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Cytotoxicity and Antimicrobial Activity of Black Cumin Reinforced with Hydroxyapatite Crystals by Green Synthesis Process: An Invitro Study

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Abstract---Introduction: *Nigella sativa* L. is an herb belonging to the Ranunculaceae family that grows in countries bordering the Mediterranean Sea. They are traditional Arab herbal medicines. The seed of *N. sativa* (black cumin or Habbatul Barakah) reported to have antibacterial, antifungal, antitumor, antihypertensive, antioxidant, hepatoprotective histamine release inhibitors. The recent development in the field of nanotechnology is by the imminent application of nano-hydroxyapatite in dentistry for remineralization of enamel. The aim of the study is to incorporate the black cumin seed extract with hydroxyapatite crystals into a new formulation for the first time to evaluate the antimicrobial and cytotoxic properties and to characterize their structural and morphological properties. Materials and method: The organisms used were *Streptococcus mutans*, *Staphylococcus aureus*, *E. Faecalis* and *Candida albicans*. Agar well diffusion method was used to assess the antimicrobial efficacy of the nanoparticles at various concentrations that ranges from 25 μ L, 50 μ L and 100 μ L. The synthesized nanoparticles were characterized by using ultraviolet double beam spectrophotometers in the wavelength range of 250-750nm. Results: Zone of inhibition was found to be highest at 100 μ L concentration against *Staphylococcus mutans* and *Candida albicans*. The mean zone of inhibition was found to be

increased as the concentrations of black cumin hydroxyapatite synthesized particles increased. Conclusion: The NS have been shown to have a variety of possible therapeutic effects on a variety of oral and dental diseases

Keywords---black cumin, green synthesis, hydroxyapatite crystals nigella sativa.

Introduction

The interest towards the medicinal herbs and plants in the use for the treatment of disease has increased worldwide. Because the medicinal herbs and plants have less side effects than the synthetic drugs(1). There are more than 500 habitats in the oral cavity and are the etiological cause for dental caries, periodontal disease and fungal disease. Many systemic diseases such as infective endocarditis, respiratory disease and cardiovascular diseases. One of the main causative agents for dental caries is streptococcus mutans and Streptococcus sobrinus. Recent investigations show that natural compounds are promising agents for dental caries prevention. Nigella sativa L. is an herb belonging to the Ranunculaceae family that grows in countries bordering the Mediterranean Sea. They are traditional Arab herbal medicines. The seed of N. sativa (black cumin or Habbatul Barakah) reported to have antibacterial, antifungal, antitumor, antihypertensive, antioxidant, hepatoprotective histamine release inhibitors(2). The seeds contain 28-36% fixed oils, alkaloids, saponins, proteins and 0.4-2.5% essential oils. The most reported active compound of N. sativa seeds is thymoquinone (TQ) and other compounds such as dithymoquinone, thymol, and thymohydroquinone(3). Shaker and Al-Wafi's experimental use of TQ in gingival inflammation in a rat model resulted in reduced gingival inflammation in the rats which are treated with TQ(4).

Tooth enamel is the most mineralized tissue of the human body. Composition of enamel is 96 wt.% inorganic material and 4 wt.% organic and water. The inorganic content of dentine is 70 wt.%. This inorganic material contains a calcium phosphate related to the hexagonal hydroxyapatite, and the chemical formula is $\text{Ca}_{10}(\text{PO}_4)_6 \cdot 2(\text{OH})$ (5). The main component of enamel is the hydroxyapatite (HA), that gives a bright white appearance and eliminates the diffuse reflectivity light by closing the small pores on the enamel. Hydroxyapatite is the most studied biomaterials in the medical field for its proven biocompatibility and as it is the main constituent of bone and teeth. It is also an important source of calcium and phosphate, which is very important for the remineralization of enamel. The inorganic components in the human body are made up of calcium and phosphate salts. Other inorganic materials such as calcium carbonates and sulphates are present in smaller quantities. Hydroxyapatite crystals represent 60–70% and 90% in weight of bone and enamel respectively.

The recent development in the field of nanotechnology is by the imminent application of nano-hydroxyapatite in dentistry for remineralization of enamel. The crystal's size ranges between 50 and 1000 nm. Nano-hydroxyapatite crystals

have a strong ability to bond with proteins and with fragments of plaque and bacteria. This is because of the size of the nanoparticles, which increase the surface area and where proteins can bind. Sangi Co. Ltd a Japanese company was the first to take an interest in hydroxyapatite, after purchasing the rights from NASA (U.S. National Aeronautics and Space Authority) in 1970. Since NASA proposed a synthetic hydroxyapatite as a repairing material. The Sangi Co. Ltd had the idea to launch toothpaste that can repair and remineralize the tooth enamel, which is the first time nano-hydroxyapatite contains toothpaste (Apadent)(6).

The current study aimed to incorporate the black cumin seed extract with hydroxyapatite crystals into a new formulation for the first time to evaluate the antimicrobial and cytotoxic properties and to characterize their structural and morphological properties.

Materials and methods

Study setting: The study was done in BLUE LAB (microbiology and biochemistry lab), Saveetha Institute of Medical and Technical Science (SIMATS), Chennai.

Ethical approval: Ethical approval was obtained from institutional review board, Saveetha University (SIMATS).

Green synthesis: Black cumin seeds are brought from a supermarket near Porur, Chennai. The black cumin seeds are first made into fine powder. Then 1g of powder is exactly weighed in the weighing machine (Figure 1a). The weighed black cumin powder is dissolved in 100mL of distilled water which is measured in a measuring glass cylinder and boiled in the heating mantle for 5-10 mins at 50 to 70° C. After boiling, 50 mL of the black cumin powder dissolved in the distilled water is filtered using Whatman filter paper no.1 with the help of a funnel. 0.5 g of hydroxyapatite crystals was added to the filtered black cumin extract in a conical glass container (figure 1b,c). This conical glass container is placed in the magnetic stirrer and stirred at 350 to 450 rpm for continuous 3 days (Figure 1d,e). Intermittent reading was taken at 1,4,28,33 hours in a double beam spectrometer to determine the amount of light of a specific wavelength absorbed by an analyte in the sample. After 3 days the sample is poured in a glass plate and placed in the hot air oven at 90 degree Celsius till it dries. After drying the glass plate is scrapped and the powder is collected in a small vial and weighted. The weighted nanoparticle is 0.466 g.

Figure 1. Green synthesis process of black cumin and hydroxyapatite powder particle

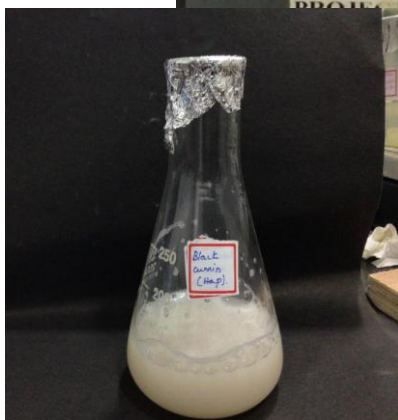
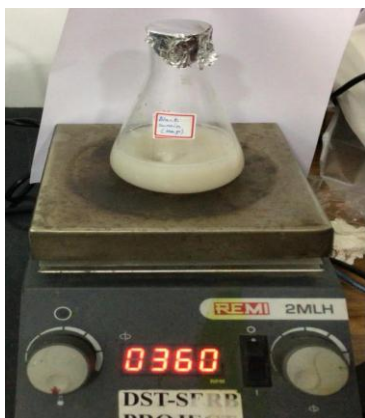


1a. 1g of power hydroxyapatite crystals



1b. 0.5 g of

1c. filtered black cumin extract



1d. Conical glass container is placed in the magnetic stirrer
1e. After 3 days the stirred composition

Cytotoxicity

0.1g of nanoparticle sample is mixed in 1 ml of distilled water. For cytotoxicity the samples are mixed in different concentrations 10 μ l, 20 μ l, 30 μ l, 40 μ l, 50 μ l with 10 nauplii in each concentration (Figure 2). The count of the live nauplii is counted in a 24 hours interval and the readings are recorded.

Figure 2. Cytotoxicity test done for black cumin- hydroxyapatite crystal particles in different concentrations 10 μ l, 20 μ l, 30 μ l, 40 μ l, 50 μ l with 10 nauplii



Media Preparation

Mueller Hinton agar for *Streptococcus mutans*, *Staphylococcus aureus*, *E.Faecalis* and SDA agar for *Candida albicans* and was prepared, sterilized and poured onto the Petri plates. The plates were allowed for solidification. Agar Well Diffusion method was used to assess the antimicrobial efficacy.

Swabbing

After solidification, the respective plates were swabbed with the oral pathogens- *Streptococcus mutans*, *Staphylococcus aureus*, *E.Faecalis* and *Candida albicans*.

Well Formation

After swabbing, three wells on each plate were formed using a gel puncher. To those three wells, green synthesized black cumin and hydroxyapatite particles were loaded in the concentration range of 25 μ L, 50 μ L and 100 μ L. The plates were then incubated at 37°C for 24 hrs for *Streptococcus mutans*, *Staphylococcus aureus*, *E.Faecalis* and 48 h for *Candida albicans*. After incubation, the zone of inhibition was measured and calculated

Figure 2: The antimicrobial activity test of green synthesized blackcumin and hydroxyapatite particle extract against *Streptococcus mutans* (a) and *Staphylococcus aureus* (b)

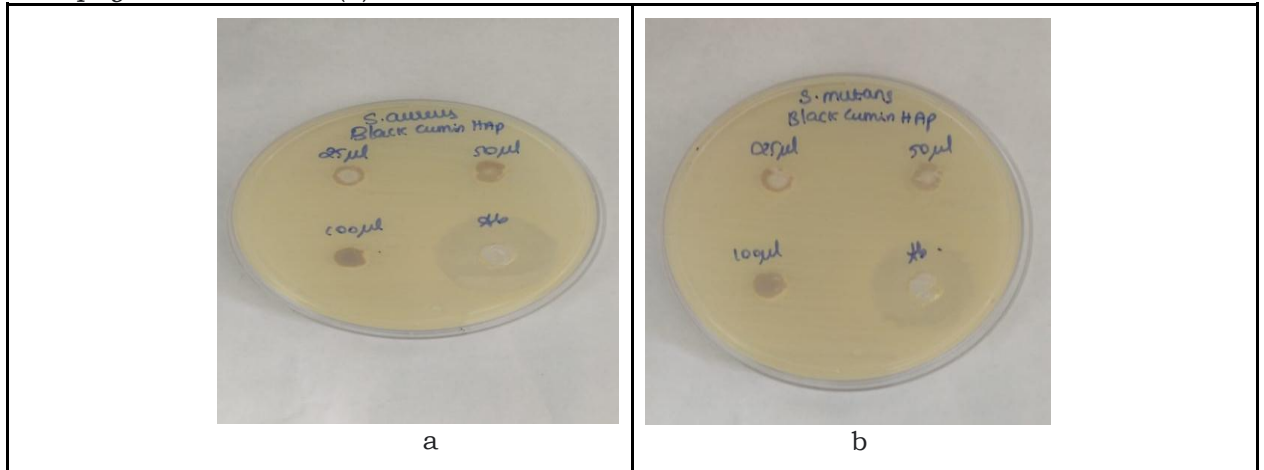
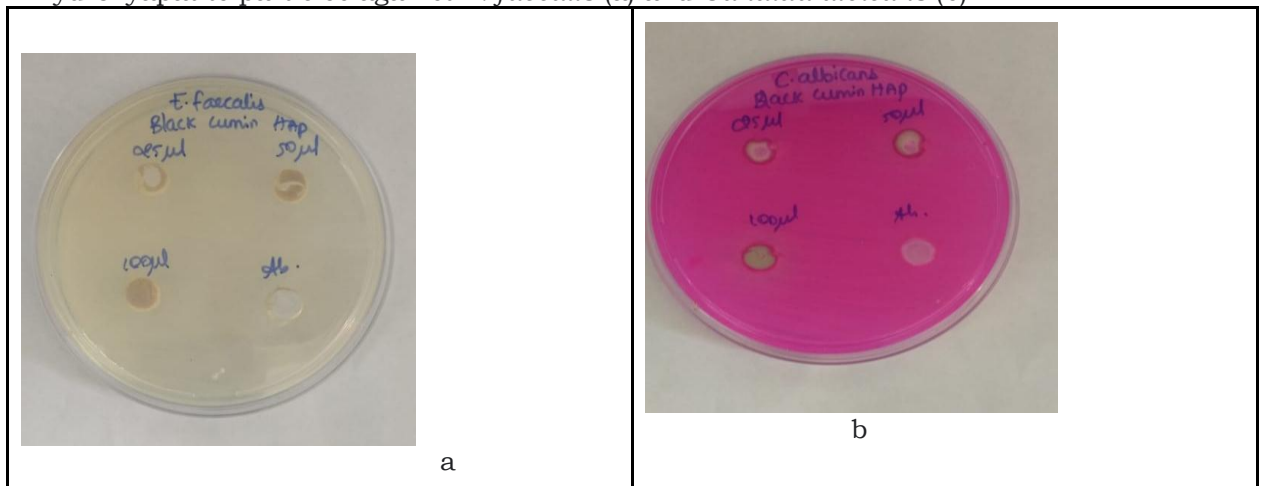


Figure 3: The antimicrobial activity test of green synthesized blackcumin and hydroxyapatite particles against *E. faecalis* (a) and *Candida albicans* (b)



Results

The results for cytotoxicity were mentioned in table 1. The results show that on day 1 all the nauplii were alive in the well plate. But on day 2 in 10 μL conc. 8 were alive, 20 μL conc. 7 were alive and in 30 μL, 40 μL and 50 μL conc. 9, 9, 8 were alive respectively. The diameter of the inhibition zone for all tested concentrations of green synthesized blackcumin and hydroxyapatite particles achieved for bacterial strains is presented in Table 2. Figure 2 depicts the antimicrobial activity of green synthesized blackcumin and hydroxyapatite particle extract against *Streptococcus mutans* and *Staphylococcus aureus*. Zone of inhibition against *Streptococcus mutans* was found at 25 μL was 9 mm, 50 μL was 10 mm and at 100 μL was 10 mm. Zone of inhibition against *Staphylococcus aureus* at 25 μL was 10 mm, at 50

μL was 11 mm and at 100 μL was 12 mm. Figure 3 depicts the antimicrobial activity of green synthesized blackcumin and hydroxyapatite particles against *E. faecalis* and *Candida albicans*. Zone of inhibition against *E. faecalis* at 25 μL was 9 mm, 50 μL was 9 mm and at 100 μL was 9 mm. Zone of inhibition against *Candida albicans* at 25 μL was 9 mm, 50 μL was 9 mm and at 100 μL was 11 mm. So, it was seen that as the concentration of the green synthesized blackcumin and hydroxyapatite particles increased, the antimicrobial activity increased.

Table 1. Cytotoxicity test of green synthesized black cumin and hydroxyapatite crystals (no of live nauplii in the end of day1 and day2)

Concentration	Day1	Day2
10 μl	10	8
20 μl	10	7
30 μl	10	9
40 μl	10	9
50 μl	10	8

(Counts of live nauplii in day1 and day2)

Table 2. Antimicrobial test and zone of inhibition of pathogens against green synthesized black cumin and hydroxyapatite

Name of the pathogen	Zone of inhibition			
	25 μl	50 μl	100 μl	Ab
S.mutans	9mm	10mm	10mm	20mm
S.aureus	10mm	11mm	12mm	30mm
E.faecalis	9mm	9mm	9mm	40mm
C.albicans	9mm	9mm	11mm	12mm

(Zone of inhibition given in millimeter)

Discussion

Owing to increased drug use, microorganisms have developed resistance to many antibiotics, reducing the effectiveness of traditional medicines. As a result, new antimicrobial agents must be discovered (7). Nanotechnology is a relatively modern technology that has ushered in a new era in the area of research. Among the various inorganic nanoparticles available, blackcumin and hydroxyapatite crystals are simple to process, inexpensive, has a wide range of dental applications, and is a safe material (8).

The results of our study showed that black cumin green synthesized with hydroxyapatite crystals has some antimicrobial property against *Streptococcus mutans* and *Staphylococcus aureus*. There is no antimicrobial activity against *E. faecalis*. There is marked antifungal activity against *Candida albicans* equal to the level of antibody. The cytotoxicity test of black cumin- hydroxyapatite reveals that it can be safely used as medicinal drug for further study .

Some researchers recently looked into the impact of natural products on the prevention of dental caries (9,10). The current study's result showed the antimicrobial activity against *S. mutans*, where the zone of inhibition is 10 mm at 100µl concentration. Only one study using a rat experimental model by Nasuti C et al., found, and aimed to look into the impact of NS on caries initiation by inhibiting microbiome formation (11). According to an animal study by Nasuti C et al., they were infected with *S. mutans* and fed a high-sugar diet. In contrast to the control groups, rats given 10 mg of TQ per kilogramme of body weight (in kg) in oral gel or drinking water had a statistically significant reduction in caries score ($p=0.02$) and plaque index ($p=0.01$). The authors credited TQ's microbial biofilm inhibition properties for the reduction in dental caries. In our investigation we found that the black cumin-hydroxyapatite crystals have anticariogenic properties as it is effective against streptococcus mutans. Harzallah et al conducted research to determine the anticariogenic activity and TQ of Tunisian NS essential oil. They used the broth microdilution method to determine the NS essential oil's minimum inhibitory concentration (MIC) and TQ for oral cariogenic pathogens (12). Many experiments have been conducted to examine the antimicrobial effect of NS. An in vitro study by Rahman M et al., was conducted to evaluate the NS's inhibitory and resistance-modifying abilities against a panel of pathogenic bacteria, including oral isolates (13). In our study there is no effect of black cumin-hydroxyapatite against *E. faecalis* showing it is a selective antimicrobial agent. According to Epanand RM et al., NS exhibited a selective antimicrobial property, as it was effective against Gram positive bacteria but ineffective against Gram negative bacteria, according to the findings(14).

Oral candidiasis is a common oral infection caused by fungi, most commonly *Candida albicans*. Study by Randhawa MA et al., and Khan MA et al., is the first of its kind since it looked at the antifungal properties of Amphotericin B, ketoconazole, and TQ in nanoparticle form against *Candida albicans* (yeasts and biofilm) in contrast to their traditional forms in vitro (15,16,17). The antifungal property in the current study showed the black cumin-hydroxyapatite is very effective against *Candida albicans*.

Black cumin green synthesized with hydroxyapatite crystals can be used as an alternative in the form of antibiotic and antifungal drugs for the prevention of oral disease. The limitations of the study are problems to extrapolate from *in vivo* doses to *in vitro* concentrations, difficulties in simulating the consequences of long term exposures. Further the study has to be carried out incorporating the thymoquinone with this green synthesized particle to form a nano formation and to check for their antimicrobial properties.

Conclusion

In conclusion the black cumin - hydroxyapatite novel formulation was effective against *S. mutans* and *S. aureus*, and *Candida albicans*. But it has no effect against *E. faecalis*. We can consider black cumin-hydroxyapatite has antibacterial activity against dental pathogens and is non toxic.

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