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Nigella sativa Mouthwash's antimicrobial properties make it a viable candidate for development of a natural alternative to chlorhexidine

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Abstract
Chlorhexidine (CX) mouthwash is effective against *Porphyromonas gingivalis*, a periodontal pathogen, but has unwanted side effects. We developed a prototype Nigella Sativa mouthwash (NSM), and measured its anti-microbial effects against *P. gingivalis in vitro*, as well as assessed its shelf life. We used the agar disc diffusion method and compared inhibition zones with antibiotics as positive controls, and to study acidity, mold, and microbial growth, we used the streaking plate method, and stored the plates in both light and dark conditions, as well as cold (4º C) and room temperature. Data were collected at zero days, seven days, and 14 days. NSM showed the same anti-microbial inhibition level as the strongest positive control. While acidity did not fluctuate measurably, mold and pellet formation were documented in all samples by 14 days, regardless of temperature or light condition. The NSM was an effective anti-microbial against *P. gingivalis*, but will need to be reformulated to suppress microbial growth in order to serve as a natural alternative to CX.

Keywords: *Nigella sativa*, periodontitis, chlorhexidine, *Porphyromonas gingivalis*, anti-bacterial agents, mouthwashes, proof of concept study

1. Introduction
The American Academy of Periodontology (AAP) defines chronic periodontitis (CP) as, "An infectious disease resulting in inflammation within the supporting tissues of the teeth, progressive attachment, and bone loss [1]. Patients with uncontrolled CP experience high rates of tooth loss and edentulism [2]. While lack of access to dental care can impact prevention, diagnosis and treatment of CP, the patient's oral hygiene habits play a strong role [3]. To prevent CP, risk factors should be addressed, such as quitting smoking and ensuring control of underlying conditions such as diabetes, and dental care should focus on plaque removal to prevent periodontal pathogens [3]. This may be done by mechanical and chemical means, and includesthe use of anti-microbial mouthwashes and other oral applications on a daily basis [3].
The primary anti-microbial mouthwashes for maintaining oral hygiene are those containing chlorhexidine gluconate (CX) [4, 5]. CX has been shown to specifically reduce the adherence of *Porphyromonas gingivalis*, an important periodontal pathogen, to epithelial cells [6] .However, CX mouthwash can cause tooth discoloration, alter taste, and in rare cases, cause parotid swelling, anaphylaxis, and other reactions [6].
Researchers are proposing natural alternatives to CX, and comparing their antimicrobial effects to CX. *Nigella sativa* (NS) is an herbaceous annual plant that is also called black seed and black cumin and is believed to have healing powers [7]. An active phytochemical compound found in the volatile oil of NS is called thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone) (TQ) [7]. Therapeutic properties of NS have identified by research include anti-microbial, analgesic, anti-oxidant, anti-cancer and anti-allergic properties, which are believed to involve TQ as a mechanism [7]. The main chemical components of NS include 32 to 40% fixed oil, 34% carbohydrates, and 16 to 20% proteins [7]. The smaller chemical components of NS include 6% water, 5.5% fiber, 1.79 to 3.74% minerals, and 0.4 to 0.45% volatile oil [7].
Chaieb and colleagues proposed that the TQ in NS would prevent biofilm formation, which is a precursor to oral plaque formation [8].Their study showed that TQ demonstrated significant bactericidal activity against *Staphylococcus aureus* and *Staphylococcus epidermides*, two oral pathogens, and other studies have demonstrated NS's antimicrobial action against other oral pathogens, including *Mutans streptococci*, *Streptococcus sobrinus*, *Streptococcus mitis*, *Enterobacter cloacae*, *Streptococcus oralis*, *Streptococcus anginosus*, *Enterococcus durans*, and *Candida* albicans [8-10].

For NS mouthwash (NSM) to be useful for controlling CP, it must inhibit the so-called "red complex" bacteria known to cause CP [11]. Of these, *P. gingivalis* is a major etiologic agent [12]; therefore, in order for an NSM to be effective, it would need to inhibit *P. gingivalis.* Those with CP would need to use NSM regularly, and would need to store it to have it on hand. One study found that when NS seeds were ground and volatile constituents exposed to air, heat and light, they were less stable and experienced higher decrement [13]. Otherwise, little is known about storing NS in different forms.

To date, no *in vitro* studies have examined NSM's bactericidal effect against *P. gingivalis*. Therefore, this study aims to formulate a prototype NSM, study its bactericidal effect against *P. gingivalis in vitro*, and determine its shelf life through quantifyingits acidic stability and its propensity over time to become contaminated with mold and microbial growth.

2. Materials and Methods

First, NSM was formulated. To understand NSM's bactericidal effect against *P. gingivalis*, an agar diffusion study was done. To test NSM's acidic stability and propensity over time to become contaminated with microbial growth, a streaking plate method was used. These procedures will be described here.

2.1 Mouthwash Components

Table 1 lists the components of the NS mouthwash.

Table 1: *N. sativa* Mouthwash Components

Ingredient	Concentration	Quantity	Mixtures
N. sativa cold pressed oil	10.0%	10 mL	A
Glycerin	10.0%	10 mL	A
0.05% cetylpyridiniumchloride	5.0%	1 mL	B
Tween 20	3.5%	3.5 mL	B
PEG 400	3.5%	10 g W/V	B
Peppermint oil	q.s.	0.03 mL	C
Food color (blue)	q.s.	0.01 mL	C
Distilled water	q.s.	To make up total volume	C

As shown in Table 1, NS is the main ingredient in the mouthwash. When NS is used in oral preparations, fillers Tween 20 and PEG-400 help solubilize the NS oil into an aqueous solution [14]. Glycerol and xylitol areroutinely-usedtaste enhancers included in concentrations generally recognized as safe (GRAS).Cetylpyridiniumchloride (CPC) is approved for use as a food preservative, and is safe for use in mouthwash in concentrations between 0.045% and 0.1% [15]. It has weak antimicrobial properties and is used in experimental mouthwashes [15]. The final ingredients – peppermint oil (for flavor), blue food coloring (for appearance), and distilled water – were added *quantum statis* (q.s.) to achieve the desired concentration and volume.

2.2 Formulation of *N. sativa* Mouthwash

Three hundred ml of liquid NSM was createdby first formulating three emulsion mixtures: A, B, and C. The mixture to which each component was assigned is included in Table 1. First, the components of each mixture were combined, then mixture A was added to mixture B, and mixture B was added to mixture C.

2.3 Agar Disc Diffusion Method

The agar disc diffusion method was used to study the NSM's bactericidal effect against *P. gingivalis* [16]. NSM's bactericidal effect was compared with that of antibiotics Amoxicillin/Clavulanic acid (AMC), Cefuroxime (CXM), and Ciprofloxacin (CIP), which are used in treating CP, as positive controls.In the agar disc diffusion method, agar plates are inoculated with the test microorganism, and then filter paper discs with a diameter of approximately 6 mm containing the test compound is placed on the agar surface [16]. The discs are incubated, and the diameters of inhibition growth zones are measured, with larger zones indicating larger inhibition zones and greater antimicrobic capacity [16].

P. gingivalis (ATCC® 33277TM) was suspended in 0.85% saline corresponding to No. 0.5 McFarland turbidity standard, and the culture was incubated at 37 °C for 18 h, and then diluted to 1/10 concentration to yield a culture density of approximately one-and-a-half × 108 CFU/mL. These subcultures were incubated at 37 °C for 24 hours. Next, one-half mL of the *P. gingivalis*inoculum was spread over the entire agar surface of the six Mueller-Hinton agar plates used in the study using a spreader. These plates were then incubated at 37 °C for five minutes, then placed in a laminar airflow cabinet.

Two plates each were assigned to each of the antibiotic conditions (AMC, CXM, and CIP, total n=six), and six plates were assigned to NSM. To prepare the discs, fiveμl of the solution was pipetted onto a five mm Whatman filter paper disc, then allowed to air dry for five to 10 minutes. Solutions contained the following amount of antibiotic: AMC 20/10 mcg, CXM 30 mcg, and CIP five mcg. Subsequent drops were added to the disc in a similar way until the required μg of substance had been added. Air dried discs were placed with tweezers onto the required area of the agar. The agar plates were then incubated at 37 °C for 24 hours. Zones of microbial inhibition were measured to the nearest mm for each disc.

2.4 Streaking Plate Method

Sample conditions for the streaking plate method differed by two variables: light level, and temperature (see Table 2).

Table 2: Sample Conditions for Streaking Plate Method

Light Level	Temperature	Number of Samples
Dark	4 ºC	4
Dark	Room Temperature	4
Light	4 ºC	4
Light	Room Temperature	4
	Total Samples	16

For the light level, either the light was on in the storage area, or the light was off and the sample wrapped to block all light. For the temperature, either samples were stored at room temperature, or they were stored at four degrees C. Varying the light and temperature levels was done to determine if either of these impacted changes in pH level and formation of pellet or mold in the samples. All samples were tested for pH and were evaluated for pellet or mold formation, at three time points: after the plate was prepared (zero days), after seven days, and after 14 days.

All NSM samples were streaked by dipping an inoculation loop into the sample and then streaking onto an agar plate. Triplicate streaks from all samples were conducted. Plates were left overnight in an incubator at 37 ºC. To determine if any mold was in the sample, the following morning (at zero days), the presence of any microbial growth/colonies was

determined by eye.

To measure the pH, oneml of each NSM sample was transferred into a one-and-a-halfml Eppendorf tube and all samples were allowed to reach room temperature before measuring the pH using a pH test strip. pH was measured thrice for each sample and recorded comparing to the color chart. Subsequently, these tubes were used to assess whether insoluble material in the samples as present in the form of precipitation or mold. Samples were centrifuged at four degrees C at 16,000 rpm on a desktop micro-centrifuge. The presence of any pellet was visually determined by eye in all samples. As described earlier, pH level, and presence of pellet and mold were determined on all samples at days zero, seven, and 14.

3. Results& Discussion

3.1 Figures and Tables

First, NSM's bactericidal effect against *P. gingivalis*was compared to the positive antibiotic controls by examining the results of the agar disc diffusion method (see Figure 1).

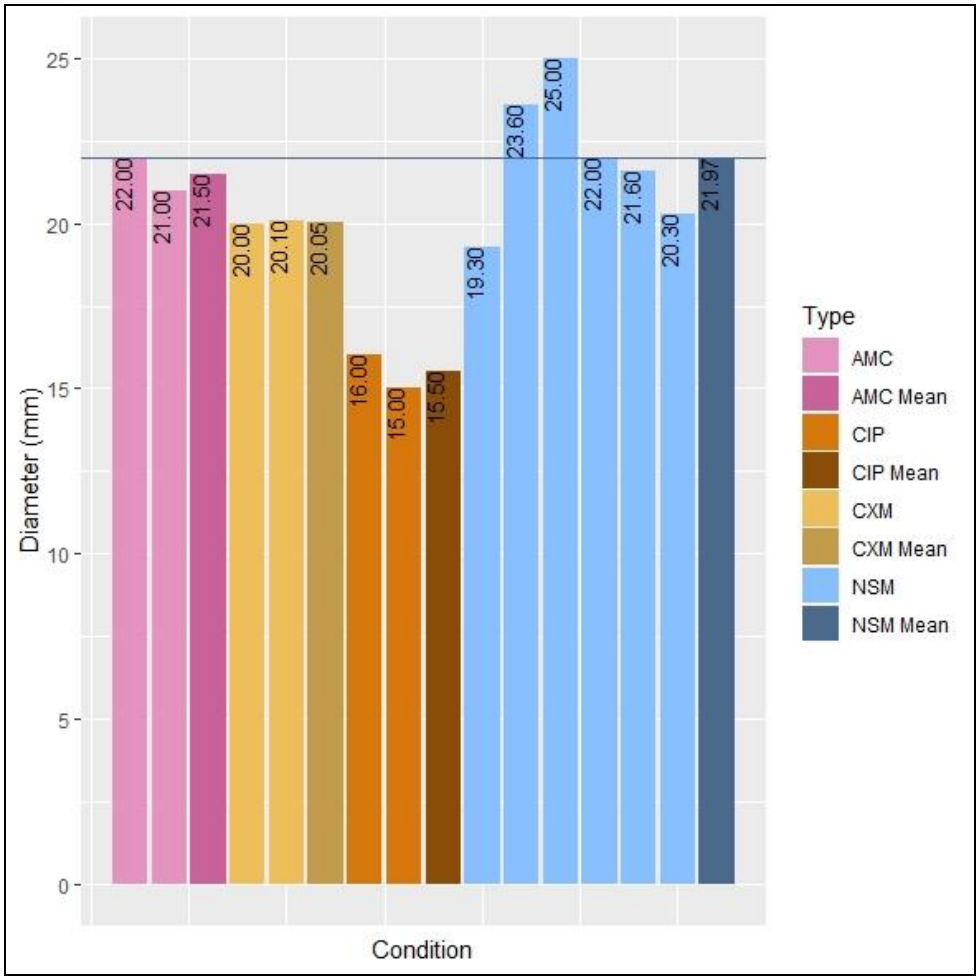

Fig 1: Results from the Agar Disc Diffusion Method

Notes: AMC = Amoxicillin/Clavulanic acid, CXM = Cefuroxime, CIP = Ciprofloxacin, NSM = N. sativa mouthwash.

In Figure 1, the results from both plates from each positive control (antibiotic) condition are graphed, along with their mean. In addition, the individual results from each of the six NSM plates are included in the chart, along with their mean. The mean diameter of inhibition for the six NSM plates was 21.97 mm; this was comparable to the AMC means, and exceeded the means for CIP and CXM.

Results from measuring pH changes and microbial growth in samples is presented in Table 3.

Table 3: Acidity and Microbial Growth Results for Streaking Plate Samples

Outcome	Condition	Temperature	0 Days	7 Days	14 Days
pH Level mode	Dark	4 ºC	6.00	6.00	6.00
	Light	4 ºC	7.00	7.00	6.00
	Dark	RT	6.00	6.00	6.00
	Light	RT	7.00	6.00	6.00
Pellet % yes	Dark	4 ºC	0%	0%	100%
	Light	4 ºC	0%	0%	100%
	Dark	RT	0%	0%	100%
	Light	RT	0%	0%	100%
Mold % yes	Dark	4 ºC	0%	100%	100%
	Light	4 ºC	0%	100%	100%
	Dark	RT	0%	100%	100%
	Light	RT	0%	0%	100%

RT = Room Temperature.

As seen in Table 3, in terms of pH level, with respect to the mode of the four samples in each condition, there appears to be no pattern of difference depending upon the condition. All samples tested at a pH of either six or seven throughout the study, which are safe pH levels for mouthwash. Pellet formation was only detected in samples at 14 days; prior to that time, no pellet formation was detected. However, at 14 days, pellet formation was detected in all samples. In terms of mold formation, no mold formation was seen in any samples until the seven-day measurement. At that point and at 14 days, all samples showed evidence of mold formation except samples stored at room temperature in the light, which at seven days showed no mold formation.

NSM was shown to be a successful bactericide against *P. gingivalis*, performing as well as AMC and better than the other positive controls tested. In terms of acidity, NSM was shown to be stable. However, the shelf life of the NSM formulation used in this study was not very long; after one week, almost all samples stored at different temperatures and in different light conditions were found to have mold. After two weeks, pellets were found in all samples. So while NSM's anti-microbial properties suggest that it is a viable alternative to CX, in order for it to be useful commercially, the formulation will need to developed such that mold and pellet formation does not occur, especially when stored in a cool, dark environment Optimally, the solution would involve strategically adding other natural anti-mold components that would extend the shelf life of NSM, even if it needed to be stored in a cool, dark place.

Different alternatives already exist. As described earlier, natural antimicrobials such as myrrh have been tested in mouthwash, so adding such components to this prototype would be an option as a next step. Other natural components have been studied in mouthwash, including Parodontax© (an herbal mouthwash containing myrrh, echinacea, and chamomile) [17], guava, pomegranate, neem, tulsi, propolis, green tea, cranberry juice, sodium bicarbonate, alum, grapefruit extract [18], and hydrogen peroxide [5].

Although there are a lot of options to try, it is possible to target this inquiry. Valerio and colleagues recently reviewed antimold microbial and plant metabolites that could be used in intelligent food packaging to deter or prevent mold growth [19]. They point to the ten genera of the most common molds contaminating food and note that *Aspergillus* and *Penicillium* species are the most common food contaminants, providing some guidance as to prioritizing additives to test [19].

This study was limited in that it only focused on one formulation of NSM, and compared its antimicrobial properties to standard positive controls. Multiple infectious agents are involved in periodontitis, but this study only focused on *P. gingivalis*. No focus was placed on the practical requirements of the mouthwash, such as color and flavor, as these will need to be addressed after the active ingredients are determined. A strength of this study is that it used standard methods, such as the agar disc diffusion method and the streaking plate method; this way, results can be compared to other studies.

4. Conclusions

In conclusion, this prototype NSM formulation was shown to be a successful antimicrobial against *P. gingivalis*in this *in vitro* study, but experienced mold and pellet growth after only a short time in storage in different conditions. The composition of the mold and pellet formation should be determined, and other natural antimicrobials should be considered as additional ingredients that could be added to extend the shelf life of this promising natural mouthwash prototype.

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