The effect of black seed oil extracts on mutans streptococci in comparison to chlorhexidine gluconate (in vitro)

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ABSTRACT

Background: The black seed or Nigella sativa has been used for centuries to promote health and fight disease. This plant has a great focus for research due to its antibacterial, antifungal, anti-tumor, and hypotensive effects. This study was conducted to assess the effect of the black seed oil extract on sensitivity of mutans streptococci and the adherence to tooth surface in comparison to chlorhexidine gluconate in vitro.

Materials and methods: Four different concentrations of black seed oil extract (1%, 5%, 10%, and 20%) were prepared using ethanol as a solvent for the evaluation of the antimicrobial activity of the black seed oil extract against mutans streptococci isolated from saliva of volunteers and compared with 0.2% chlorhexidine gluconate using agar diffusion test, followed by determination of the Minimum Bactericidal Concentration (MBC) of the black seed oil extract. Three concentrations (1%, 5%, and 10%) were used in the adherence study whereby a stainless steel wires were threaded from one end in the roots of previously cleaned, polished and sterilized first premolars, which were then immersed in 10 ml of the agent for 2 minutes, followed by washing with sterilized deionized water. The teeth were then immersed in 10ml Brain Heart Infusion Broth and inoculated with 2% of bacterial isolates and incubated aerobically at 37°C for seven days. A positive score was given to the microbial growth on wire, teeth and bottle indicating a non-effective treatment and vice versa.

Results: The study showed inhibition zones for black seed oil extract which were found to be increased as the concentration of the extract increased. The MBC of the black seed oil extract against mutans streptococci was 10%. The results also showed that the oil extract was effective in inhibiting the adherence of mutans streptococci to tooth surface at a concentration of 10%.

Conclusion: The black seed oil extract has a bactericidal effect against mutans streptococci at a concentration of 10% and can inhibit the adherence of these microorganisms to tooth surface.

Key words: Nigella sativa, mutans streptococci, adherence. (J Bagh Coll Dentistry 2012; 24(4):126-131).

INTRODUCTION

Dental caries is one of the most common chronic infectious diseases in the world (1). There are three major hypotheses for the etiology of dental caries: the specific plaque hypothesis, the nonspecific plaque hypothesis, and the ecological plaque hypothesis (2,3). The specific plaque hypothesis has proposed that only a few specific species, such as Streptococcus mutans and Streptococcus sobrinus, are actively involved in the disease. On the other hand, the nonspecific plaque hypothesis proposed that caries is the outcome of the overall activity of the total plaque microflora, which is comprised of many bacterial species (2,4). The ecological plaque hypothesis suggests that caries is a result of a shift in the balance of the resident microflora driven by changes in the local environmental conditions (4). Regarding the specific theory, the abilities of mutans streptococci to adhere firmly to tooth surfaces in the presence of sucrose and to form acids by fermenting various dietary sugars have been associated with its caries-inducing potential (5).

Natural compounds have been recently investigated as promising agents for the prevention of dental caries. An aromatic plant, black cumin (Nigella sativa) is widely grown in different parts of the world and the seeds of black cumin have been used to promote health for centuries especially in Middle East and Southeast Asia. The seeds of Nigella sativa contain a yellowish volatile oil (0.5-1.6%), a fixed oil (35.6-41.6%), proteins (22.7%), amino acids, minerals and vitamins. Many biological activities of Nigella sativa seeds have been reported, including: antibacterial, antifungal, anti-tumor, and hypotensive effects (6). Thymoquinone is the bioactive constituent of the volatile oil of Nigella sativa. Moreover, it has been reported that thymoquinone has antibacterial potency and its activity can enhance antibiotic actions (7).

Previous Iraqi studies had been conducted to study the effect of the different extracts of black seed on oral bacteria (8,9). These studies found that the aqueous (8) and oil (9) extracts exhibited antimicrobial action on bacteria isolated from the root canals. This study was conducted to assess the effect of different concentrations of black seed oil extract on the sensitivity of mutans streptococci (MS) and the adherence of the bacteria to tooth surface in comparison to chlorhexidine gluconate (CHX).
MATERIALS AND METHODS
Stimulated saliva samples were collected under standard conditions to obtain five microbial samples. Dental students aged 18-22 years old and with no medical history were selected to participate in the study. Each individual was instructed to chew a piece of Arabic chewing gum (0.4-0.5gm) for five minutes to stimulate salivary flow as much as possible. Methods of isolation and identification of mutans streptococci were according to those described by Holbrook and Beighton and Finegold and Baron. Saliva was collected in sterilized screw capped bottles. The collected saliva was homogenized by vortex mixer for two minutes. Ten-fold serial dilutions were prepared using sterile normal saline. Two dilutions were selected and inoculated on Mitis-Salivarius Bacitracin Agar (MSB Agar), the selective media for mutans streptococci, which was prepared according to the manufacturer’s instructions. 0.1ml was withdrawn from dilutions of $10^{-1}$ and $10^{-2}$ using adjustable micropipette with disposable tips and then spread in duplicate by using sterile microbiological glass spreader on the plates of MSB agar. The plates were then incubated anaerobically by using a gas pack supplied in an anaerobic jar for 48 hours at 37°C followed by aerobic incubation for 24 hours at 37°C. A single colony from mutans streptococci was transferred to 10 ml sterile Brain Heart Infusion Broth (BHI-B) and then incubated aerobically for 24 hours at 37°C to activate the inoculums. The purity of the isolates was checked by inoculation of 0.1 ml of the isolates from BHI-B suspensions on media by spreader as mentioned before, and then a selective colony was transferred to 10 ml of sterile BHI-B and incubated aerobically for 24 hours at 37°C. Identification of mutans streptococci was carried out by 4 stages:-

a) Colony morphology.
b) Morphological test of bacterial cell.
c) Biochemical test.
d) Identification system for mutans streptococci of Analytic Profile Index (API) 20 strep.

Figure 1 shows a rough type colony of mutans streptococci on MSB agar.

The antimicrobial activity of the black seed oil extract was assessed in this study using the agar diffusion test. Four different concentrations were prepared from the stock 100% cold pressed black seed oil extract using ethanol as a solvent. The concentrations prepared were: 1%, 5%, 10%, and 20%. Bacterial isolates of mutans streptococci were spread on Brain Heart Infusion Agar (BHI-A).

Figure 1: Rough type colony of mutans streptococci on Mitis Salivarius Bacitracin agar

Wells of equal sizes and depths were prepared in the agar using Kork porer for the evaluation of the antimicrobial effect of the different concentrations of the black seed oil extract and 0.2% chlorhexidine gluconate. Each well was filled with 50μl of a concentration that was prepared from the stocks of the extract. Single control well in the agar plates was filled with 100% ethanol to evaluate the antimicrobial effect of alcohol alone. Plates left for 15 minutes at room temperature and then incubated aerobically for 24 hours at 37°C. Inhibition zones diameters were measured using a scientific ruler. The resistance of the isolates to the tested agents was indicated when there were no zones of inhibition.

The alcohol inhibition zone diameter was subtracted from the total values of the inhibition zones diameters of the different concentrations of the oil extract.

To determine the minimum bactericidal concentration (MBC) of the black seed oil extract, all the concentrations of the black seed oil extract that revealed inhibition zones were mixed with BHI-A to get 25ml of agar and extract then poured into Petri dishes and allowed to harden and inoculated with 0.1ml from the activated isolates of mutans streptococci. All these Petri dishes were incubated for 24 hours at 37°C including the control plates (negative control which contained BHI-A with microbial inoculums without the addition of the extract and the positive control plates which contained BHI-A and different concentrations of the oil extract without microbial inoculums). Each Petri dish was checked and examined for microbial growth. The minimum bactericidal concentration (MBC) was
determined as the lowest concentration of the extract that killed the microorganisms.

To study the effect of the black seed oil extract on the adherence of mutans streptococci to tooth surface, three concentrations were used: the minimum bactericidal concentration (MBC) and two concentrations just lower than the MBC. These three concentrations were compared with 0.2% chlorhexidine gluconate, control positive (broth and bacteria without agent), control negative (broth and agent without bacteria) and the solvent alone. A stainless steel wire was threaded from one end in the root of a previously cleaned, polished and sterilized first premolar (Figure 2).

The teeth were then immersed in 10 ml of the agent for 2 minutes except for control positive. The wires and the teeth were then washed with sterilized deionized water and dried, immersed in 10ml Brain Heart Infusion Broth containing 5% sucrose (pH=7). The study and control tubes were incubated with 2% of bacterial isolates and incubated aerobically at 37°C for seven days. A positive score was given to the microbial growth on wire, teeth and bottle indicating a non-effective treatment and vice versa as shown in Figure 3. This method was described by Al Bazaz, 2010.[10]

![Image](image_url)

**Figure 2: Stainless steel wires threaded in one end in the roots of previously cleaned, polished and sterilized first premolars.**

![Image](image_url)

**Figure 3: Microbial growth after aerobic incubation (positive score) in the left tube and non-effective treatment (negative score) in the right tube.**

**RESULTS**

Table 1 shows the diameters of the inhibition zones of the different concentrations of the black seed oil extract against mutans streptococci which were found to be increased as the concentration of the extract increased. Different concentrations of the black seed oil extract and chlorhexidine gluconate in relation to mutans streptococci showed statistically highly significant difference as shown in Figure 4. Table 2 is showing LSD test between different concentrations of the black seed oil extract, statistically a high significant difference found between all the concentrations used. T-test was performed to compare each concentration used of black seed oil extract and chlorhexidine, statistically highly significant difference (p 0.001) was shown and the sensitivity of mutans streptococci was higher to black seed oil extract at concentrations (10%,20%) than chlorhexidine (Table 3).

**Table 1: Mean values of the inhibition zones (in mm) produced by the different concentrations of the black seed oil extract against mutans streptococci.**

<table>
<thead>
<tr>
<th>Extract</th>
<th>Concentration (%)</th>
<th>Mean diameter of inhibition zone (mm)± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black seed oil extract</td>
<td>1</td>
<td>12.66 ± 0.82</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>14.48 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>17.44 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>20.05 ± 0.73</td>
</tr>
<tr>
<td>Chlorhexidine</td>
<td>0.2</td>
<td>16.00 ± 0.34</td>
</tr>
</tbody>
</table>

F value = 424.12, P value = 0.000 (highly significant), df =4
Table 2: LSD test between different concentrations of black seeds oil extract (Agar well diffusion method)

<table>
<thead>
<tr>
<th>Conc. of oil extract</th>
<th>5%</th>
<th>10%</th>
<th>20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%</td>
<td>MD=-1.73*</td>
<td>MD=-4.71*</td>
<td>MD=-7.78*</td>
</tr>
<tr>
<td>5%</td>
<td></td>
<td>MD=-2.98*</td>
<td>MD=-6.05*</td>
</tr>
<tr>
<td>10%</td>
<td></td>
<td></td>
<td>MD=-3.06*</td>
</tr>
</tbody>
</table>

*P<0.001 (highly significant), MD (mean difference)

Table 3: t-test between each concentration of black seed oil extract and chlorhexidine (CHX)

<table>
<thead>
<tr>
<th>Conc. of oil extract</th>
<th>CHX 0.2%</th>
<th>t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%</td>
<td>0.2%</td>
<td>11.81*</td>
</tr>
<tr>
<td>5%</td>
<td>0.2%</td>
<td>11.43*</td>
</tr>
<tr>
<td>10%</td>
<td>0.2%</td>
<td>-9.77*</td>
</tr>
<tr>
<td>20%</td>
<td>0.2%</td>
<td>-25.19*</td>
</tr>
</tbody>
</table>

*P<0.001 (highly significant)

Figure 4: Bar Chart Graph showing the mean diameter of inhibition zones of the different concentrations of the black seed oil extract and chlorhexidine gluconate against mutans streptococci.

The results of this study showed that the MBC of the black seed oil extract for mutans streptococci was 10%. This concentration showed no bacterial growth when mixed with the agar and inoculated with the mutans streptococci, while the concentrations of 1% and 5% still showed bacterial growth when they were mixed with the agar and inoculated with the mutans streptococci, i.e., these concentrations could be regarded inhibitory but not bactericidal.

The results also showed that the black seed oil extract was effective in prevention the adherence of the mutans streptococci to tooth surface at the concentration of 10% as shown in Table (4).

Table 4: The effect of the different concentrations of the black seed oil extract, chlorhexidine gluconate, and ethanol on the adherence of mutans streptococci.

<table>
<thead>
<tr>
<th>Agents</th>
<th>Adherence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black seed oil extract 10% (MBC)</td>
<td>-ve</td>
</tr>
<tr>
<td>Black seed oil extract 5%</td>
<td>+ve</td>
</tr>
<tr>
<td>Black seed oil extract 1%</td>
<td>+ve</td>
</tr>
<tr>
<td>Control positive</td>
<td>+ve</td>
</tr>
<tr>
<td>Control negative</td>
<td>-ve</td>
</tr>
<tr>
<td>Chlorhexidine 0.2%</td>
<td>-ve</td>
</tr>
<tr>
<td>Ethanol</td>
<td>+ve</td>
</tr>
</tbody>
</table>

DISCUSSION
Interest in medicinal plants has burgeoned due to increased efficiency of the new plant-derived drugs and the growing interest in natural products. Because of the concerns about the side effects of conventional medicine, the use of natural products as an alternative to conventional treatment in healing and treatment of various diseases has been
on the rise in the last few decades. The black seed or *Nigella sativa* is a complex substance of more than 100 compounds, some of which have not yet been identified or studied. A combination of fatty acids, volatile oils and trace elements are believed to contribute to its effectiveness(13).

In this study, the cold pressed oil extract of the black seed was used rather than other types of extracts because this slow mechanical process protects the highly sensitive and precious nutrients which make the black seed so valuable particularly the highly sensitive essential fatty acids. Moreover, the oil is 35% more concentrated than the raw seeds, and the volatile oils are fully extracted.

In this study, the MBC was determined for the black seed oil extract but not for chlorhexidine gluconate since the latter is traditionally used in a concentration of 0.2% in the mouth rinses containing chlorhexidine gluconate.

The antimicrobial effect exhibited by the black seed oil extract against mutans streptococci in this study could be attributed to the presence of thymohydroquinone in the chemical composition of the black seed oil extract. Thymohydroquinone had been isolated by El-Fatatry from the oil of *Nigella sativa* and was found to have high activity against gram positive microorganisms (14). In addition, it could be attributed to the presence of other volatile oils including niggellone, thymoquinone, thymol, carvacrol, α & β-pinene, d-limonene, d-citronellol, p-cymene and 2-(2-methoxypropyl)-5-methyl-1,4-benzenediol in the chemical composition of the cold-pressed black seed oil extract which might be responsible for its antimicrobial effect.

In this study, the use of the black seed oil extract in a concentration of 20% produced an inhibition zone of 20mm against mutans streptococci. A previous Iraqi study by Majeed (2006) (9) showed that the use of the fully concentrated cold pressed black seed oil extract produced inhibition zones of 20.8mm and 16.8mm against *Streptococcus mutans* when tested in the direct contact and indirect vapor-forming methods, respectively.

The MBC of the black seed oil extract for mutans streptococci was found to be 10%, which is in agreement with the results of Majeed (2006) (9) who found that the 10% concentration was considered as the MBC of black seed oil extract against intra canal microbes using broth macrodilution method for determination of the MBC.

The adherence of mutans streptococci to tooth surface was found to be prevented at a concentration of 10% of black seed oil extract. This finding suggests that the black seed oil extract could be used as an antibacterial agent in a concentration of 10% to prevent bacterial colonization on the tooth surface as 0.2% chlorhexidine gluconate, with the added advantages that the black seed oil extract is biocompatible and can enhance the cellular immunity.

There is no available research dealing with the effect of black seed oil extract on the adherence of mutans streptococci to tooth surface to compare with. However, the methanolic extract was found to exhibit antiplaque action by potently inhibiting mutans streptococci thus preventing dental caries (15). In addition, a marked decrease in the number of intracanal microbes was found by Khattab and Omar (2006) (16) following the application of the black seed oil for pulpotomy treatment of non-vital primary molars, and they reported that *Nigella Sativa* oil proved to be a potent antibacterial agent capable of killing anaerobic microbes as well as streptococci. In addition, it decreased the number of colonies of *Staphylococcus aureus*.

Other concentrations of black seed oil extract may be tested in further studies with different types of extracts. It is very important to develop guidelines for all procedures adopted in evaluating the antibacterial activity of black seed and analyze extracts of black seed from different regions for the actual ingredient which is responsible for their antibacterial activity. There is also an urgent need that a standard method should be devised for extract preparation.

REFERENCES
8. Nader M, Ali W, Al Thwaini A. Effect of *Nigella sativa* (black seeds), salvadora persica (siwak) and...


