Original Article

Comparative Evaluation of Antibacterial Efficacy, Molecular Docking of Ethanolic Extract of Blackseed, Seaweed and Calcium Hydroxide Intracanal Medicament with Enterococcus Faecalis Antigens

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ABSTRACT

Aim: To evaluate the inhibitory effect of ethanolic extract blackseed, seaweed, and calcium hydroxide intracanal medicament with Enterococcus faecalis biofilm. To study the binding interaction between the active components of blackseed and seaweed against the enterococcal surface protein of (*E. faecalis*) by molecular docking. **Materials and Methods:** The ethanolic extracts of blackseed and seaweed were prepared using the Soxhlet apparatus. They were divided into three groups, namely, |Group I: Calcium hydroxide, Group II: Blackseed, and Group III: Seaweed. The antibacterial activity of the three groups was detected employing various concentrations ranging from 250, 125, and 62.5 µg/ml and based on the zone of inhibition. The inhibitory potential of medicaments to inhibit *E. faecalis* growth at various stages and kinetics plate were assessed following biofilm architecture evaluation by crystal violet biofilm assay. With the Swissdock suite, the molecular docking procedure was carried out. PyMOL version 4.1.5 was the program used for visualization. Since enterococcal surface protein (Esp) is primarily involved in the formation of biofilms, it was chosen as the target protein of *E. faecalis*. Based on their chromatographic investigations, Group II Thymoquinone (TQ) and Group III Ledenoxide were chosen as ligands. **Results:** The percentage of inhibition of *E. faecalis* biofilm was analyzed as statistically significant observed within groups. On post-hoc analysis, significant differences were present between the groups ($P < 0.05$). Molecular docking reveals binding energies of thymoquinone (Group II) and ledenoxide (Group III) against the enterococcal surface protein of *E. faecalis* were −6.90 Kcal/mol and −6.44 Kcal/mol, respectively. **Conclusion:** Compared to seaweed, black seed extract exhibited higher antibacterial activity against the *E. faecalis* biofilm in microbial inhibition and molecular interaction.

Keywords: *Antibiofilm, E. faecalis, molecular docking*

Introduction

The primary factor contributing to the failure of root canal therapy is the development of bacterial biofilm and its by-products in nonvital root canal systems, which result in apical periodontitis.[1] Chemo-mechanical preparation is used to eliminate the maximum possible bacteria lodged inside the root canal system.[2] However, it has been shown that it is impossible to achieve complete disinfection through cleaning and shaping.[3] *E. faecalis* plays an important role in the etiology of persistent periradicular lesions post the endodontic treatment.[4] *E. faecalis* can withstand harsh environmental conditions and maintain their viability for extended periods.[5]

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Calcium hydroxide ($Ca[OH]_2$) was first introduced in 1920 as a crucial pulp therapy agent by Herman, and intracanal medication was subsequently employed.[6] Calcium hydroxide is easily identified by its white color, high pH, and low solubility in liquids. The calcium and hydroxy ions that were separated from the $Ca(OH)_2$ have bactericidal and bacteriostatic properties.[7] It is currently more affordable and accessible.[8]

Nigella sativa, commonly called blackseed, which has a lot of medical potential was not explored for endodontic purposes.[9] It contains several energetic active ingredients, such as alkaloids, flavonoids, thymoquinone (TQ), saponins (alpha-hederin), proteins, and fatty acids, which can be used to treat a variety of illnesses. These oil extracts have anti-tumor, anti-inflammatory, antibacterial, and anti-mutagenic qualities, among others.

Stoechospermum marginatum, otherwise called brown seaweeds, is a naturally occurring organic and inorganic content that is beneficial to human health. Seaweeds are easily found in seawater, which contains one million bacterial cells per millimeter. Because algae are exposed to osmosis, UV light, and high salinity, they have exceptionally good biological and antibacterial qualities and can thrive in any type of environment.[10-12]

One computer-based technique to assess the interaction of medications is molecular docking. It is a preliminary, time-saving and effective process at the molecular level. The mechanism of protein to ligand interaction is evaluated, and their binding energies are valued as positive or negative.[13]

The current study aims to comparatively evaluate the molecular docking effect of calcium hydroxide and the extracts of brown seaweed (*Stoechospermum marginatum*) and black seed (Nigella sativa) against the persistent microbe *E. faecalis.*

Materials and Methods

Collection of seaweeds and preparation of ethanolic extract

Fresh brown seaweeds, s*toechospermum marginatum* were collected at Mandapam, Tuticorin district, R.K Algae Centre, Tamil Nadu, India. The collected samples were cleaned (to remove surface salts) by using sterile distilled water. At 50°C an hour, it was dried and milled in an electrical blender. Each 250g of powder was kept under No. 1 Whatman filter. The analytical-grade ethanol in the sample was used in the Soxhlet apparatus for 72 hours. The extracts were concentrated to be solvent-free by evaporation in a rotary vacuum evaporator at a temperature of <40°C. The crude extract was stored at 4°C for further analysis.

Preparation of black seed ethanolic extract

The black cumin seeds, *Nigella sativa* (Jothi herbals shop at Chidambaram town, Tamil Nadu), were prepared in the same way as previously explained in seaweed preparation.

Calcium hydroxide

The calcium hydroxide was purchased from the prime dental company and was used in this study.

Micro-organisms

Enterococcus faecalis strain (MTCC 439) was obtained in freeze-dried form from the microbial collection (MTCC), Chandigarh, India. The organism was revived into a live culture in the laboratory setting.

Antibacterial efficacy: Agar well diffusion method

0.5 McFarland standard suspension was adjusted to give 10^6 CFU/ml (final inoculum). A culture of inoculum was made on the dry surface of the Muller–Hinton agar plate (Himefia). The bacterial wells with diameter of 6mm was made using a sterile cork borer. The wells were loaded with 20 µl of the test samples with different concentrations (1000 µg, 500 µg, 250 µg, and 125 µg prepared with 10% Dimethyl sulfoxide (DMSO)). 20 µg of Gentamycin was taken as a positive control. After overnight incubation (24 h at 37°C), plates were observed in the zone of inhibition around the wells.

Micro-broth dilution method

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. The MICs of crude extracts were determined in brain heart infusion (BHI) by using a modified resazurin micro-titre plate assay.

Biofilm assay by using crystal violet quantitative assay

Procedure

According to Toledo-Arana *et al.*[14] (2001) with some modifications, the biofilm was quantified to detect the effect of the test medicament on the biofilm. In a bacterial suspension, a tube consisting of 1×10^6 CFU/ml of *E. faecalis* in BHI broth was supplemented with 1% (wt./vol) sucrose. Various concentrations of test materials were incubated for three days in a 37°C environment. Media and test material with their respective concentrations were refilled every 24 hours. The cells containing planktonic were removed 72 hours post incubation by aspiration methods. At pH 7.4, using phosphate buffered saline solution (PBS) twice to remove the unbounded microbial cells and other compounds from medicated wells. Next, 125 µl of 0.1% crystal violet solution was added to each well to stain

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biofilm-forming cells and incubated for 10 min at room temperature. In 95% ethanol at 37°C for 30 minutes, the medicated plates were shaken and kept under for absorbance recorded at 595 nm. The assay was performed in triplicate.

Molecular docking

All docking calculations were performed using the Swissdock suite. The docking process is divided into four steps: preparation of the protein, preparation of the ligand, generation of the receptor grid, and docking of the ligand. PyMOL version 4.5.0 was used as visualisation software. The target protein of *E. faecalis*, the enterococcal surface protein (Esp), was selected because of its importance in biofilm formation. Based on chromatographic studies, thymoquinone of Group II and ledene oxide of Group III were selected [Figure 1].

Procedure

From the research collaboratory for structural bioinformatics (RCSB) protein data bank (PDB), the protein structure of Esp (PDB ID:60RI) was used for this procedure. The molecular structures of the ligands thymoquinone (Pub Chem CID: 10281) 100 and ledene oxide (Pub Chem CID: 534497) 101 were obtained from the PubChem database. The target protein and the ligands were downloaded in PDB and mol2 format, respectively. Preparation of the target protein and the ligands includes the elimination of water molecules, addition of charges, identification of the active binding site of the target protein, and calibration.

The 'DOCK' command is given to find the best protein-ligand pose. The binding energy is expressed in Gibbs free energy (ΔG) [Figure 2].

Results

The zones of inhibition in different concentrations (1000 µg/ml; 500 µg/ml; 250 µg/ml; and 125 µg/ml) of all groups against *E. faecalis* were analyzed. Post-hoc analysis shows the significance formed by the difference between each concentration of test samples. The minimum inhibitory concentration of each test compound was determined to be 125 µg/ml for Group I, 62.5 µg/ml for Group II, and 250 µg/ml for Group III. There is a statistically significant difference between the test groups at their minimum inhibitory concentrations [Table 1].

The percentage of inhibition was analysed and showed that Group I (mean-74.2367), Group II (mean-52.1333), and Group III (mean-38.3167) at a concentration of 250 µg. At 125 µg concentration, the percentage of inhibition is shown by Group I (mean-60.31), Group II (mean-40.33), and Group III (mean-24.50). At 62.5 µg concentration, the percentage of inhibition was Group I (mean-50.08), Group II (mean-39.09), and Group III (mean-23.02) [Table 2].

The docking results showed that the binding energy of Group II had a Delta G value of −6.90 kcal/mol and that of Group III had a Delta G value of −6.44 kcal/mol [Table 3].

Discussion

In a complex ecology, with less nutrient supply, *E. faecalis* can adapt to environments accordingly. In most of the studies, the growth of *E. faecalis* forms biofilm, which is the root cause of the failure

Table 1: Intergroup comparison of minimum inhibitory concenteration

Dependent variable	MIC (µg/ml)	*P*
Group I vs Group II	125/62.5	0.000*
Group II vs Group III	62.5/250	0.000*
Group III vs Group I	250/125	0.000*

*$P<0.005$

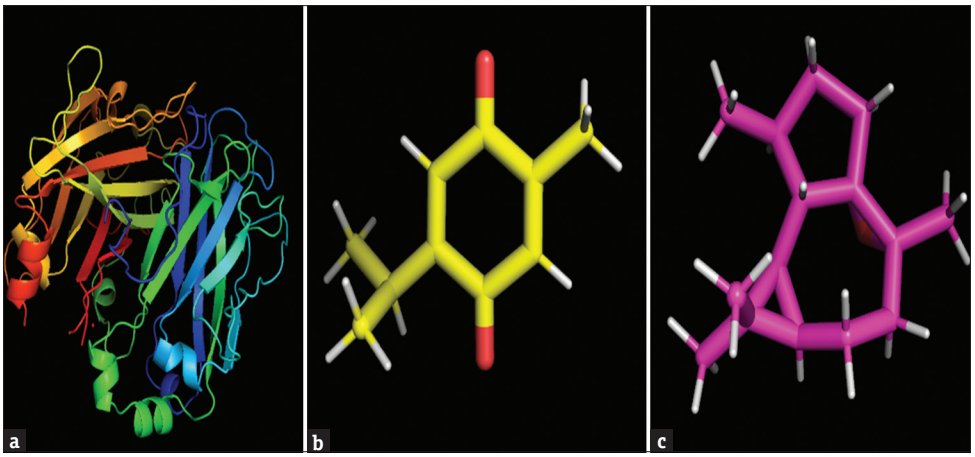

Figure 1: The molecular structure of protein target-Enterococcal surface protein (a) and ligands, thymoquinone (b), and ledene oxide (c)

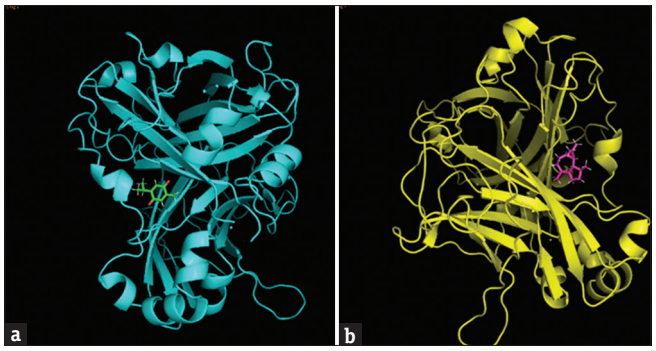

Figure 2: Comparing the binding energy ((a) and (b)) using molecular docking

of endodontic treatment. Hence, the *E. faecalis* biofilm model was taken against effect by using test medicaments in this study.[15]

The molecular docking through SWISSDOCK software detected the level of binding energy between the target protein of the bacteria and the active component of the plant extract, narrowed down from their respective chromatographic studies. The active components of Group II and Group III were docked against the Esp of *E. faecalis*. Due to difficulties in parametrization, molecular docking could not be performed with hydroxyl ions of Group I.

The molecular docking results showed that the binding energy of Group II had a Delta G value of −6.90 kcal/ mol and that of Group III had a Delta G value of −6.44 kcal/ mol. The results of molecular docking show that Group II has more affinity for the target protein.

The results of Group I correlate with the study done by Chai *et al*.[16] The study compares calcium hydroxide with six different antibiotics in a membrane filter model. The biofilm was completely inhibited by calcium hydroxide as indicated by the results. Because of the *in vitro* plate model, the pH of calcium hydroxide was high. The antibacterial activity of calcium hydroxide is due to the pH gradient caused by the release of hydroxyl ions. Estrela *et al*.[17] stated that this high pH causes chemical injury to the organic components and transport proteins or through the destruction of phospholipids or unsaturated fatty acids of the cytoplasmic membrane during the peroxidation process, which is a saponification reaction.

Kokoska *et al*.[18] reported that TQ is the main component of black seed. TQ has a potent growth inhibitory effect against gram-positive bacteria, with MICs ranging from 8 to 64 µg/ml. In his study, he also concluded that the solvent extraction method is more effective in bringing out a broad spectrum of antimicrobial properties. The results of Group II correlate with this study.

Table 2: Intergroup comparison of percentage inhibition of tested compounds against *E. faecalis* biofilm

Concentration	Study groups	*n*	Mean	*P*
250 µg/ml	Group I	3	74.2367	0.01*
	Group II	3	52.1333	
	Group III	3	38.3167	
125 µg/ml	Group I	3	60.31	0.01*
	Group II	3	40.33	
	Group III	3	24.50	
62.5 µg/ml	Group I	3	50.08	0.01*
	Group II	3	39.09	
	Group III	3	23.02	

*P<0.05

Table 3: Comparing the binding energy of calcium hydroxide, thymoquinone (blackseed), and ledene oxide (brown seaweed) through molecular docking

Test organism	Target protein	Herbal compound	Active compound	Highest affinity
E. faecalis	Nil	Calcium hydroxide	Nil	Nil
E. faecalis	Esp	Blackseed	Thymoquinone	−6.90 cal/mol
E. faecalis	Esp	Brown seaweed	Ledene oxide	−6.44 cal/mol

In regards to the anti-biofilm activity, Chaieb *et al*.[19] their study proved that TQ prevents about 90% of the plaque biofilm formation in various bacterial strains, including *E. faecalis*. TQ not only reduces the number of living cells but also inhibits the oxidative activity of microbial cells present in the biofilms.

One of the most significant resistance mechanisms in pathogens is the expression of efflux pumps (EPs). The efflux pumps present in the bacterial cell membranes are responsible for the extrusion of antibiotics as well as the limitation of the intracellular build-up of hazardous substances like metals, detergents, antibiotics, and antimicrobial peptides.[20] Paster *et al*.[21] showed that TQ effectively inhibits the activity of these efflux pumps in bacteria.

The results of Group III are supported by Anjali *et al*.,[22] who revealed the presence of major bioactive compounds that exhibit antibacterial activity to be ledene oxide and 1-heptatricotanol. In addition, the bactericidal activity of the brown algae could be due to the presence of free fatty acids (cyclopentane tridecanoic acid and tetradecanoic), which consequently enhance its overall defensive action against the gram-positive and gram-negative bacteria. Chinnababu *et al*.[23] reported that the antibacterial activity of S. marginatum was mainly due to the various phytonutrients that may affect the growth and metabolism of bacteria.

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Both Shankar *et al*. and Hufnagel *et al*. claim that *E. faecalis* may have an ecological advantage over other pathogens due to its ability to form biofilms. The enterococci surface protein is encoded by the Esp gene and may be involved in the colonization and persistence of *E. faecalis* during infections. It is most likely the mediator of the pathogen's initial contact with host surfaces during the biofilm-forming process.[24,25] Because of the ligands' strong affinity for the protein, Esp may be a useful target, and inhibiting it may have an impact on the formation of biofilms.

Similar research was conducted by Geeta *et al*.,[26] which examined ledermix and licorice's antibacterial efficacy against *E. faecalis* using the molecular docking method in conjunction with microbial inhibition. Licorice exhibited the highest antibacterial activity in both microbial inhibition and molecular docking, according to the findings.

Conclusion

A molecular docking study showed that blackseed had more affinity towards the enterococcus surface protein when compared to brown seaweed. The study shows that black seed and brown seaweed natural extracts can be used as intracanal medicaments. Further studies will focus on biocompatibility and clinical trials as an intracanal medicament.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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