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# ANTIBACTERIAL ACTIVITY OF *NIGELLA SATIVA* L. SEED OIL IN WATER EMULSION AGAINST DENTAL CARIOGENIC BACTERIA

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**Keywords:**

*Nigella sativa*, Oil-in-water emulsion, Antimicrobial, Dental cariogenic bacteria

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**ABSTRACT:** Our previous study highlighted the antimicrobial activity evaluation of *Nigella sativa* seed oil extract that was obtained from supercritical fluid extraction using carbon dioxide (SCFE-$CO_2$) on Gram positive pathogenic bacteria. The aim of this study was to formulate oil-in-water (o/w) emulsion of previous *N. sativa* seed oil extract and to investigate its antimicrobial activity against *Streptococcus mutans* and *Streptococcus sanguis* as the most common causative bacteria of dental caries. Oil-in-water emulsion containing 10% *N. sativa* seed oil extract (DLBS1355) exhibited good inhibition activity on both *S. mutans* and *S. sanguis*. Time-kill assay and biofilm inhibition assay were conducted using emulsion with 0.5% *N. sativa* seed oil extract content. The results showed bactericidal and biofilm inhibition activities on both tested bacteria. Antimicrobial activity of the emulsion was found higher on *S. sanguis* than *S. mutans*. Results of the present study showed that emulsification process did not significantly affect the antimicrobial activity of *N. sativa* seed oil extract and the formulated *N. sativa* oil-in-water emulsion could further be used as an antimicrobial agent against dental cariogenic bacteria.

**INTRODUCTION:** Dental caries and dental periodontitis are the most common dental-associated diseases especially in Asian countries. Compared to other Asian countries, Indonesia has higher prevalence of dental caries in children.[1] The prevalence of dental caries in Indonesia was increased about 10% in 2007-2013 that was predicted due to social economy factors in the rural area and the change of human eating habits. [2-3] Gram positive bacteria belongs to Streptococcus genus such as *Streptococcus mutans, Streptococcus salivarius, Streptococcus sanguis* and *Streptococcus sorbinus* have been found to play an important role in human dental decay. [4-7]

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The potency of some Indonesian medicinal plants has been widely highlighted by several studies for their therapeutic effect as anti-inflammatory, anticancer, hepatoprotector, and antidiabetic. [8-11] Natural-based remedies have also been recently recommended to substitute the uses of fluoride and other chemical compounds addressed for oral health. Exhibiting good antimicrobial activity, *Nigella sativa* L. seed, known as black cumin, has been widely used as food preservatives. [12-13] Antimicrobial activity of *N. sativa* seed against various strains of Streptococcus has also been reported previously. [14-15]

Anti-inflammatory, anticancer, antioxidant, immunomodulation, antiglycemic and hepatoprotective activities are some biological activities of *N. sativa* seed crude extract or essential oil that have been scientifically revealed, with thymoquinone as the main bioactive constituent.[16-19]

Previously, our study has been successfully designed the optimum extraction method of *N. sativa* seed oil extract using supercritical fluid extraction using carbon dioxide (SCFE-$CO_2$) and investigated its antimicrobial activity. The results showed that *N. sativa* seed oil extract was found to be more effective to inhibit the growth of Gram positive pathogenic bacteria. [20] In the present study, we evaluated the antimicrobial activity of oil-in-water (o/w) emulsion of previously obtained *N. sativa* seed oil extract, which was named as DLBS1355, against two most common causative dental cariogenic bacteria, *Streptococcus mutans* and *Streptococcus sanguis*.

## MATERIALS AND METHODS:

**Chemical and culture media:** Bacterial strains were obtained from American Type Culture Collection (ATCC). All bacterial media and chemicals were purchased from Merck (Germany) unless explained elsewhere and Kolliphor HS15 was obtained from Megasetia Agung Kimia (Indonesia).

**Preparation of DLBS1355 emulsion:** DLBS1355 o/w emulsion was prepared using Kolliphor HS15 and propylene glycol as surfactant. DLBS1355 o/w emulsion was made by mixing 1.4 g Kolliphor HS15 and 0.2 g propylene glycol for 20-30 min using magnetic stirrer at 60 °C. Then, 2 g of DLBS1355 was added and further mixed for 10 min. Purified water was added to obtain the final volume of 20 ml DLBS1355 o/w emulsion which contains 10% (w/v) *N. sativa* seed oil extract.

**Bacterial strains and culture condition:** Two strains of dental cariogenic bacteria belonging to Streptococcus genus were used in this study. *Streptococcus mutans* ATCC 35668 and *Streptococcus sanguis* ATCC 10556 were cultivated into brain heart infusion broth (BHIB) at 37 °C for 24 h prior to usage.

**Antimicrobial activity evaluation:** Preliminary evaluation of antimicrobial activity of DLBS1355 o/w emulsion was done by disk diffusion method according to the general guide from Clinical and Laboratory Standards Institute (CLSI) (2003). An approximately 106 CFU/ml of *S. mutans* and *S. sanguis* culture were respectively spread onto Mueller Hinton Agar, thereafter each sterile disk was added with 10 µl of DLBS1355 o/w emulsion, and then were placed on agar. All plates were then incubated at temperature of 37 °C for 48 h. Presence of clear zone around the disk was considered as inhibition zone and the diameter was measured.

**Minimum inhibitory concentration (MIC) determination:** The minimum inhibitory concentration (MIC) was determined by broth dilution method using a 96-microwell plate. The 24 h culture of each *S. mutans* and *S. sanguis* (approximately 107CFU/ml) were respectively inoculated into BHIB supplemented with various concentrations of DLBS1355 o/w emulsion. MIC was determined as the concentration that firstly exhibited a reduction on the visual growth.

**Time kill assay:** Time kill assay was assessed in broth medium as described in previous study21 with modification. Both *S. mutans* and *S. sanguis* were inoculated separately on BHIB and incubated for 5 h to obtain the log-phase culture (107-8 CFU/ml). DLBS1355 o/w emulsion was then added into the medium and incubated at temperature of 37 °C. The viable cells of *S. mutans* and *S. sanguis* were enumerated at 0, 15, 30, 75 and 120 min after treatment.

**Inhibition of biofilm formation:** The ability of the emulsion to inhibit the formation of bacterial biofilm was evaluated by method as described previously22 with minor modification. The biofilm formation inhibition assay was performed on 96-well microplate. *S. mutans, S. sanguis* and mixed culture of *S. mutans* and *S. sanguis* were respectively inoculated into BHIB that supplemented by glucose and o/w emulsion containing 0.5% *N. sativa* seed oil extract and incubated for 72 h. *S. mutans* and *S. sanguis* cultivated into BHIB without emulsion were determined as negative control. After incubation, the planktonic cells were discarded and biofilm formed on the bottom of the well was washed twice using sterile purified water. The biofilm was evaluated qualitatively by adding 0.01% crystal violet (v/v) for 30 min. The remaining crystal violet was discarded and washed twice with water.

**Scanning electron microscopy (SEM) analysis:** To investigate the effect of DLBS1355 o/w emulsion on bacterial cellular morphology and growth, visualization using scanning electron microscope was performed. *S. mutans* and *S. sanguis* were respectively cultured into emulsion supplemented BHIB with 0.5% *N. sativa* seed oil extract as the final concentration. After incubation, cells were separated from media by centrifugation and washed twice using sterile phosphate-buffered saline (PBS). A solution of 10% formaldehyde in PBS was used for fixation followed by gradual dehydration using increased concentration of ethanol. The dehydrated cells were air-dried and then coated with gold for further visualization using JEOL JSM 6510 scanning electron microscope.

## RESULTS:

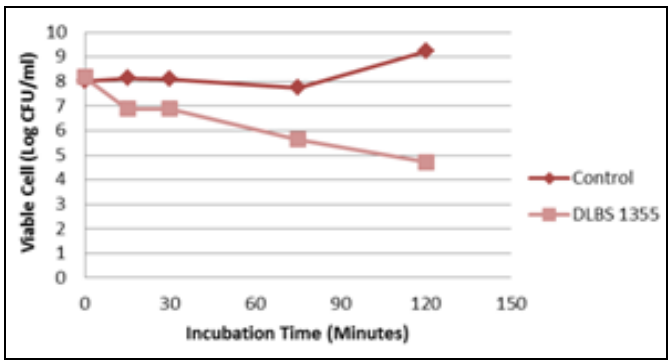
**Antimicrobial evaluation:** Disk diffusion assay was used in preliminary investigation of the antimicrobial activity of DLBS1355 o/w emulsion against *S. mutans* and *S. sanguis*. Optimization inhibition activity of the *N. sativa* seed oil extract against some Gram positive pathogenic bacteria was done in previous study. [20] Concentration of *N. sativa* seed oil extract used in optimization of inhibition activity in previous study was 3-10% of final concentration. Therefore, DLBS1355 o/w emulsion containing 10% *N. sativa* seed oil extract was used in this study. A marked inhibition zone was found in this study indicating a strong growth inhibition activity of DLBS1355 o/w emulsion against *S. mutans* and *S. sanguis* (**Table 1**). Emulsion without *N. sativa* seed oil extract did not inhibit *S. mutans* and *S. sanguis* growth (data not shown). The inhibition activity of DLBS1355 o/w emulsion shown in this study was similar to that obtained in our previous study.[20]

**TABLE 1: ANTIBACTERIAL ACTIVITY EVALUATION OF DLBS1355 O/W EMULSION**

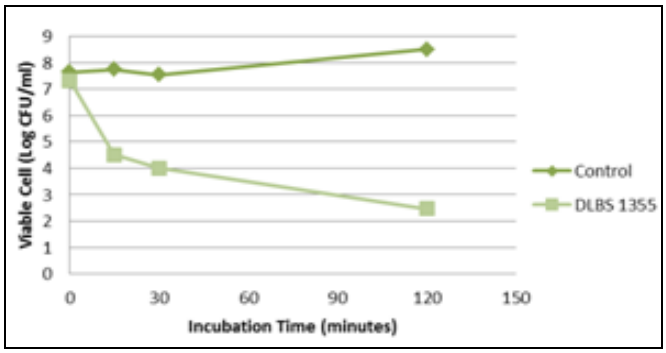
| Bacteria | Diameter of IZ (mm) * | MIC (%) ** |
|---|---|---|
| *S. mutans* | 32.75 ± 8.38 | 0.110 |
| *S. sanguis* | 52.00 ± 1.92 | 0.037 |

IZ: Inhibition zone; MIC: Minimum inhibitory concentration
*Conducted using DLBS1355 o/w emulsion containing 10% *N. sativa* seed oil extract
** Concentration of *N. sativa* seed oil extract in emulsion

**Time kill assay:** Time kill assay was performed at a 5-fold concentration of the highest MIC. Time kill assay of DLBS1355 o/w emulsion containing 0.5% *N. sativa* seed oil extract exhibited bactericidal effect against *S. mutans* (**Fig. 1a**) and *S. sanguis* (**Fig. 1b**). After 2 h of administration, the number of viable cells was reduced significantly. The bactericidal effect was found to be more effective against *S. sanguis* compared to *S. mutans.* Significant reductions on the number of viable cells were observed during the first 15 min for *S. sanguis*, and 75 min for *S. mutans*.




**a**




**b**

**FIG. 1: TIME KILL ASSAY OF DLBS1355 O/W EMULSION CONTAINING 0.5% *N. SATIVA* OIL EXTRACT AGAINST (a) *S. MUTANS* AND (b) *S. SANGUIS***

**Biofilm formation inhibition assay:** The effect of DLBS1355 o/w emulsion on biofilm formation was evaluated qualitatively using crystal violet staining. Compared to negative control, which was the untreated biofilm, the effect of DLBS1355 o/w emulsion containing 0.5% *N. sativa* seed oil extract was clearly seen on the inhibition of biofilm formation of both *S. mutans* and *S. sanguis* (**Fig. 2**). Similar to that shown on planktonic cells, the emulsion showed a more effective inhibition on the biofilm formation of *S. sanguis* compared to *S. mutans*, as shown by diameters of inhibition zone (**Table 1**).

The completely inhibited biofilm formation of *S. sanguis* indicates a significant reduction of the viable cells caused by DLBS1355 o/w emulsion.

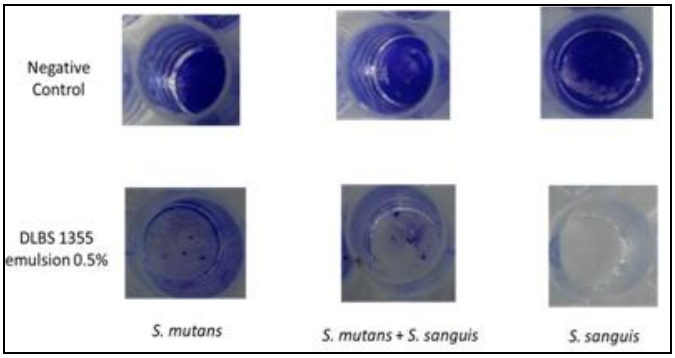



**FIG. 2: BIOFILM FORMATION INHIBITION ABILITY OF DLBS1355 O/W EMULSION VISUALIZED USING CRYSTAL VIOLET**

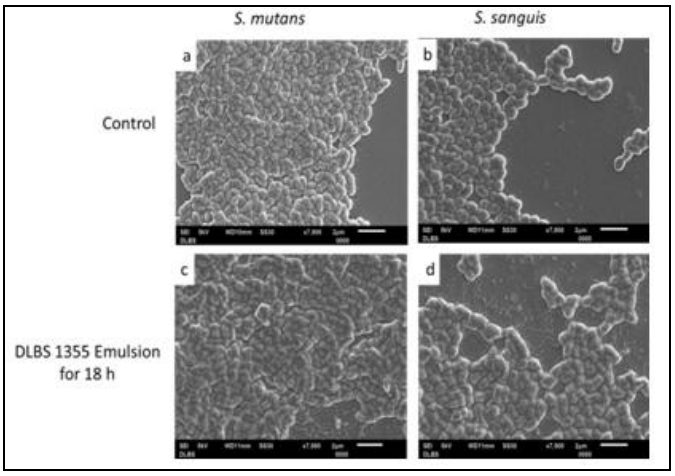



**FIG. 3: EFFECT OF DLBS1355 O/W EMULSION ON *S. MUTANS* (a, c) AND *S. SANGUIS* (b, d) CELLULAR MORPHOLOGY VISUALIZED USING SEM**

**Scanning electron microscopy (SEM) analysis:** The effect of DLBS1355 o/w emulsion on the cellular morphology of *S. mutans* and *S. sanguis* was examined using scanning electron microscope as shown in **Fig. 3.** After the growth of bacteria in DLBS1355 o/w emulsion-supplemented BHIB for 18 h, significant morphology alteration was found on *S. mutans* and *S. sanguis* (**Fig. 3c-d**). Cellular damages were clearly seen on the bacterial surface. The cell wall and cytoplasmic membrane disruption that were possibly caused by the emulsion activity allowed the cytoplasm and cellular constituents to be discharged from the cell.

**DISCUSSION:** Broad spectrum antimicrobial activity of *N. sativa* L. seed has been well-documented previously. However, our previous study and other studies have been highlighted on the higher antibacterial activity of *N. sativa* seed extract and oil extract on Gram positive bacteria. [20, 23-27] Furthermore, the antibacterial activity of *N. sativa* seed extract and its constituents against some strains of Streptococcus associated with dental caries has been reported as well.[14, 28] Consistent to the results of previous studies, the present study demonstrated a strong growth inhibition and bactericidal activity of DLBS1355 o/w emulsion against *S. mutans* and *S. sanguis*, with a slightly higher activity on *S. sanguis*.

The strong antibacterial activity showed in this study further indicated that emulsification process did not significantly affect antibacterial activity of *N. sativa* seed oil extract showed in previous study. As one of widely used lipophilic compound delivery systems, oil emulsification is able to enhance their solubility in aqueous-based system, increase the compound stability and somehow improve the biological activity. [29-33] One important virulence factor of oral Streptococci is its biofilm formation ability during its attachment on solid surfaces. The bacterial biofilms are more difficult to remove compared to planktonic bacterial cells. [34-35] Therefore, the evaluation of biofilm formation inhibition activity was also conducted in this study. DLBS1355 o/w emulsion, which contains 0.5% *N. sativa* seed oil extract, effectively inhibited biofilm formation of *S. mutans*, *S. sanguis* and mixed culture of *S. mutans* and *S. sanguis*.

The inhibition effect of *N. sativa* seed oil extract on biofilm formation of Gram positive bacteria was also reported previously with a concentration-dependent manner. [24] The fundamental enzymes involved on Streptococcus biofilm formation, glucosyltransferase and fructosyltransferase, were reported to be inhibited by synthetic antimicrobial agents and several essential oils of medicinal plants, including *N. sativa*. [36-40] Those compounds were also able to inhibit the adhesion of the Streptococcus cells and further affected the biofilm oxidative activity. [24, 41]

Inhibition effect on bacterial growth together with cell adhesion and physiological activity leads to the inhibition of biofilm formation. Antagonism effects on biofilm formation were commonly found on some strains of Streptococcus genus.

A study mentioned that the formation of *S. mutans* biofilm was suppressed by the presence of *S. oligofermentans.* [42] In this study, the biofilm formed by *S. mutans* cultured together with *S. sanguis* was slightly inhibited compared to the biofilm from single culture of *S. mutans* (**Fig. 2**), which was similar to the result of the previous study. The most suggested bacteriostatic and bactericidal mechanisms of natural antimicrobial agents including *N. sativa* are related to the integrity and function of the cell wall and cytoplasmic membrane. Peptidoglycan layer damage and cell wall leakage caused by several medicinal plants active compounds leading to morphological changes on gram positive pathogenic bacteria were reported previously. [43-44]

In this study, we also observed the significant morphological alteration on the cell growth of *S. mutans* and *S. sanguis* in DLBS1355 o/w emulsion-supplemented BHIB compared to untreated cells (**Fig. 3**). Supported by the results of previous studies mentioned above, the irregular apparent cell layer of *S. mutans* and *S. sanguis* observed in this study revealed the effect of DLBS1355 o/w emulsion on the structure and integrity of the bacterial cell wall. This cell wall structure-related effect may explain a higher antibacterial efficacy of our *N. sativa* seed oil extract on Gram positive bacteria than Gram negative bacteria. In addition, inhibition of cell wall synthesis gene expression was also reported to cell wall integrity, inhibition of ATP production and cell growth were as also reported due to the phenolic content of DLBS1355 [45]. Facing the multi-drug resistant pathogenic bacteria, discovery and development of new antimicrobial agents have already become a global concern.

This study highlighted the promising antimicrobial activity of *N. sativa* seed oil extract formulated in o/w emulsion against dental cariogenic bacteria and their biofilms, thus it could further be used for oral remedies to prevent and/or cure the common oral diseases. The antibacterial effect synergism of *N. sativa* seed essential oil with antibiotic was reported in several previous studies, therefore it may be used in combination with antibiotic. [28, 46] Further investigation is needed in order to investigate the cytotoxic effect of *N. sativa* seed o/w emulsion regarding to human use.

**CONCLUSION:** Based on the result of the present study, it can be concluded that *N. sativa* oil extract (DLBS1355) formulated in oil in water (o/w) emulsion possesses antimicrobial activity against dental cariogenic bacteria. It further showed the potential use of DLBS1355 o/w emulsion to prevent and/or cure the common oral diseases. Further study is needed to investigate its cytotoxicity regarding to human use.

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