anti-oxidant effects through its superoxide scavenging ability is considered to be one of the most important properties.[58] It was also tested for its effects on bacterial adherence to epithelial tissues and was found to prevent the attachment of bacteria to host tissues.[59] It showed a high potency to prevent bacterial biofilm formation by decreasing the metabolic oxidative activity of the cells. Moreover, it has a potent growth-inhibiting activity against gram-positive bacteria with minimum inhibitory

concentration (MIC, which is defined as the concentration that completely inhibited visible cell growth during a 24 hrs. incubation period at 37°C) ranging from 8 to 64 μg/mL.[60, 61] In addition thymoquinone induced a selective antimicrobial activity against oral pathogens; its synergistic effect resulted in at least a 4-fold potentiation of the tested antibiotics and antiseptics.[62] It was shown to have anticariogenic activity against *S. mutans*, and this may be a promising approach for the prophylaxis of dental caries. Thymoquinone inhibits dental plaque formation at a concentration of less than 100 μg/mL (IC_{50} =20 μg/mL).[63] The antibacterial activity of thymoquinone and its biofilm inhibition potencies were investigated on 11 human pathogenic bacteria. This study showed that thymoquinone exhibited a significant bactericidal activity against various human pathogenic bacteria especially Gram-positive *cocci* (*Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* CIP 106510). Additionally, thymoquinone prevented cell adhesion to glass slide surfaces.[61]

Thymoquinone has potent anti-oxidant effects through its superoxide scavenging ability, which is considered to be one of the most important properties.[58] Oxidative stress is a condition that results from a lack of equilibrium between the intracellular production of free radicals and the antioxidants that regulate oxidative reactions by inhibiting, delaying or hampering the oxidation of substances. This disequilibrium between pro-oxidants and antioxidants can be disrupted by an increase in the amount of free radicals, or by a reduction in the amount of anti-oxidative substances, and can result in lipid per- oxidation, DNA damage and the degradation of cellular proteins. It was demonstrated that a series of inflammatory diseases, including diabetes mellitus, atherosclerosis, and chronic inflammatory lung disease, are related to oxidative stress.[22]

Cancer has emerged as one of the top diseases in many countries, with its worldwide incidence rate increasing annually.[67] A cure for this disease is desperately needed as the cost of treatment is not cheap and the complications from this disease invariably lead to fatal outcomes. A number of studies have demonstrated the anticancer effect of thymoquinone, an active ingredient from *Nigella sativa*, in many different types of malignancies.[68].

Furthermore, it prevented periodontal inflammation and decreased alveolar bone loss in experimental periodontitis in rats.[64] Another study showed that thymoquinone can accelerate new bone formation in the rapid maxillary expansion procedure, which will help in future orthodontic treatment.[65] In summary, thymoquinone is a relatively safe compound, particularly when given orally to rats and with the absence of side effects in humans.[52, 66]

Natural products have been used for thousands of years in folk medicine for various purposes worldwide and any plant products have been successfully incorporated into dentifrice or mouthwash in many countries.[70] While the antimicrobial effects of *Nigella sativa* extract were assessed in several studies, to the best of our knowledge, none of these studies tested the potential power of *Nigella sativa* to reduce the effect of dental plaque biofilm, which plays a major role in dental caries and gingival inflammation. Therefore, the objective of this study is to investigate the inhibitory effect of *Nigella sativa* extracts on dental plaque formation to control dental caries initiation and gingival inflammation.

II. Specific Aim and Hypothesis:

The aim of the study is to evaluate the antibacterial and anti-inflammatory activities of thymoquinone, the active ingredient of *Nigella sativa*, against plaque and cariogenic bacteria and to determine if it will prevent the initiation of dental caries and improve overall gingival health.

We hypothesized that thymoquinone will prevent caries initiation through inhibition of bacterial attachment and prevent gingivitis by preventing plaque formation.

III. Materials and Methods

This is a prospective randomized animal study, and was conducted at Tufts University School of Dental Medicine. Tufts Medical Center Institutional Animal Care and Use Committee (IACUC) approved all experiments. In this study we initiated caries by orally challenging the rats with *S. mutans* and caries inducing diet to test thymoquinone effect on caries and plaque in a rat's gingivitis model.

Experimental Design

A. Sample Size Calculation

A sample size of sixteen rats (4 per group) would provide an 80% power to detect a 1-unit difference in each outcome (plaque index, bleeding on probing, caries score and bacteria sample collection), between the four groups, assuming a common standard deviation of 0.75 units (nQuery Advisor, 7.0).

B. Animals

Sixteen 21-day old, male Fisher rats were purchased from Taconic (Hudson, NY). Animals were housed in individual cages under controlled temperature of 72.5°F and constant humidity of 27%, under 12-hour intervals of light/dark, and were fed *ad libitum*. Animals were randomized into two groups. The first group was composed of 4 rats to serve as a negative control group that was fed from age 21 days until age 60 days, normal standard diet and was not subjected to microbial challenge. The second group composed of 12 rats that were fed a high

sucrose diet in order to promote caries formation and challenged with orally applied *S. mutans*. The tested groups were exposed to thymoquinone from the first day to the end of the experiment (5^{th} week). At weaning BOP was assessed and samples were collected. At baseline (after one week) the same outcomes were measured then rats were orally challenged with *S. mutans* and introduced to caries promoting diet. By the 5^{th} week the outcomes (BOP and sample collection) were measured immediately after the animals were euthanized. The plaque index and the caries risk rating were assessed from the maxillary arch samples after three days from embedding them in the fixative.

The amount of consumed diet and the animals' weight were measured every 3-4 days. (Harlan laboratories, food composition are listed in Appendices A and B).

C. Cariogenic Bacteria

S. mutans bacteria (ATCC® 25175TM) was purchased from ATCC. Bacteria was activated by rehydrating it in 0.5 mL of sterile saline, re-suspending it in 5 mL of brain infused agar broth then incubated overnight at 37 ºC. The suspension was centrifuged for ten minutes, then the pellet was re-suspended in 1 mL sterile saline and applied to the rats' maxillary and mandibular teeth using a Microbiologics KWIK-STIK™ applicator.

D. Thymoquinone Preparations

Thymoquinone crystals were purchased from Sigma Aldrich (St. Louis, MO). Two forms of preparation were used at the beginning of the study: topical gel and intraperitoneal injections.

The protocol was modified after the start of the study where the intraperitoneal injections group was eliminated (due to lethality) and was replaced by a group that received TQ in drinking water.

1. Thymoquinone as a topical gel

All materials used in preparation of the gel are pharmaceutical grade. A plain mucoadhesive oral gel was formulated as a vehicle for medicated gels. A TQ mucoadhesive gels was also prepared as described below.

- **Formulation of mucoadhesive gel**

The plain gel has the following composition (for 10 gm). Hydroxypropylmethycellulose (HPMC) 4 %w/w, probylparaben 0.1 w/w, methyl paraben 0.1w/w, distilled water to 10 gm. All gels (10 g batches) were prepared at room temperature in glass vessels (50 mL) using a stirrer with attached metal blade. Preparation was performed under complete aseptic conditions. The gel was evaluated for pH, viscosity, stability, and microbial limits using appropriate analytical procedures. Gel viscosity was determined by using a Brookfield Cone & Plate Rheometer. The Microbial Limits Test <61> in USP 29 was used to determine the total microbial count and for the presence of yeasts and molds. The active ingredient was determined using HPLC analysis after appropriate extraction procedure. The prepared gels showed appropriate pH, viscosity, and absence of pathogenic microorganism and the homogeneity was acceptable.

- **Preparation of plain gel**

Plain mucoadhesive gel with specified pH was prepared. The pH was adjusted to 7.4 using phosphate buffer. To about 8 g of purified water, all soluble additives were dissolved, and then appropriate amount of the polymers (HPMC) was added slowly and allowed to fully hydrate for at least 3 hours then the weight was adjusted to 10 gm.

- **Preparation of medicated gel (0.2% w/w TQ)**

Twenty mg of TQ was dissolved in propylene glycol (1 mL). The plain gel was then added to the TQ solution in geometric dilution and appropriate mixing was carried out. The weight of the medicated gel was adjusted to 10 gm. These gels were mixed for appropriate time to produce homogenous medicated gels.

2. Thymoquinone in drinking water

TQ was first dissolved in 2 mL of propylene glycol, mixed by vigorous vortexing for 20 seconds, than added into 1L of drinking water. The water was divided equally to two separate plastic water bottles provided with sipper tubes. Each rat was housed in a separate cage and the amount of water consumed was recorded daily. The water was changed 3 times a week.

E. Study Groups

Group 1: rats were fed standard normal diet (control diet) and received no treatment (Negative control, n=4) (Figure 4).

Group 2: rats were fed caries inducing diet and orally challenged with *S. mutans* but received no treatment (Positive control, n=4).

Group 3: rats were fed caries inducing diet and orally challenged with *S. mutans*. Tested with thymoquinone in oral gel applied daily over the teeth and gum (Test group 1, n=4).

Group 4: rats were fed caries inducing diet and orally challenged with *S. mutans*. Tested with thymoquinone in drinking water (Test group 2, n=4).

F. Measurements

The outcomes (BOP and sample bacterial collection) were measured when the rats were anesthetized with isoflurane for 3 minutes. At weaning (1st day), at baseline (one week) and at the end of the experiment (5th week).

1. Microbiological Parameters

a. Subgingival Plaque Collection

The subgingival bacteria were collected from one site (buccal site) of each of the four experimental teeth. After isolation of the sample sites with cotton rolls, subgingival plaque samples were collected using the paper point technique.[71] Sterile paper points (ISO #40 taper 0.02 mm/mm) were inserted gently using sterile dental tweezers into gingival sulcus until tissue resistance. After 20 seconds, paper points were removed carefully without touching the adjacent tissues and placed into a sterile container containing brain heart infusion for microbiological examination. All samples were taken by the same investigator in order to standardize the sampling procedure.

b. Subgingival plaque samples processing

Samples were processed immediately after collection. The vial was mixed by vigorous vortexing for 20 seconds. A 2 μL aliquot was inoculated onto blood agar plates, containing pancreatic digest of casein 14.5 g/L, papaic digest of soybean meal 5 g/L, sodium chloride 5 g/L, agar 14 g/L, growth factors 1.5 g/L, and defibrinated sheep blood 5% (Fisher Scientific, Waltham, MA, USA). All plates were incubated in a standard incubator at 37°C for 24 hrs. After incubation, the number of colony forming units per 100 μL (CFU/100 μL) was determined.

2. Periodontal parameters and caries evaluation

a. Bleeding on probing (BOP)

Bleeding on probing was measured from four surfaces (buccal, lingual, mesial, and distal) from each experimental tooth (upper right central incisor, upper left 1st molar, lower left central incisor, and lower right 1st molar), using a sterile probe with 0.45 mm tip diameter (PCPUNC15; Hu Friedy, Chicago, IL, USA). The probe was inserted into the gingival sulcus, until reaching tissue resistance, for 10 seconds, then score as 1 for bleeding and 0 for no bleeding.[59] Then, the percentage of BOP was calculated by adding the number of bleeding sites divided by the number of sites evaluated then multiplying the score by 100 (Figure 5).

b. Caries detection

The presence of caries in the maxillary teeth was evaluated using a caries detector dye. The maxilla was separated carefully, cleaned from all the attached muscles, and then fixed in 10% neutral buffered formalin (pH 7.4) for 24 hours. All maxillary fissures were cleaned and food derbies removed, then all cavity walls and surfaces of samples were stained with caries

detector (Sable, Ultradent Products, Inc) applied for 10 seconds. The samples were rinsed with tap water, dried and viewed under a stereo-microscope (Olympus, SZ16) at 60x power, equipped with a digital camera. Ratings were given from 1 to 28, representing the sum of the carious grooves per rat. Four caries risk rates were assigned: 1-7, 8-14, 15-21 and 22-28 (Figure 6). Two independent investigators performed the caries evaluation and the mean was taken for statistical analyses.

c. Plaque detection

The same samples were used to measure the presence of plaque in the maxillary teeth and was evaluated using a plaque disclosing dye. Samples were used to measure the percentage of plaque by O'Leary Plaque Control Record.[72] Plaque disclosing dye was used in several other studies to test for the presence of plaque. [73, 74] Plaque disclosing dye (Reveal, Henry Schein Inc. Melville, NY) was applied to all surfaces for 10 seconds then rinsed, dried and viewed under a stereo microscope (Olympus, SZ16) at 60x power, equipped with a digital camera. The occlusal surface of each tooth was divided into four surfaces: mesial, distal, buccal and lingual and each surface scored as 1 for presence of plaque or 0 for the absence of plaque. The index is calculated by dividing the number of plaque containing surfaces by the total number of available surfaces * 100 (Figure 7). Two independent investigators performed the plaque evaluation from microscopic pictures of all maxillary teeth in the 4 rats of each group and the mean was taken for statistical analyses. There were small differences in the evaluation between the two investigators after explaining the concept of caries and plaque dye to the independent investigator and all the results were convergent.

3. Histological Studies

The mandibles from each animal were dissected, cleaned, and fixed in 10% neutral buffered formalin (pH 7.4) for 24 hours. Samples were prepared to detect any signs of inflammation and caries. Mandibular arches were decalcified prior to paraffin embedding by placing the samples in the Cal-Rite Decalcifying/Fixation Solution, which is a mixture of formic acid and formaldehyde, allowing simultaneous decalcification and further fixation for three weeks. Then the samples were tested for decalcification using ammonium hydroxide. After decalcification, the specimens were dehydrated in graded alcohol solutions, and embedded in paraffin wax. Serial 6-10 μm thick sections were prepared and stained with hematoxylin and eosin.[75]

4. Data Presentation and Statistical Analyses

The data were tested for normality using the Shapiro-Wilk test prior to further statistical analysis. The data were not normally distributed and therefore were expressed as medians ± IQR (interquantile range). Data were analyzed using SAS software package version 9.2 (SAS Institute, Cary, NC). Differences among the groups were evaluated using Kruskal-Wallis tests (non-parametric test) to test the changes as effect of TQ. The variables are BOP, PI, and caries rating and microbiological cultures. Since the sample size was small and the data not normally distributed, the Mann-Whitney U test (non-parametric test for pairwise comparison) was used. P values < 0.05 were considered to be statistically significant.

IV. Results

Effect of Thymoquinone on Bacterial Count

Bacterial sampling was performed at baseline and after 5 weeks. There were no differences between the groups at baseline (Figure 8). After 5 weeks of consuming a high sucrose diet, samples from animals in the negative as well as the positive control groups (Figure 9). showed high bacterial colonies (Median [IQR]), 12.5 (7.0-21.0) and 30.0 (11.0-43.8), respectively. Bacterial counts in the oral gel treated group were significantly lower (1.0[0.0-4.3]) compared to both the positive (30.0[11.0-43.8]) and negative control (12.5[7.0-21.0]) groups (p=0.03). Similarly, bacterial counts in the group treated with TQ in the drinking water group (0.5[0.0-1.0]) was significantly lower compared to both positive and negative control groups (p=0.03 for both). (Table 1,2). Effect of TQ treatment on subgingival bacteria induced by high sucrose diet consumption and orally applied *S mutans*. *Denotes statistically significant difference compared to the negative control group. #Denotes statistically significant difference compared to the positive control group.

Effect of Thymoquinone on Bleeding on Probing

At baseline, BOP was not detected in any of the tested animals (data not shown), after 5 weeks of consuming high sucrose diet, BOP was seen in both the negative and positive control groups. In contrast, animals that received TQ either as an oral gel or mixed with their drinking water; BOP was absent (Figure 10). BOP was significantly higher in the positive control group (Figure 11), compared to the negative control group: (Median [IQR]), positive group (40.7[37.6-

48.4]) versus (31.1[21.3-35.9]) in the negative control group (P=0.02). Following TQ treatment, BOP in the oral gel group was significantly lower (6.3[0.0-21.9]) compared to both the negative (p=0.04) as well as the positive control group (p=0.02). Similarly, BOP in the group receiving TQ in the drinking water was significantly lower (3.1[0.0-6.3]) compared to both the negative and positive control (p=0.02 for both) groups (Table 3, 4).

Effect of Thymoquinone on Caries Development

(Figure 12) shows unstained representative mandibular teeth removed from each of the experimental groups. The samples from the negative control group and the oral gel TQ treated group show the presence of incipient caries. Samples from the positive control group show changes in teeth morphology and presence of extensive tooth decay. In contrast, samples from the group that received TQ in the drinking water were caries-free. The presence of caries was further evaluated in the maxillary teeth using a commercially available detector dye. As shown in the representative microscopic photographs in (Figure 13), caries were detected in samples from the negative as well as the positive control groups, whereas samples from the TQ treated groups were caries-free. (Figure 14) depicts averaged data collected by two independent investigators. Caries scores were significantly higher in the positive control group (18.5[17.3-20.5]) versus (10.0[7.8-13.8]) in the negative control group (P=0.02). Caries scores in the oral gel treated group l (5.0[0.8-7.0]) were significantly lower compared to both the negative (p=0.04) as well as the positive control groups (p=0.02). Similarly, caries scores in the group that received TQ in the drinking water were significantly lower (0.5[0.0-2.5]) compared to the negative as well as the positive control (p=0.02 for both), (Tables 3,4).

Effect of Thymoquinone on Plaque Score

The plaque score was assessed according to the O'Leary method using the maxillary arches and a commercially available dye.[76] As shown in the representative photographs in (Figure 15), considerable plaque was detected in samples from the negative and positive control groups, whereas samples from the TQ treated groups had minimal (oral gel group) or no detectable (drinking water group) plaque. (Figure 16) shows averaged data collected by two independent investigators. The plaque index in the positive control group (21.5[21.0-23.5]) was significantly higher compared to the negative control group (12.5[11.3-35.9]) (P=0.02). TQ treatment significantly lowered the plaque index. The plaque index in the oral gel treated group (6.0[4.0-8.8]) was significantly lower compared to both the negative as well as the positive control group (p=0.02 for both). Similarly, the group that received TQ in the drinking water had significantly lower plaque index values (4.0[3.3-4.8]) compared to the negative control group and positive control group (p=0.02 for both), (Tables 3, 4).

Effect of Thymoquinone on Body Weight

Body weight was measured every 3 to 4 days for the whole duration of the study. There were no differences in body weight between the different groups either at baseline or after completion of the study (Figure 17).

Histological Findings

Histological examination of mandibular samples confirmed what was observed clinically.

(Figure 18) shows the effect of thymoquinone on the gingiva. The positive control group showed an increase in connective tissue thickness and sub epithelial edema. These changes were not found in samples prepared from the TQ treated groups. These findings confirmed the clinical bleeding on probing test, which correlated with the severity of gingivitis. (Figure 19) shows inflammation present in the periodontal ligaments. The positive control group demonstrated numerous dilated congested vessels and increased cellularity compared to the negative control. In TQ treated groups those changes were less evident especially in animals that received TQ in drinking water. Dental caries were observed histologically as cavitation, loss of cusp tip, and presence of residual debris; while on the other hand, TQ treated groups, the tooth similar more or less to those of the negative control (Figure 20).

V. Discussion

Several natural products are marketed for oral and dental use to satisfy the shift to usage of natural products from pharmaceutical products. These alternative products can be either dental products with natural ingredients or herbal products. Different herbs have been included in dental products such as: bloodroot, carawy, chamomile, echinacea, peppermint, rosemary, thyme, aloe vera, green tea, fennel, ginger, *Salvadora persica* (miswak extract), clove oil, eucalyptus and *Nigella sativa* extracts.

Nigella sativa has long been used in folk medicine to promote good health and to treat diseases. According to recent studies, it was suggested that extracts of *Nigella sativa*, TQ in particular, have many therapeutic effects, including antioxidant[77], anti-inflammatory[78], and antibacterial[62], anticancer.[79] Because numerous studies reported that TQ inhibits bacterial growth and tissue inflammation, we hypothesized that TQ may play a role in preventing caries onset and progression of gingivitis. Therefore, we aimed to explore the effects of oral administration of thymoquinone on oral diseases in an experimental rat model.

In the present study, caries rate was measured using a caries detecting dye, an accepted method for bench studies. Our results showed that caries rate in the TQ treated groups were significantly lower compared to both the negative as well as the positive control groups (not treated with thymoquinone). This result is similar to the *in vitro* study, which found that TQ has an antimicrobial activity against cariogenic bacteria and its growth inhibition effects on human epithelial cell lines.[80] Also, this finding confirmed another *in vitro* study which showed that

Nigella sativa extract has the highest inhibition zone and inhibited the growth of two types of cariogenic bacteria (*Streptococcus mutans* and *Streptococcus miti*).[81]

We also investigated the potential of TQ to counteract the damaging effects of plaque on gingival health by measuring periodontal and microbiological parameters. We found that administration of TQ, both as a gel as well as mixed in drinking water, significantly decreased plaque formation most likely due to the known antimicrobial and biofilm inhibition potencies of TQ which was previously investigated on 11 human pathogenic bacteria. TQ exhibited a significant bactericidal activity against various human pathogenic bacteria especially gram-positive *cocci* (*Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* CIP 106510). TQ also prevented cell adhesion to glass slide surfaces.[61]

Furthermore, our data showed a significant decrease in bacterial counts in the TQ treated groups compared to both the negative and positive control groups. This result is supported by in vitro studies which showed that TQ possesses a selective antibacterial activity against oral bacteria. Therefore, the authors suggested that TQ could be used as a source of natural products with resistance-modifying activity.[62]

Our data also showed a significant decrease in bleeding on probing, clinically as well histologically with improvement in gingival health. In both cases, TQ treated rats showed no bleeding on probing and minimal signs of inflammation. These findings are in agreement with previous published reports showing that TQ diminishes periodontal inflammation and alveolar bone loss due to its anti-inflammatory and antioxidant properties.[64]

The data from the present study confirmed the direct relationship between consumption of dietary sugars and the initiation of dental caries initiation. [82] The caries scores, plaque scores,

and BOP showed statistically significantly higher values in the positive group, fed a high sucrose diet, when compared to animals consuming a regular diet (negative control). These findings are consistent with those reported by Sidi, et al.[83] Investigators conducted a crossover study, which involved two 3-week experimental periods to examine the influence of frequent sugar intake on experimental gingivitis and it was reported that frequent sugar intake resulted in increased gingival inflammation, as measured by gingival bleeding on probing.[83].

TQ was shown to be a relatively safe compound in experimental animals, especially when given orally.[66] In our study, TQ (10 mg/kg body weight) was used daily for 5 weeks and we found no changes in body weight or animal behavior. Our findings confirm the reported 50% lethal dose (LD50) levels of orally administered thymoquinone are 10 times higher than the LD50 levels of intraperitoneally administered TQ, in both mice and rats.

There are certain limitations of this study. First, the study was conducted on animals and as such extrapolation to the human condition warrants further investigation. Although the embryonic development in animals is closely related to that of humans and can be used to study the causes of human diseases and efficiency of treatments with predictive validity, animal studies do not reliably predict human outcomes. Second, while molars in rats are similar in anatomic configuration and structure to human molars, they are also smaller, so it was difficult to perform thorough periodontal parameters evaluation.[84] Also the limited mouth opening prevented access to the posterior teeth at such an age (21 days pups). Another consideration is that all rats had access to food and drinking water after applying the oral gel and this may have reduced the topical effect of the treatment.

VI. Conclusion

Within the limitations of our study we can conclude that:

1. An increase in the use of TQ can prevent caries caused by high sugar diet consumption.
2. TQ may counteract plaque formation by its biofilm inhibition properties.
3. Oral administration of TQ can help in preventing gingival inflammation, especially considering its potent anti- inflammatory properties.

Clinical Implications and Future Studies

Since high sugar diet consumption is a risk factor for caries and gingival disease, dental practitioners should enhance knowledge of children parents and patients and develop clinical strategies for earlier detection to prevent caries and gingival disease progression. The effectiveness of the home oral hygiene preventive methods are influenced by the individual's manual dexterity and motivation. Because of the difficulty to ensure adequate removal of plaque by mechanical means, there is a great interest in the use of antimicrobial agents to replace or to be adjuncts to the mechanical approaches. Our study results showed that TQ counteracted the effect of caries, plaque formation and gingival disease. Therefore, intake of *Nigella sativa* dietary supplements or TQ mixed with oral paste or mouthwash could be recommended for patients who have bad oral hygiene or patients with special needs to increase their immunity and reduce the presence of oral pathogenic bacteria.

In this study, we evaluated the effect of high sucrose diet, in conjunction with bacterial challenge, on caries and gingival health by measuring the caries score, periodontal parameters, and microbiological analyses. As sucrose has an adverse effect on oral host defense, further study of these taxa is warranted and may lead to new therapeutic approaches to prevent caries and periodontal disease. Further studies are needed to elucidate the cellular mechanisms/mediators involved in TQ action with emphasis on inflammatory cytokines. In addition, clinical trials over longer periods of time are needed to build up strong evidence for caries and plaque and the effect of TQ on caries incidence and gingival health. Lastly, pharmacokinetics studies for determination of TQ level in serum using HPLC are needed to

monitor its level in blood for precise calculations of absorptive level and effective dose.

References

1. Beighton D, Lynch E. Comparison of selected microflora of plaque and underlying carious dentine associated with primary root caries lesions. Caries Res 1995;29(2):154-8.
2. Hebbal M, Ankola A, Metgud S. Caries risk profile of 12 year old school children in an Indian city using Cariogram. Med Oral Patol Oral Cir Bucal 2012.
3. Konig KG. [Etiology of caries with emphasis on the role of sugars]. Dtsch Zahnarztl Z 1987;42(10 Suppl 1):S20-9.
4. Yamashita Y, Bowen WH, Burne RA, Kuramitsu HK. Role of the Streptococcus mutans gtf genes in caries induction in the specific-pathogen-free rat model. Infect Immun 1993;61(9):3811-7.
5. Marsh PD, Bradshaw DJ. Dental plaque as a biofilm. J Ind Microbiol 1995;15(3):169-75.
6. Makinen KK, Philosophy L. The role of sucrose and other sugars in the development of dental caries; a review. Int Dent J 1972;22(3):363-86.
7. Dowd FJ. Saliva and dental caries. Dent Clin North Am 1999;43(4):579-97.
8. Rybakov AI. [Sugar in the pathogenesis of dental caries]. Vestn Akad Med Nauk SSSR 1989(3):71-4.
9. Petersen PE, Bourgeois D, Ogawa H, Estupinan-Day S, Ndiaye C. The global burden of oral diseases and risks to oral health. Bull World Health Organ 2005;83(9):661-9.
10. Petersen PE. The World Oral Health Report 2003: continuous improvement of oral health in the 21st century--the approach of the WHO Global Oral Health Programme. Community Dent Oral Epidemiol 2003;31 Suppl 1:3-23.
11. Rockville M. Oral Health in America: A Report of the Surgeon General. US Dept of Health and Human Services, National Institutes of Health, National Institute of Dental and Craniofacial Research 2000;2.
12. Bagramian RA, Garcia-Godoy F, Volpe AR. The global increase in dental caries. A pending public health crisis. Am J Dent 2009;22(1):3-8.
13. Yabao RN, Duante CA, Velandria FV, et al. Prevalence of dental caries and sugar consumption among 6-12-y-old schoolchildren in La Trinidad, Benguet, Philippines. Eur J Clin Nutr 2005;59(12):1429-38.
14. Brown A. Caries prevalence and treatment needs of healthy and medically compromised children at a tertiary care institution in Saudi Arabia. East Mediterr Health J 2009;15(2):378-86.
15. Marsh PD. Dental plaque as a microbial biofilm. Caries Res 2004;38(3):204-11.
16. Dental plaque revisited--oral biofilms in health and disease. J Ir Dent Assoc 2000;46(3):105.
17. Davey ME, O'Toole G A. Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev 2000;64(4):847-67.
18. PD. M. Dental plaque as a biofilm and a microbial community - implications for health and disease. BMC Oral Health. 2006

19. Sbordone L, Bortolaia C. Oral microbial biofilms and plaque-related diseases: microbial communities and their role in the shift from oral health to disease. Clin Oral Investig 2003;7(4):181-8.
20. Diamanti-Kipioti A, Gusberti FA, Lang NP. Clinical and microbiological effects of fixed orthodontic appliances. J Clin Periodontol 1987;14(6):326-33.
21. Owens J, Addy M, Faulkner J, Lockwood C, Adair R. A short-term clinical study design to investigate the chemical plaque inhibitory properties of mouthrinses when used as adjuncts to toothpastes: applied to chlorhexidine. J Clin Periodontol 1997;24(10):732-7.
22. Abou Sulaiman AE, Shehadeh RM. Assessment of total antioxidant capacity and the use of vitamin C in the treatment of non-smokers with chronic periodontitis. J Periodontol 2010;81(11):1547-54.
23. Marsh PD. Microbiological aspects of the chemical control of plaque and gingivitis. J Dent Res 1992;71(7):1431-8.
24. Saekel R. China's oral care system in transition: lessons to be learned from Germany. Int J Oral Sci 2010;2(3):158-76.
25. Blinkhorn AS, Davies RM. Caries prevention. A continued need worldwide. Int Dent J 1996;46(3):119-25.
26. Moynihan PJ. The role of diet and nutrition in the etiology and prevention of oral diseases. Bull World Health Organ 2005;83(9):694-9.
27. Loe H, Theilade E, Jensen SB. Experimental Gingivitis in Man. J Periodontol 1965;36:177-87.
28. Trahan L, Bareil M, Gauthier L, Vadeboncoeur C. Transport and phosphorylation of xylitol by a fructose phosphotransferase system in Streptococcus mutans. Caries Res 1985;19(1):53-63.
29. Lynch E. Antimicrobial management of primary root carious lesions: a review. Gerodontology 1996;13(2):118-29.
30. Niederman R. Glass ionomer and resin-based fissure sealants - equally effective? Evid Based Dent 2010;11(1):10.
31. Samuels N, Grbic JT, Saffer AJ, Wexler ID, Williams RC. Effect of an herbal mouth rinse in preventing periodontal inflammation in an experimental gingivitis model: a pilot study. Compend Contin Educ Dent 2012;33(3):204-6, 08-11.
32. Lopez R, Fernandez O, Baelum V. Social gradients in periodontal diseases among adolescents. Community Dent Oral Epidemiol 2006;34(3):184-96.
33. CoutinhoTCL T. Gingivitis prevalence in children. . RGO 1997;45(3):170-4.
34. Armitage GC. Development of a classification system for periodontal diseases and conditions. Ann Periodontol 1999;4(1):1-6.
35. Masamatti SS, Kumar A, Virdi MS. Periodontal diseases in children and adolescents: a clinician's perspective part. Dent Update 2012;39(8):541-4, 47-8, 51-2.
36. Goodson JM, Palys MD, Carpino E, et al. Microbiological changes associated with dental prophylaxis. J Am Dent Assoc 2004;135(11):1559-64; quiz 622-3.

37. Page RC. The etiology and pathogenesis of periodontitis. Compend Contin Educ Dent 2002;23(5 Suppl):11-4.
38. Craig RG. Destructive periodontal diseases, systemic inflammation, and atherosclerotic complications: the emerging role of the dental profession. J Calif Dent Assoc 2009;37(11):773-7.
39. Bakdash B. Current patterns of oral hygiene product use and practices. Periodontol 2000 1995;8:11-4.
40. Mariotti A. Dental plaque-induced gingival diseases. Ann Periodontol 1999;4(1):7-19.
41. Wolf DL, Lamster IB. Contemporary concepts in the diagnosis of periodontal disease. Dent Clin North Am 2011;55(1):47-61.
42. Schour I, Massler M. Gingival disease in postwar Italy (1945) prevalence of gingivitis in various age groups. J Am Dent Assoc 1947;35(7):475-82.
43. Muhlemann HR. Psychological and chemical mediators of gingival health. J Prev Dent 1977;4(4):6-17.
44. Loe H, Silness J. Periodontal Disease in Pregnancy. I. Prevalence and Severity. Acta Odontol Scand 1963;21:533-51.
45. E B Hancock RJC, T J O'Leary. The relationship between gingival crevicular fluid and gingival inflammation. A clinical and histologic study. Journal of Periodontology 1979;50((1)):13-9.
46. Engelberger T HA, Kallenberger A, Rateitschak KH. Correlations among Papilla Bleeding Index, other clinical indices and histologically determined inflammation of gingival papilla. J Clin Periodontol. 1983;10((6)):579-89.
47. Silness J, Loe H. Periodontal Disease in Pregnancy. Ii. Correlation between Oral Hygiene and Periodontal Condtion. Acta Odontol Scand 1964;22:121-35.
48. Breuer MM CR. The relationship between gingivitis and plaque levels. J Periodontol. 1989;60((4)):172-5.
49. Müller HP HA, Eger T. Site-specific association between supragingival plaque and bleeding upon probing in young adults. Clin Oral Investig. 2000;4((4)):212-8.
50. Ower P. The role of self-administered plaque control in the management of periodontal diseases: 2. Motivation, techniques and assessment. Dent Update 2003;30(3):110-6.
51. Khan MA. Chemical composition and medicinal properties of Nigella sativa Linn. Inflammopharmacology 1999;7(1):15-35.
52. Salem ML. Immunomodulatory and therapeutic properties of the Nigella sativa L. seed. Int Immunopharmacol 2005;5(13-14):1749-70.
53. Ali BH, Blunden G. Pharmacological and toxicological properties of Nigella sativa. Phytother Res 2003;17(4):299-305.
54. Randhawa MA, Alghamdi MS. Anticancer activity of Nigella sativa (black seed) - a review. Am J Chin Med 2011;39(6):1075-91.

55. Toppozada HH, Mazloum HA, el-Dakhakhny M. The antibacterial properties of the Nigella sativa l. seeds. Active principle with some clinical applications. J Egypt Med Assoc 1965;48:Suppl:187-202.
56. Morsi NM. Antimicrobial effect of crude extracts of Nigella sativa on multiple antibiotics-resistant bacteria. Acta Microbiol Pol 2000;49(1):63-74.
57. Zaoui A, Cherrah Y, Mahassini N, et al. Acute and chronic toxicity of Nigella sativa fixed oil. Phytomedicine 2002;9(1):69-74.
58. Akhondian J, Kianifar H, Raoofziaee M, et al. The effect of thymoquinone on intractable pediatric seizures (pilot study). Epilepsy Res 2011;93(1):39-43.
59. Hull Vance S, Benghuzzi H, Tucci M. Inhibition of bacterial attachment to kidney epithelial cells using thymoquinone - biomed 2010. Biomed Sci Instrum 2010;46:69-74.
60. Kokoska L, Havlik J, Valterova I, et al. Comparison of chemical composition and antibacterial activity of Nigella sativa seed essential oils obtained by different extraction methods. J Food Prot 2008;71(12):2475-80.
61. Chaieb K, Kouidhi B, Jrah H, Mahdouani K, Bakhrouf A. Antibacterial activity of Thymoquinone, an active principle of Nigella sativa and its potency to prevent bacterial biofilm formation. BMC Complement Altern Med 2011;11:29.
62. Kouidhi B, Zmantar T, Jrah H, et al. Antibacterial and resistance-modifying activities of thymoquinone against oral pathogens. Ann Clin Microbiol Antimicrob 2011;10:29.
63. Tsuneo ea. Studies on Dental Caries Prevention by Traditional Medicines (Part VII) : Screening of Ayurvedic Medicines for Anti-plaque Action. . The Japaness socity of pharmacognosy 1985;39(2):146-53.
64. Ozdemir H, Kara MI, Erciyas K, Ozer H, Ay S. Preventive effects of thymoquinone in a rat periodontitis model: a morphometric and histopathological study. J Periodontal Res 2012;47(1):74-80.
65. Kara MI, Erciyas K, Altan AB, et al. Thymoquinone accelerates new bone formation in the rapid maxillary expansion procedure. Arch Oral Biol 2012;57(4):357-63.
66. Al-Ali A, Alkhawajah AA, Randhawa MA, Shaikh NA. Oral and intraperitoneal LD50 of thymoquinone, an active principle of Nigella sativa, in mice and rats. J Ayub Med Coll Abbottabad 2008;20(2):25-7.
67. Greenlee RT, Murray T, Bolden S, Wingo PA. Cancer statistics, 2000. CA Cancer J Clin 2000;50(1):7-33.
68. Banerjee S, Padhye S, Azmi A, et al. Review on molecular and therapeutic potential of thymoquinone in cancer. Nutr Cancer 2010;62(7):938-46.
69. El-Mahmoudy A, Shimizu Y, Shiina T, et al. Successful abrogation by thymoquinone against induction of diabetes mellitus with streptozotocin via nitric oxide inhibitory mechanism. Int Immunopharmacol 2005;5(1):195-207.
70. Van der Weijden GA, Timmer CJ, Timmerman MF, et al. The effect of herbal extracts in an experimental mouthrinse on established plaque and gingivitis. J Clin Periodontol 1998;25(5):399-403.

71. Tanner AC, Goodson JM. Sampling of microorganisms associated with periodontal disease. Oral Microbiol Immunol 1986;1(1):15-22.
72. Kitahara K, Kanayama K, Ito S, et al. [Studies on O'Leary's plaque control record in initial periodontal treatment. 1. Relation between PCR and different brushing methods in 1982-1986]. Nihon Shishubyo Gakkai Kaishi 1990;32(1):299-308.
73. Greggianin BF, Oliveira SC, Haas AN, Oppermann RV. The incidence of gingival fissures associated with toothbrushing: crossover 28-day randomized trial. J Clin Periodontol 2013;40(4):319-26.
74. Nayak PA, Nayak UA, Mythili R. Effect of Manuka honey, chlorhexidine gluconate and xylitol on the clinical levels of dental plaque. Contemp Clin Dent 2010;1(4):214-7.
75. Fukuzato Y, Matsuura T, Ozaki K, et al. Morphological study on dental caries induced in WBN/KobSlc rats (Rattus norvegicus) fed a standard laboratory diet. Lab Anim 2009;43(4):376-81.
76. O'Leary TJ, Drake RB, Naylor JE. The plaque control record. J Periodontol 1972;43(1):38.
77. Ragheb A, Attia A, Eldin WS, et al. The protective effect of thymoquinone, an anti-oxidant and anti-inflammatory agent, against renal injury: a review. Saudi J Kidney Dis Transpl 2009;20(5):741-52.
78. Woo CC, Kumar AP, Sethi G, Tan KH. Thymoquinone: potential cure for inflammatory disorders and cancer. Biochem Pharmacol 2012;83(4):443-51.
79. Gali-Muhtasib H, Roessner A, Schneider-Stock R. Thymoquinone: a promising anti-cancer drug from natural sources. Int J Biochem Cell Biol 2006;38(8):1249-53.
80. Hanene Jrah Harzallah a, Bochra Kouidhi b, Guido Flamini c, Amina Bakhrouf b, Touhami Mahjoub. Chemical composition, antimicrobial potential against cariogenic bacteria and cytotoxic activity of Tunisian Nigella sativa essential oil and thymoquinone. Food Chemistry 2011;129(2011).
81. Najah A. Mohammed PD. Effect of Nigella Sativa L. extracts against Streptococcus mutans and Streptococcus mitis in Vitro. J Bagh College Dentistry 2012;24((3)).
82. Michalek SM, McGhee JR, Shiota T, Devenyns D. Virulence of Streptococcus mutans: cariogenicity of S. mutans in adult gnotobiotic rats. Infect Immun 1977;15(2):466-71.
83. Sidi AD, Ashley FP. Influence of frequent sugar intakes on experimental gingivitis. J Periodontol 1984;55(7):419-23.
84. Xu Y, Wei W. A comparative study of systemic subantimicrobial and topical treatment of minocycline in experimental periodontitis of rats. Arch Oral Biol 2006;51(9):794-803.
85. Selwitz RH, Ismail AI, Pitts NB. Dental caries. Lancet 2007;369(9555):51-9.

List of Tables and Figures

Table 1: Comparison of bacterial counts (Kruskal-Wallis test)

Variable	Negative control		Positive control		TQ in Oral-gel		TQ in water		P-value
	Median	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**	
Bacterial Count (Baseline)	1.0	0.0-2.8	0.0	0.0-0.8	0.5	0.0-5.5	3.0	0.8-4.5	*0.358*
Bacterial Count (End)	12.5	7.0-21.0	30.0	11.0-43.8	1.0	0.0-4.3	0.5	0.0-1.0	***0.008***

Bacterial Count (Difference)	11.5	4.8-20.5	29.5	11.0-43.5	0.0	-1.5-0.8	-2.0	-4.3- -0.5	***0.006***

*The data are presented as median (interquartile range) and was analyzed using the Kruskal-Wallis test.

Table 2: Pair-wise comparisons of bacterial counts (Mann-Whitney U test)

Variable	Negative Control	Positive Control	TQ in Oral Gel	TQ in Water
Bacterial Count (Baseline)	1.0 (0.0-2.8)	0.0 (0.0-0.8) [1]P =0.48	0.5 (0.0-5.5) [1]P =1.00 [2]P =0.48	3.0 (0.8-4.5) [1]P =0.34 [2]P =0.11 [3]P =0.68
Bacterial Count (End)	12.5 (7.0-21.0)	30.0 (11.0-43.8) [1]P =0.34	1.0 (0.0-4.3) **[1]P=0.03** **[2]P=0.03**	0.5 (0.0-1.00) **[1]P=0.03** **[2]P=0.03** **[3]P =**0.68
Bacterial Count (Difference)	11.5 (4.8-20.5)	29.5 (11.0-43.5) [1]P =0.34	0.0 (-1.5-0.8) **[1]P=0.03** **[2]P=0.03**	-2.0 (-4.3- -0.5) **[1]P=0.03** **[2]P=0.03** [3]P =0.20

[1]P: Statistical significance versus negative control group; [2]P: Statistical significance versus positive control group.

Table 3: Comparison of caries, plaque and BOP (Kruskal-Wallis tests)

Variable	Negative control		Positive control		TQ in Oral-gel		TQ in water		P-value
	Median	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**	
Caries Score	10.0	7.8-13.8	18.5	17.3-20.5	5.0	0.8-7.0	0.5	0.0-2.5	***0.005***
Plaque Score	12.5	11.3-13.8	21.5	21.0-23.5	6.0	4.0-8.8	4.0	3.3-4.8	***0.004***
BOP	31.1	21.3-35.9	40.7	37.6-48.4	6.3	0.0-21.9	3.1	0.0-6.3	***0.006***

Table 4: Pair-wise comparisons of caries, plaque and BOP (Mann-Whitney U test)

Variable	Negative Control	Positive Control	TQ in Oral Gel	TQ in Water
Caries Score	10 .0 (7.8-13.8)	18.5 (17.3-20.5) **[1]P=0.02**	5.0 (0.8-7.0) **[1]P=0.04** **[2]P=0.02**	0.5 (0.0-2.5) **[1]P=0.02** **[2]P=0.02**
Plaque Score	12.5 (11.3-13.8)	21.5 (21.0-23.5) **[1]P=0.02**	6.0 (4.0-8.8) **[1]P=0.02** **[2]P =0.02**	4.0 (3.3-4.8) **[1]P=0.02** **[2]P =0.02**
BOP	31.1 (21.3-35.9)	40.7 (37.6 -48.4) **[1]P=0.02**	6.3 (0.0-21.9) **[1]P=0.04** **[2]P=0.02**	3.1 (0.0-6.3) **[1]P=0.02** **[2]P=0.02**

[1]P: Statistical significance versus negative control group; [2]P: Statistical significance versus positive control group.

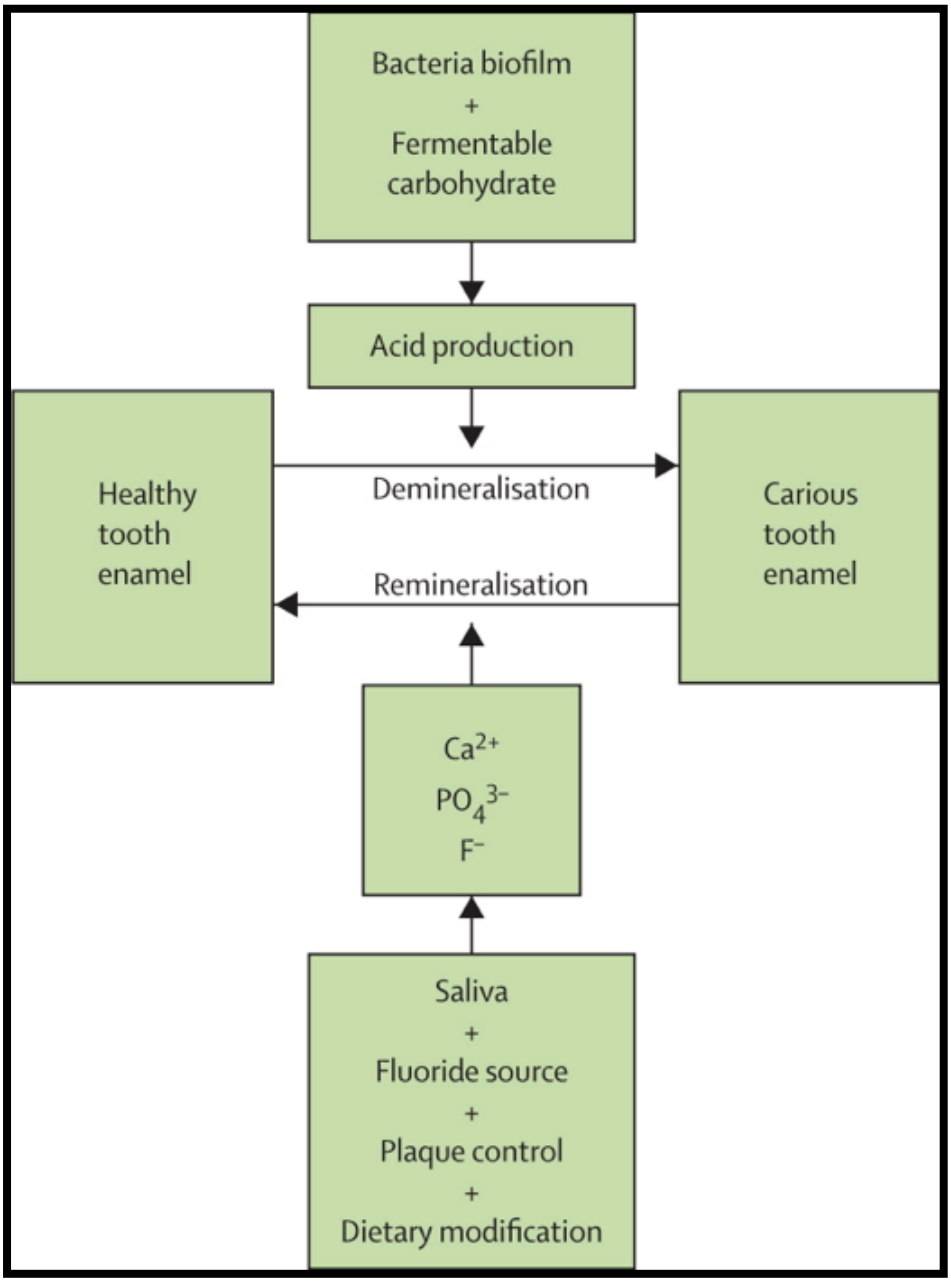


Figure 1. Diagram of the caries process as regular flux of demineralization (destruction) and remineralization (repair)[85]

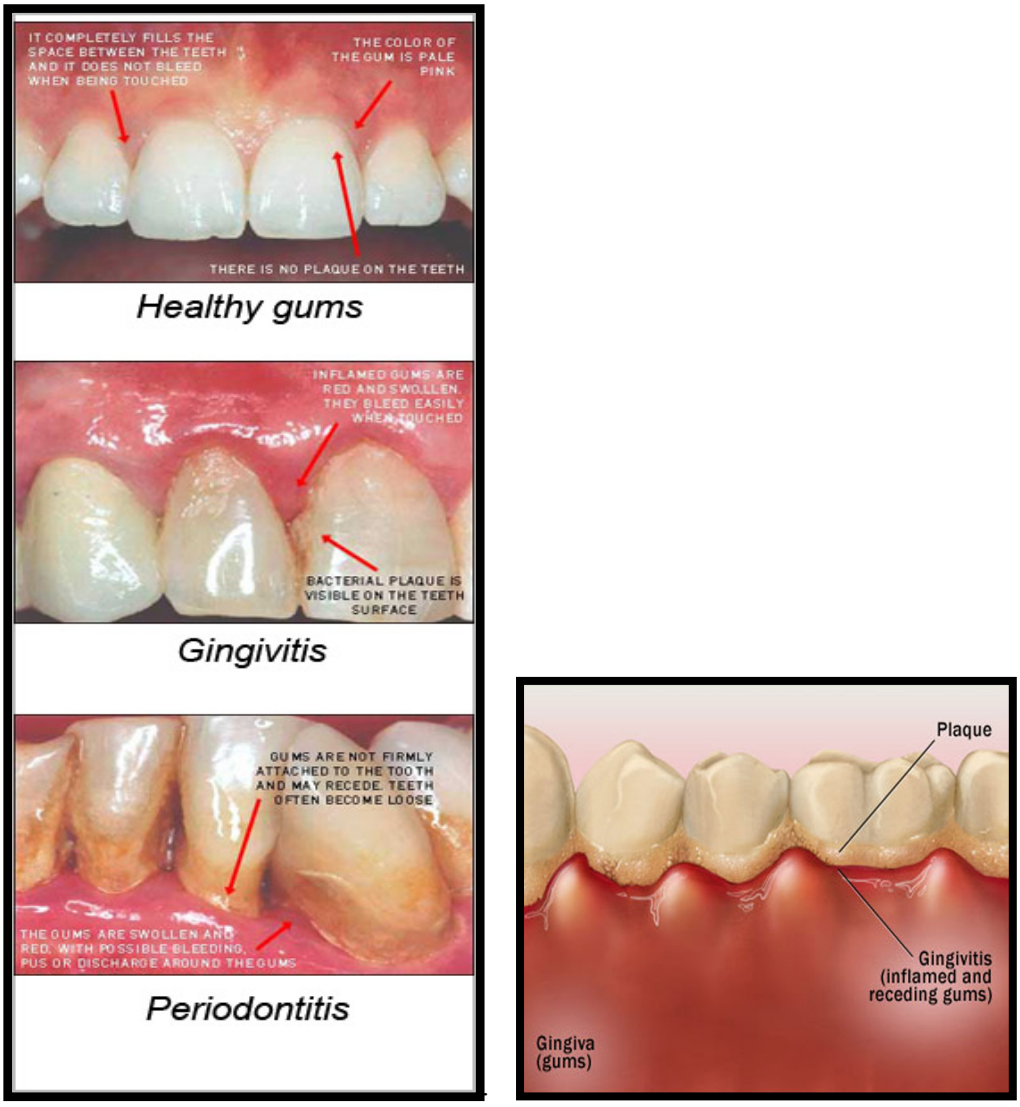


Figure 2. Plaque induced gingivitis.

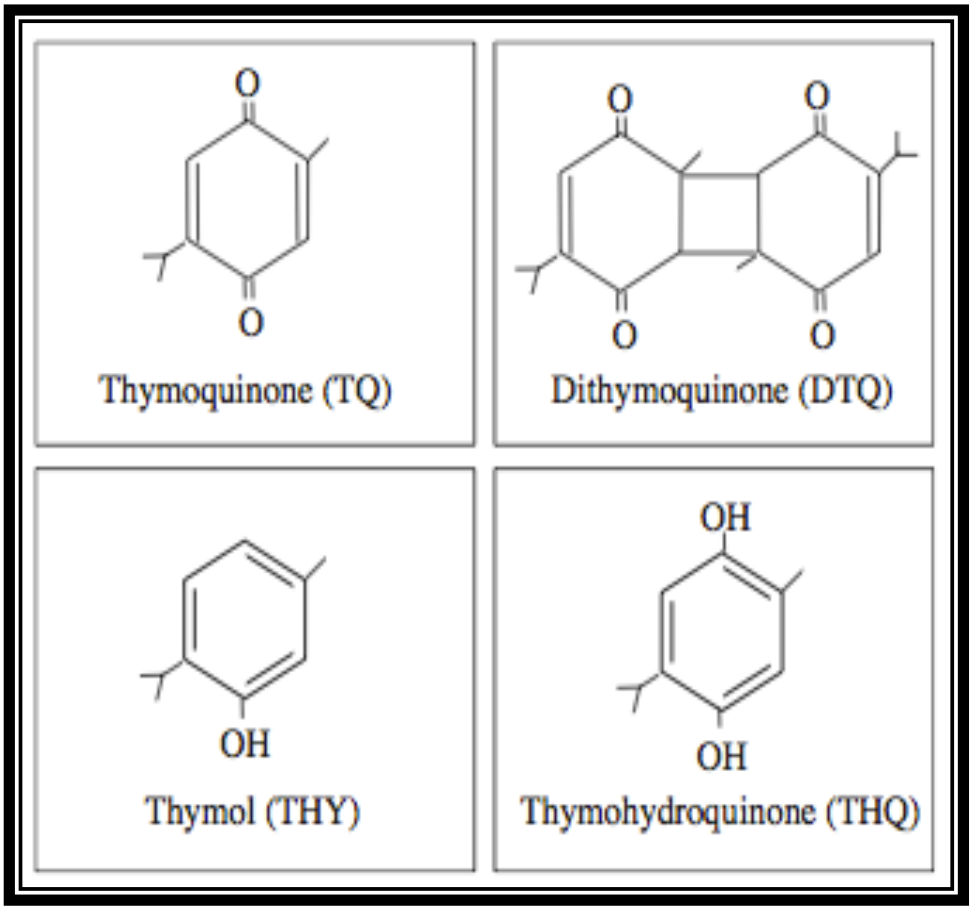


Figure 3.Chemical structure of N. sativa ingredients[52].

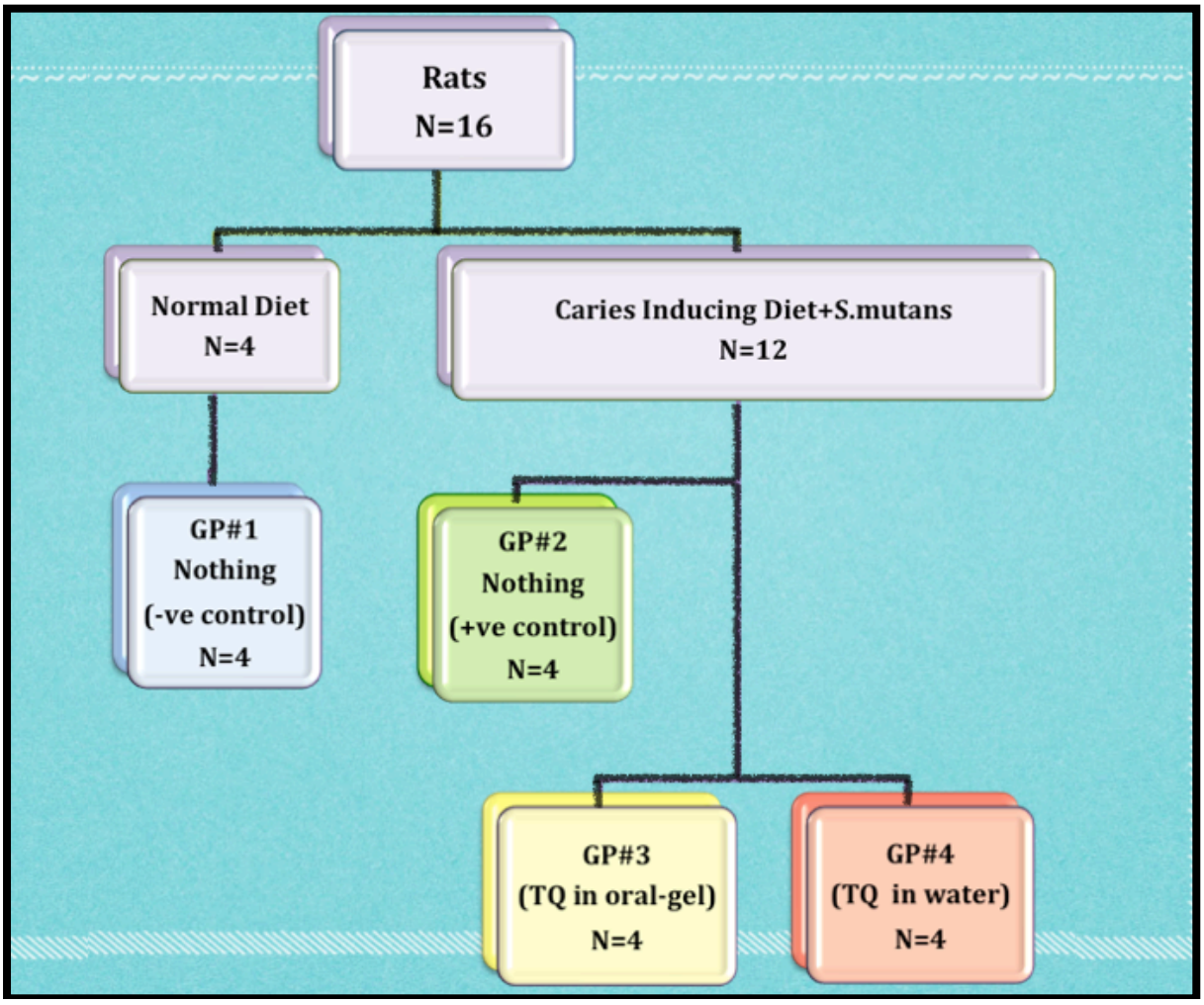


Figure 4. Experimental design flow chart.

0	No bleeding
1	Bleeding

Bleeding On Probing (BOP)

Calculation:

$$(\%)\ BOP = \left(\frac{\text{Number of bleeding sites}}{\text{Number of sites evaluated}}\right) \times 100$$

Figure 5. Bleeding on probing.

Number of Carious Grooves
1-7
8-14
15-21
22-28

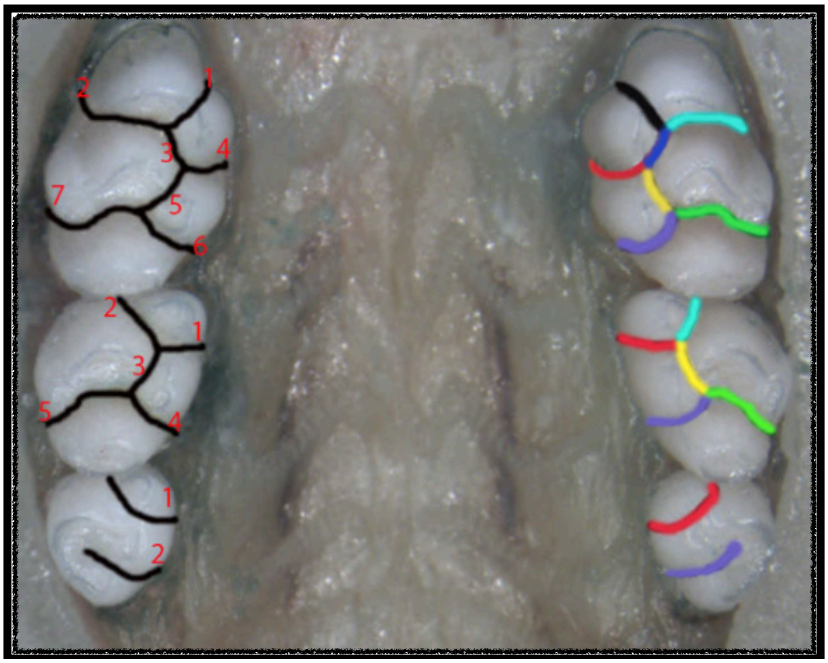
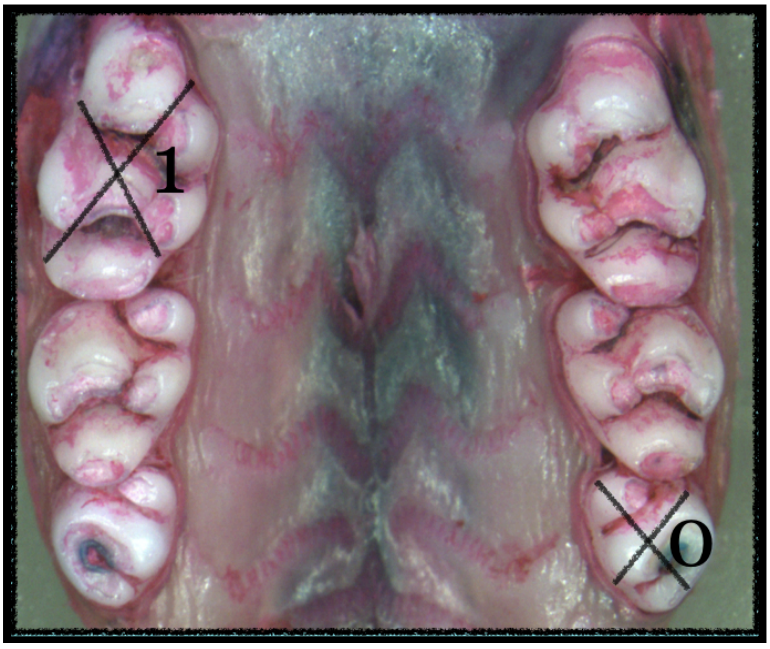


Figure 6. Caries Risk Rating.



$$\text{Plaque Index} = \frac{\text{(The number of plaque containing surfaces)} \times 100}{\text{(The total number of available surfaces)}}$$

Figure 7. Plaque control record (O'Leary Plaque index)[72].

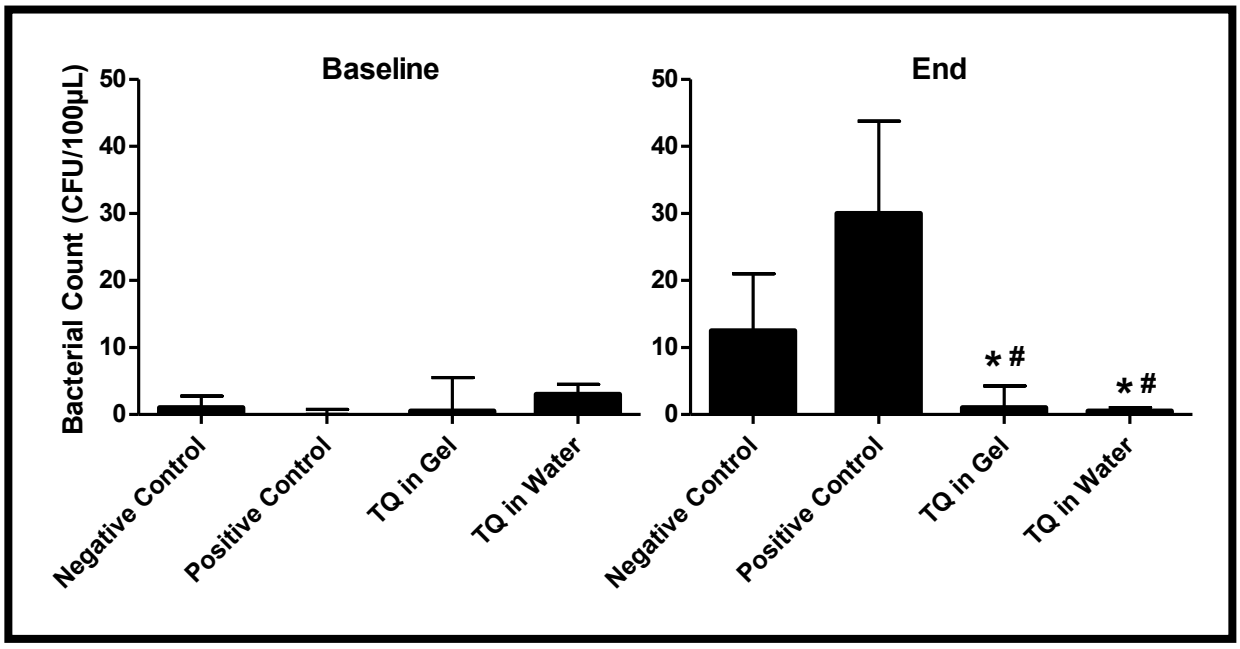


Figure 8. Effect of TQ treatment on subgingival bacteria induced by high sucrose diet consumption and orally applied *S mutans*. Data are expressed as medians ± interquartile range. *Denotes statistically significant difference compared to the negative control group. #Denotes statistically significant difference compared to the positive control group.

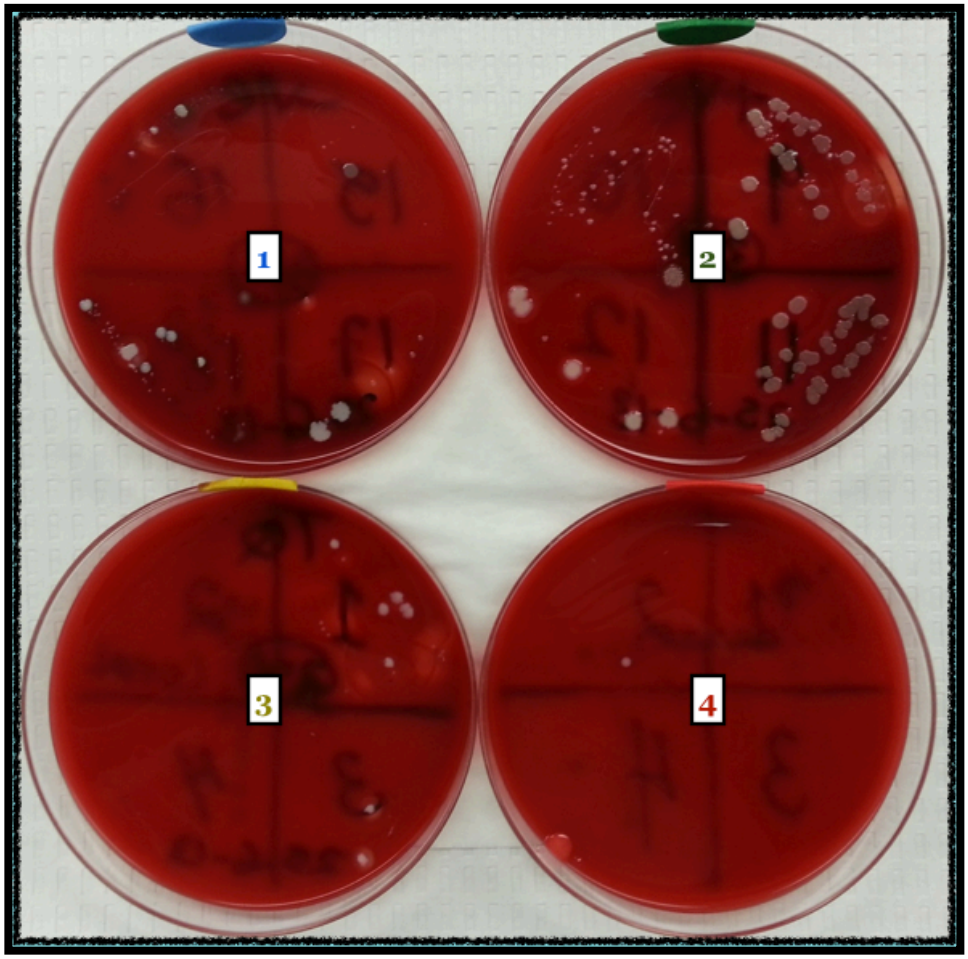


Figure 9. Effect of TQ treatment on subgingival bacteria induced by high sucrose diet consumption and orally applied *S mutans*. Photographs depict examples of subgingival bacterial cultures. #1: negative control group, #2: positive control group, #3: TQ in oral gel group, and #4: TQ in drinking water.

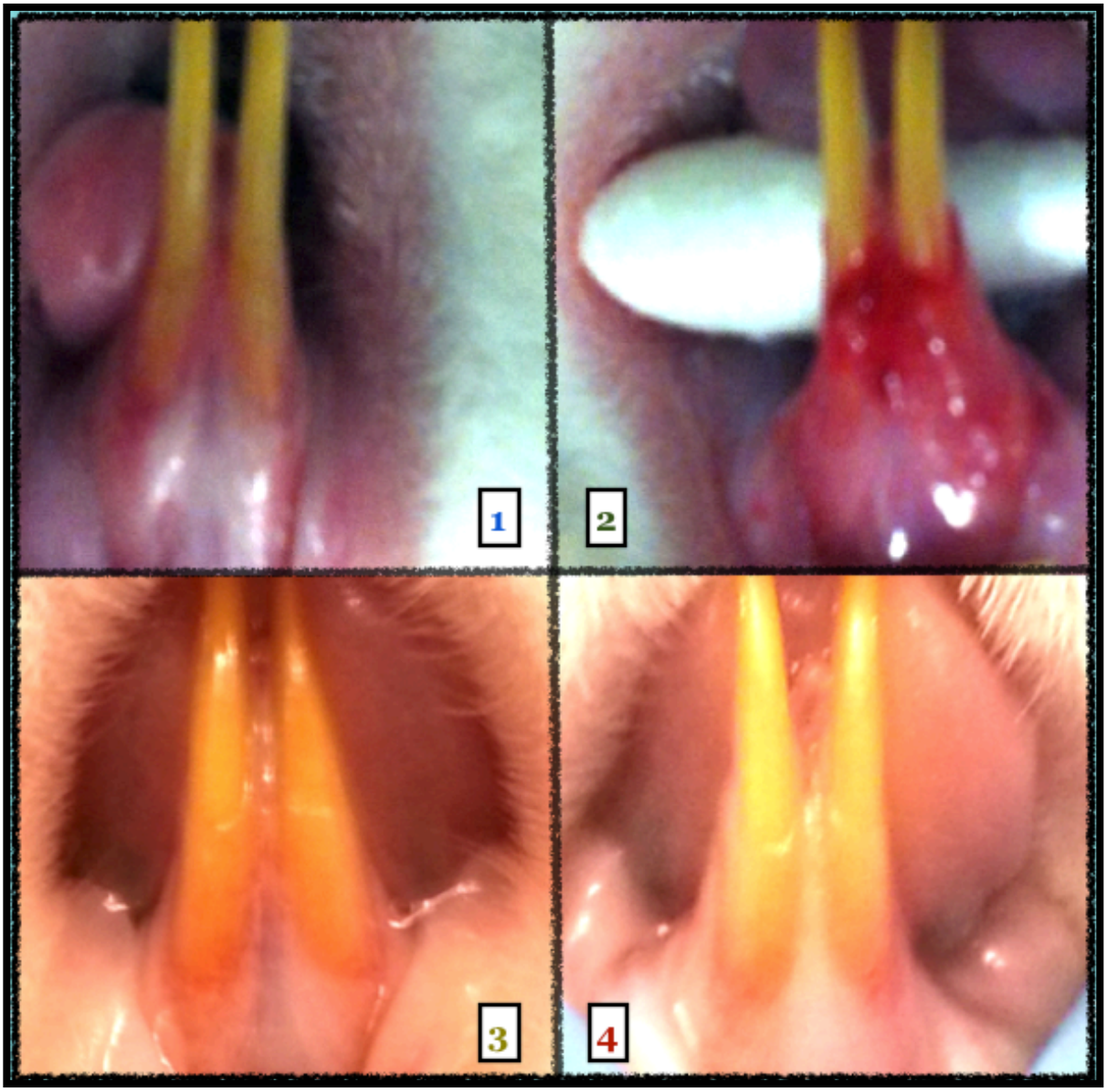


Figure 10. Effect of TQ treatment on bleeding on probing induced by high sucrose diet consumption and orally applied *S mutans*. #1: Negative control group shows moderate inflammation: redness, oedema, glazing and bleeding on probing. #2: positive control group shows severe inflammation, marked redness, oedema, ulceration and tendency to spontaneous bleeding. #3: TQ in oral gel group and #4: TQ in drinking water reveal mild inflammation, slight changes in color and no bleeding on probing with an overall normal healthy gingiva.

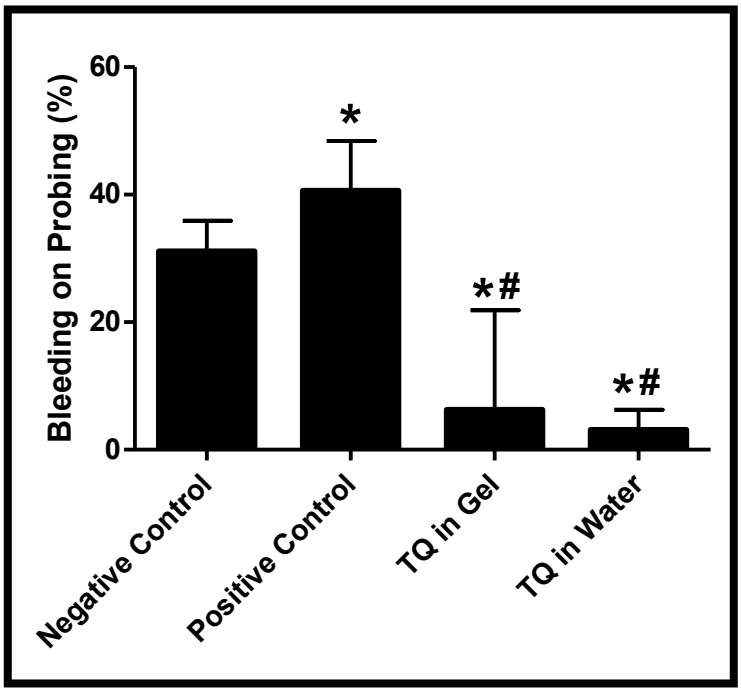


Figure 11. Effect of TQ treatment on bleeding on probing induced by high sucrose diet consumption and orally applied *S mutans*. Data are expressed as medians ± interquartile range. *Denotes statistically significant difference compared to the negative control group. #Denotes statistically significant difference compared to the positive control group.

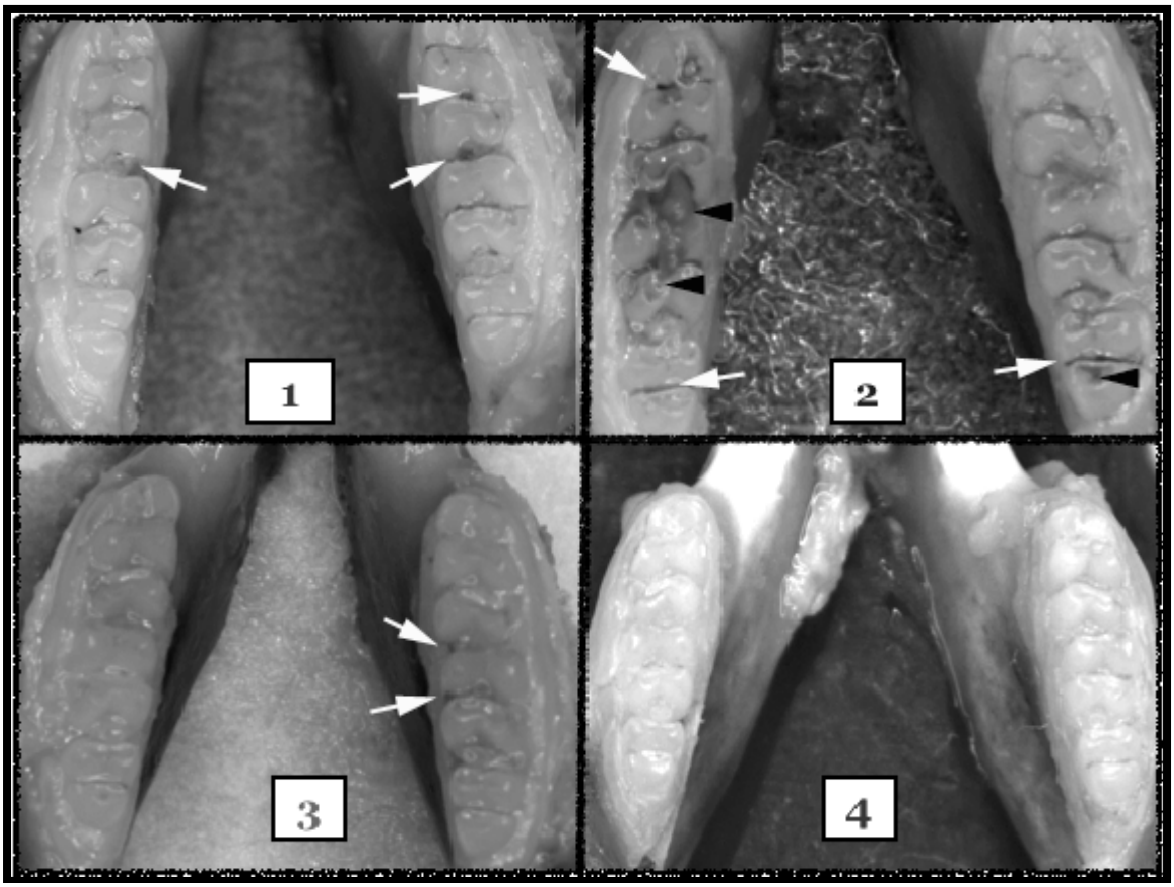


Figure 12. Representative images of unstained mandibular teeth. The samples from the negative control group (#1) and the oral gel TQ treated group (#3) show the presence of insipient caries. Samples from the positive control group (#2) show changes in teeth morphology and presence of extensive tooth decay. In contrast, samples from the group that received TQ in the drinking water (#4) were caries-free.

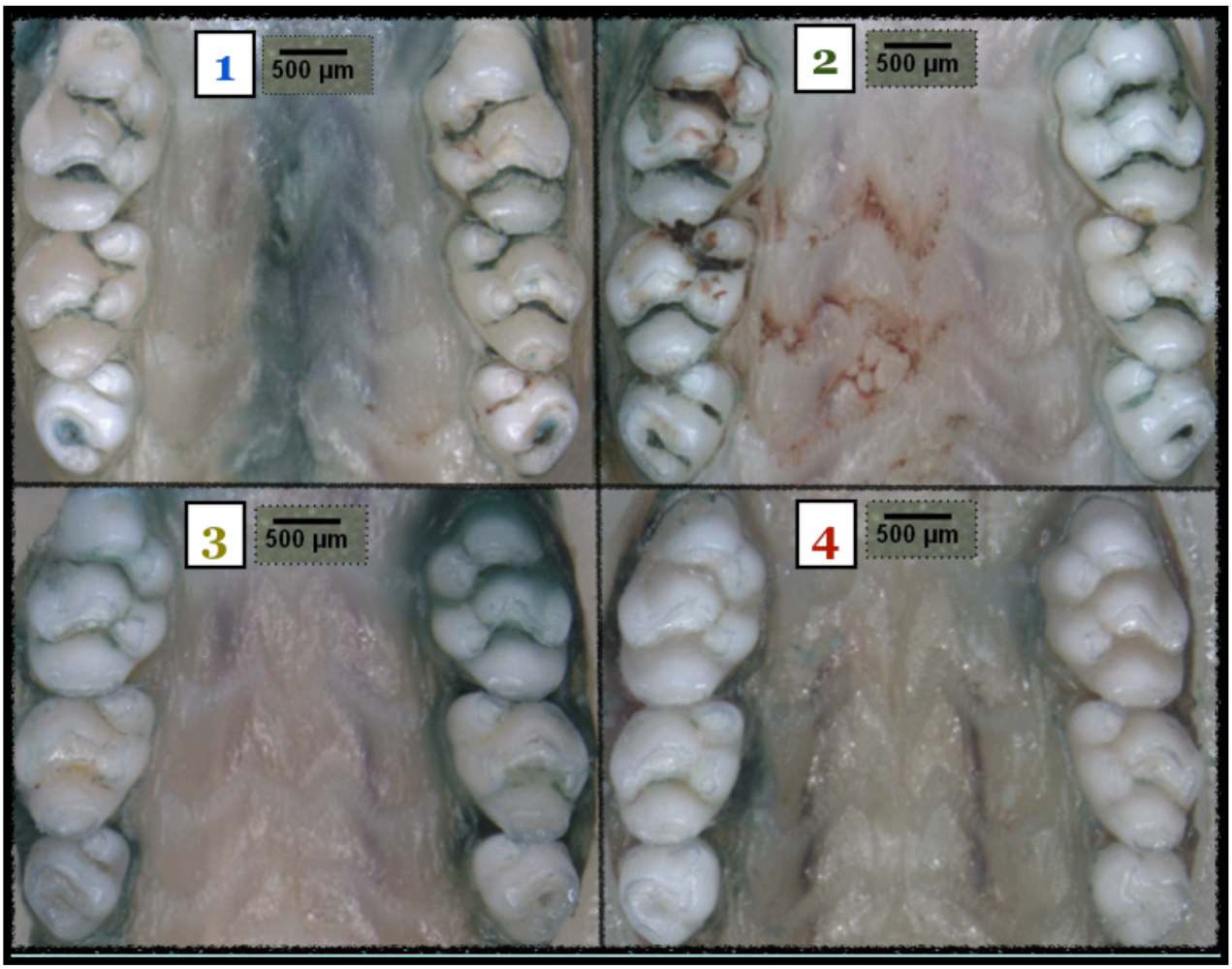


Figure 13. Effect of TQ treatment on caries induced by high sucrose diet consumption and orally applied *S mutans*. Microscopic pictures show the carious grooves stained with caries indicator dye between the groups #1 negative control shows mild caries score, #2 positive control has moderate caries, #3 TQ in oral gel shows low caries, and #4 TQ in drinking water shows no caries.

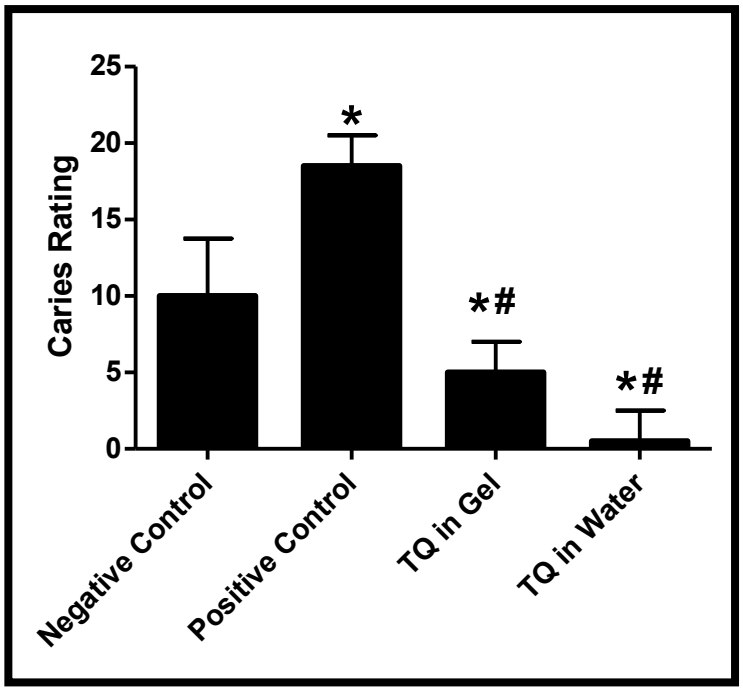


Figure 14. Effect of TQ treatment on caries induced by high sucrose diet consumption and orally applied *S mutans*. Data are expressed as medians ± interquartile range. *Denotes statistically significant difference compared to the negative control group. #Denotes statistically significant difference compared to the positive control group.

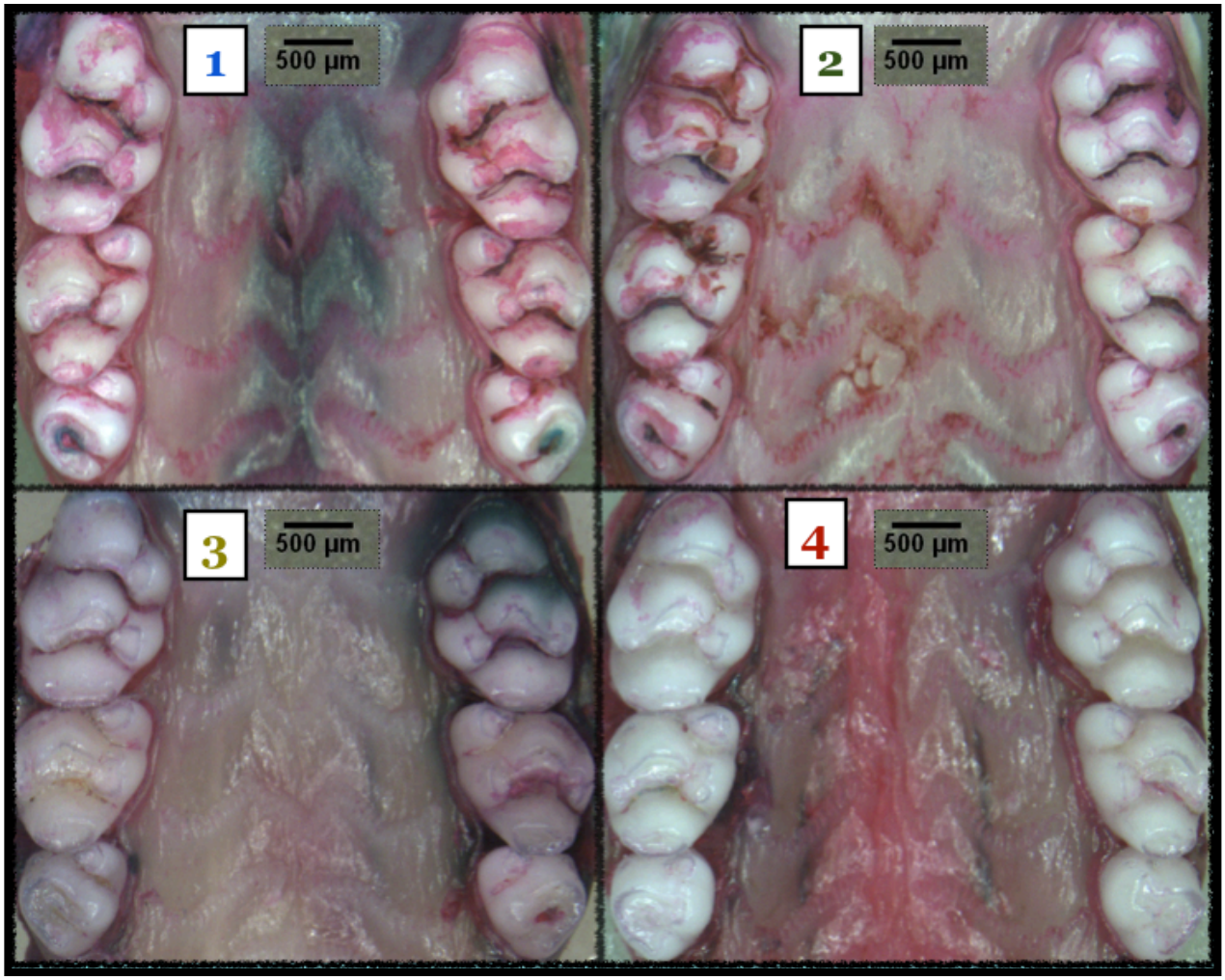


Figure 15. Effect of TQ treatment on plaque formation induced by high sucrose diet consumption and orally applied *S mutans*. Microscopic pictures shows the plaque areas with the plaque indicator dye between the groups, #1 negative control shows moderate layer of plaque covering 70% of the teeth surface,#2 positive control revels abundant plaque covering 100% of the teeth surfaces with stain. Group #3 TQ in oral-gel slows thin layer of plaque covering 25% of the teeth surfaces and Group #4 TQ in the water shows no plaque.

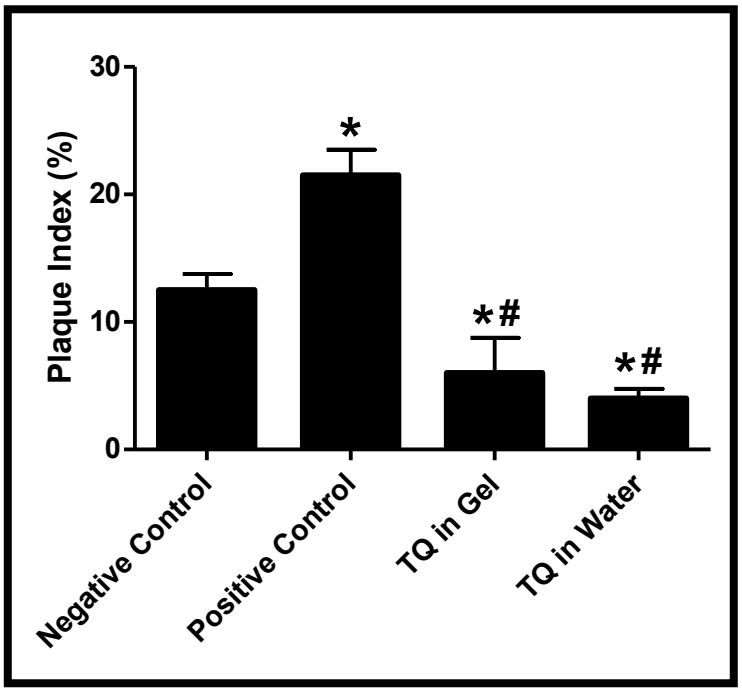


Figure 16. Effect of TQ treatment on plaque formation induced by high sucrose diet consumption and orally applied *S mutans*. Data are expressed as medians ± interquartile range. *Denotes statistically significant difference compared to the negative control group. #Denotes statistically significant difference compared to the positive control group.

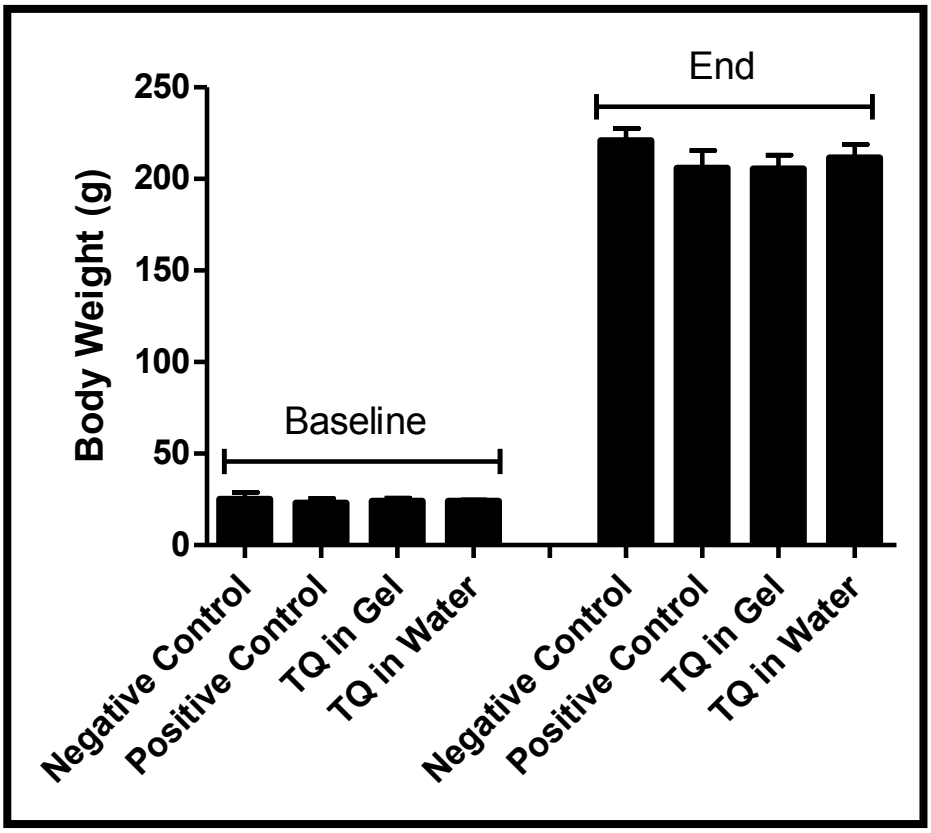


Figure 17. Effect of high sucrose diet and TQ treatment on body weight. Data are expressed as medians ± interquartile range. There are no significant differences among the groups in body weight at baseline or at the end of the 5 weeks treatment period.

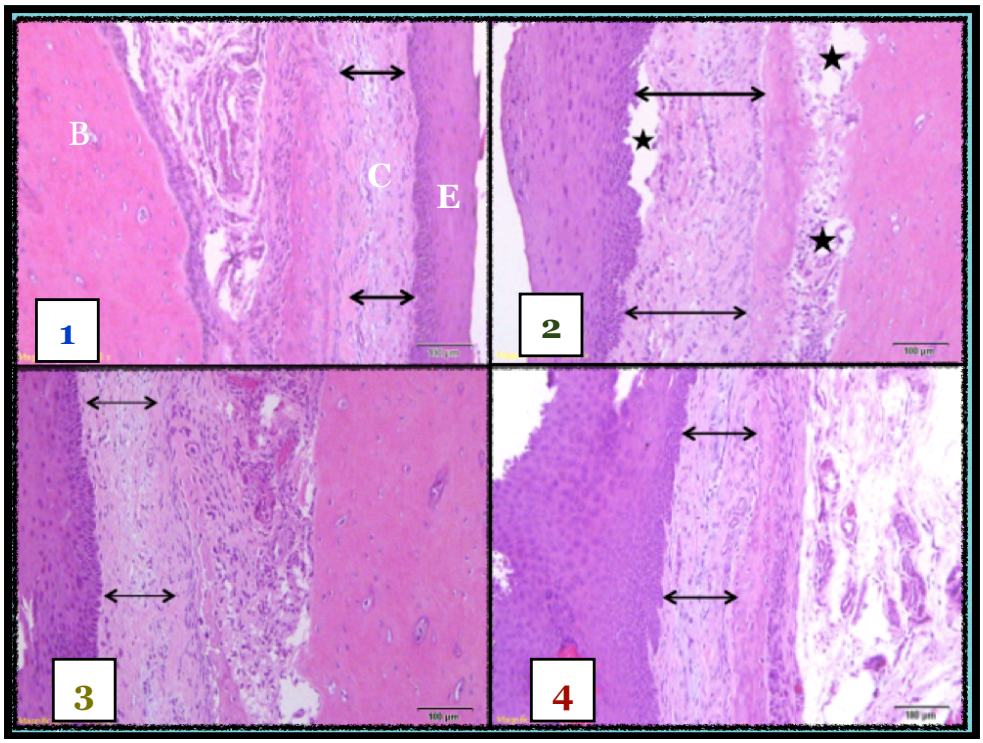


Figure 18. Effect of TQ on gingival inflammation. Representative sections of the gingiva (Buccal site) in the mandible. Compared to samples from the negative control group (#1), samples from the positive control group (#2) exhibited raised epithelium due to edema (filled stars) with increased thickness of the connective tissue layer (double head arrows) most likely because of inflammation. Both TQ treated groups (#3 and #4) showed improvement and preserved normal features similar to those seen in the negative control group samples.
***E,** Epithelial tissue; C**,** Connective tissue; B, Bone

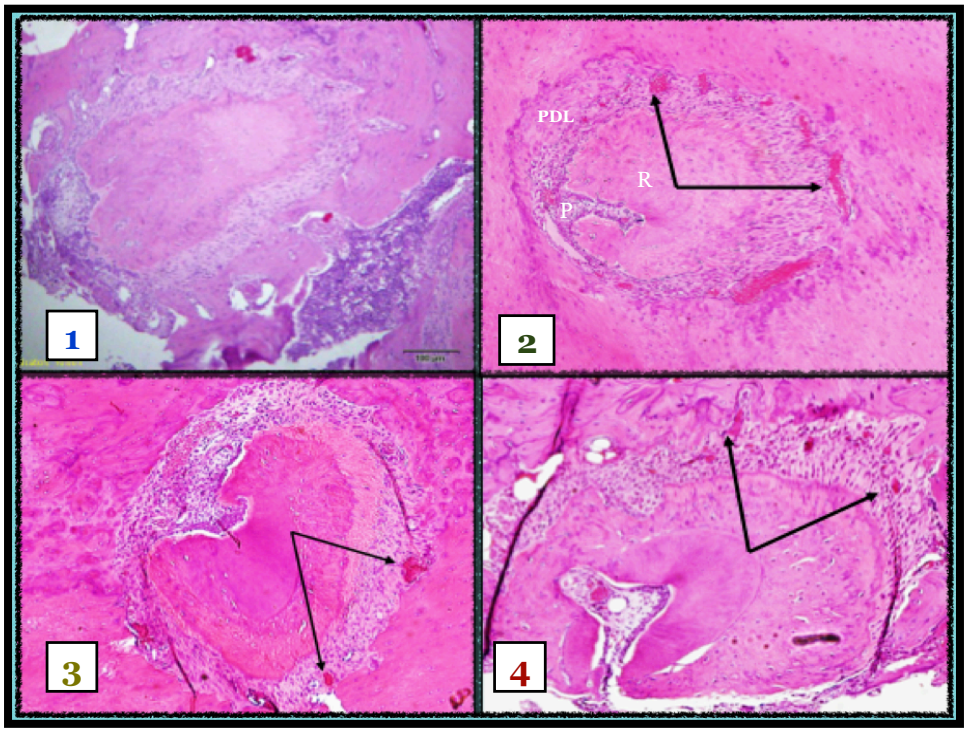


Figure 19. Effect of TQ on inflammation. Cross section in the mandible shows signs of inflammation in the PDL and dilatation of blood vessels.
*R, Root; P, pulp; PDL, periodontal ligament.

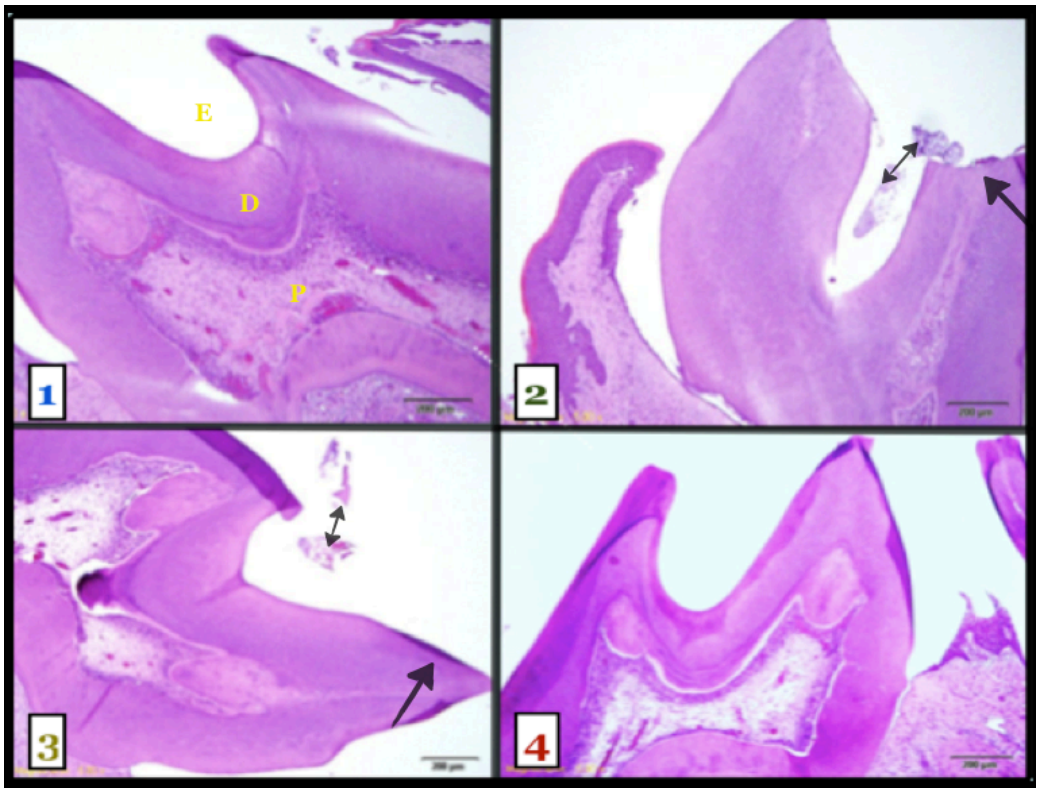


Figure 20. Effect of TQ on caries development. Dental caries was observed histologically as cavitation, loss of cusp tip (single arrow), presence of residual debris (double arrow), in the positive control)#2, in contrast, TQ in the oral-gel #3 has no cavitation with pointed cusp tip (single arrow), less debris (double arrow) and #4 TQ in water intake the tooth more or less similar to those of negative control #1.

*E, Enamel; D, Dentin; P, Pulp.

Teklad Custom Research Diet Data Sheet

TD.04541 Purified Control Diet

Formula	g/Kg
Casein	200.0
DL-Methionine	3.0
Sucrose	160.0
Corn Starch	375.99
Maltodextrin	150.0
Cellulose	10.0
Corn Oil	50.0
Mineral Mix, AIN-93G-MX (94046)	35.0
Calcium Phosphate, dibasic	3.5
Vitamin Mix, AIN-93-VX (94047)	10.0
Choline Bitartrate	2.5
TBHQ, antioxidant	0.01

Footnote

A control for TD.02366, without orotic acid, and to reduce sucrose content (replaced with cornstarch and maltodextrin).

Selected Nutrient Information[1]

	% by weight	% kcal from
Protein	17.7	18.6
Carbohydrate	65.8	69.1
Fat	5.2	12.3
Kcal/g	3.8	

[1] Values are calculated from ingredient analysis or manufacturer data

Teklad Diets are designed & manufactured for research purposes only.

Speak With A Nutritionist

- **(800) 483·5523**
- **askanutritionist@harlan.com**

Harlan Laboratories · PO Box 44220 · Madison, WI 53744-4220
www.harlan.com

Key Features

- **Purified Diet**
- **Control**
- **Complex Carbohydrate**
- **Rodent**

Key Planning Information

- *Products are made fresh to order*
- Store product at 4°C or lower
- Use within 6 months (applicable to most diets)
- Box labeled with product name, manufacturing date, and lot number
- Replace diet at minimum once per week
  More frequent replacement may be advised
- Lead time:
  · 2 weeks non-irradiated
  · 4 weeks irradiated

Product Specific Information

- 1/2" Pellet or Powder (free flowing)
- Minimum order 3 Kg
- Irradiation not advised
  · Contact a nutritionist for recommendations

Options (Fees Will Apply)

- Rush order (pending availability)
- Irradiation (see Product Specific Information)
- Vacuum packaging (1 and 2 Kg)

International Inquiry

·*Outside U.S.A. or Canada*·

- **askanutritionist@harlan.com**

Place Your Order (U.S.A. & Canada)

· *Place Order* · *Obtain Pricing* ·
· *Check Order Status* ·

- **(800) 483·5523**
- **(608) 277·2066** *facsimile*
- **tekladinfo@harlan.com**

Helping you do research better

03/18/09 BDB

Appendix A. Harlan -regular control rat diet composition

Teklad Custom Research Diet Data Sheet

TD.06129 High Sucrose Diet

Formula	g/Kg
Casein	200.0
DL-Methionine	3.0
Sucrose	650.0
Corn Starch	25.99
Corn Oil	50.0
Cellulose	20.0
Mineral Mix, AIN-93G-MX (94046)	35.0
Calcium Phosphate, dibasic	3.5
Vitamin Mix, AIN-93-VX (94047)	10.0
Choline Bitartrate	2.5
TBHQ, antioxidant	0.01

Footnote

Modification of TD 02366. Contains approx. 66.7% sucrose (65% added + 1.7% from vitamin and mineral mixes) and 2.6% corn starch.

Selected Nutrient Information[1]

	% by weight	% kcal from
Protein	17.7	18.0
Carbohydrate	69.1	70.2
Fat	5.2	11.9
Kcal/g	3.9	

[1] Values are calculated from ingredient analysis or manufacturer data

Teklad Diets are designed & manufactured for research purposes only.

Speak With A Nutritionist

- **(800) 483·5523**
- **askanutritionist@harlan.com**

Harlan Laboratories · PO Box 44220 · Madison, WI 53744-4220
www.harlan.com

Key Features

- **Purified Diet**
- **Carbohydrates**
- **Sucrose**

Key Planning Information

- *Products are made fresh to order*
- Store product at 4°C or lower
- Use within 6 months (applicable to most diets)
- Box labeled with product name, manufacturing date, and lot number
- Replace diet at minimum once per week
  More frequent replacement may be advised
- Lead time:
  · 2 weeks non-irradiated
  · 4 weeks irradiated

Product Specific Information

- 1/2" Pellet or Powder (free flowing)
- Minimum order 3 Kg
- Irradiation not advised
  · Contact a nutritionist for recommendations

Options (Fees Will Apply)

- Rush order (pending availability)
- Irradiation (see Product Specific Information)
- Vacuum packaging (1 and 2 Kg)

International Inquiry

·*Outside U.S.A. or Canada*·

- **askanutritionist@harlan.com**

Place Your Order (U.S.A. & Canada)

· *Place Order* · *Obtain Pricing* ·
· *Check Order Status* ·

- **(800) 483·5523**
- **(608) 277·2066** *facsimile*
- **tekladinfo@harlan.com**

Helping you do research better

06/23/09 DHR

Appendix B. Harlan high sucrose rat diet composition.