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Original Article

Synthesis and Characterization of Omega-3 Fatty Acid-Infused Microspheres: An *in vitro* Exploration of their Cytotoxic, Anti-Inflammatory, and Antioxidant Virtues

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ABSTRACT

Background: Omega-3 fatty acids are vital polyunsaturated fats that are fundamental to maintaining overall health and well-being. **Objectives:** This research focused on the preparation of omega-3 fatty acid microspheres and evaluated their cytotoxicity, anti-inflammatory and antioxidant properties. **Materials and Methods:** Omega-3 fatty acid microspheres were developed using the water-in-oil-in-water (w/o/w) double emulsion method. The resulting microspheres were analyzed for their surface characteristics using Scanning Electron Microscopy (SEM). Various concentrations (25, 50, and 100 µg/mL) were subjected for characterization. Cytotoxicity of the synthesized microspheres was assessed using brine shrimp lethality assay. Additionally, their anti-inflammatory and antioxidant properties were assessed through protein denaturation and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays, respectively. **Results:** Particle size analysis showed that over 90% of the produced microspheres ranged from 32 to 55 µm. Nauplii lethality was assessed over two days. At 25 µg/mL, no mortality was observed on either day. However, on day 2, mortality was 20% at both 50 µg/mL and 100 µg/mL concentrations. Additionally, protein denaturation and DPPH assays indicated a significant increase in inhibition percentages as the concentration of omega-3 fatty acid microspheres increased. **Conclusion:** The omega-3 fatty acid microspheres developed in this study was non-cytotoxic and exhibited outstanding anti-inflammatory and antioxidant activities, making them an ideal candidate for local drug delivery applications.

Keywords: Antioxidant, Fatty acid, Free radical, Inflammation.

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INTRODUCTION

Periodontal diseases, particularly periodontitis, contribute significantly to the global disease burden. They have devastating effects on oral health and are closely linked to general health, impacting both individual well-being and public health systems. As a result, they lead to substantial socio-economic consequences and incur massive healthcare costs worldwide.[1] Periodontal diseases, including gingivitis and periodontitis, rank among the most prevalent diseases in humans. Gingivitis is an inflammatory condition triggered by plaque biofilms and is characterized by redness, swelling, changes in the contour of the gums, and bleeding upon gentle probing. It does not involve attachment loss or alveolar bone resorption. In contrast, periodontitis is a chronic, multifactorial inflammatory disease caused by microbial imbalances and an improper host response. It is marked by the progressive destruction of the tissues that support teeth, including the loss of attachment and resorption of alveolar bone.[2]

The etiopathogenesis of periodontitis involves complex interactions between microbial factors and the host immune response. The process begins with the formation of a biofilm on the tooth surface, primarily composed of gram-negative anaerobic bacteria.[3,4] The accumulation of plaque triggers an immune response, characterized by the infiltration of neutrophils and macrophages to the gingival tissue. Persistent bacterial presence and immune activation lead to a dysregulated host response, causing chronic inflammation which is characterized by increased production of proinflammatory mediators and reactive oxygen species. This ongoing cycle of inflammation and oxidative stress results in the progressive destruction of periodontal tissues.[5,6] Even though bacteria are the primary etiological factor in periodontitis, various risk factors also influence the disease's progression and severity, making treatment challenging.[7,8]

Mechanical debridement is considered the gold standard for the non-surgical treatment of periodontitis. This procedure involves the meticulous cleaning of the root surfaces to remove plaque, and

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bacterial toxins.[9] Although debridement is effective in reducing inflammation and improving periodontal health, its success can be limited by various factors such as anatomical challenges and bacterial virulence.[10] As a result, additional therapeutic strategies, including antibiotics, have been employed to enhance treatment outcomes. However, the rising concern of antimicrobial resistance has prompted the exploration of alternative treatments, such as local drug delivery systems with anti-inflammatory and antioxidant properties.[11]

Omega-3 fatty acids are a group of essential polyunsaturated fats that are vital for numerous bodily functions and overall health. The multifaceted mechanisms by which omega-3 fatty acids exert their beneficial effects extend far beyond the well-known regulation of triglycerides, encompassing profound impacts on the brain, neurodevelopment, cancer, diabetes, rheumatoid arthritis, irritable bowel disease, and cardiovascular health. Central to these wide-ranging benefits is the potent anti-inflammatory action of omega-3 fatty acids, which underlies much of their therapeutic potential.[12] However, the influence of omega-3 fatty acids is not limited to inflammation alone; these essential fatty acids also play a crucial role in modulating oxidative stress. By enhancing the expression of genes responsible for antioxidative enzymes, omega-3 fatty acids orchestrate a finely tuned interplay of regulatory mechanisms that achieve an optimal balance in the body's oxidative status.[13] This intricate regulation underscores the complex and powerful nature of omega-3 fatty acids in promoting health and preventing disease across multiple physiological systems.

In light of the burgeoning interest in innovative therapeutic modalities for periodontitis, the development of local drug delivery systems incorporating omega-3 fatty acids emerges as a particularly promising avenue. Despite this intriguing potential, a thorough review of the existing literature reveals a notable paucity of empirical studies exploring this specific application. This gap underscores the need for rigorous investigation into the feasibility and efficacy of omega-3 fatty acid-based local drug delivery systems. In response to this research void, the present study was meticulously designed to formulate and characterize microspheres incorporating omega-3 fatty acids.

MATERIALS AND METHODS

Fabrication of Omega-3 Fatty Acid Microspheres

The water-in-oil-in-water (w/o/w) double emulsion method was used to generate microspheres. A water-in-oil (w/o) emulsion was created by emulsifying 250 µL of 100 µM omega-3 fatty acid in 2.5 mL of 10% polymer solution in Dichloromethane (DCM) for 10 sec over an ice bath using a tip sonicator with an output power of 15 W. The w/o emulsion containing the dispersed omega-3 fatty acid droplets in DCM was gradually added to 20 mL of 1% polyvinyl alcohol while being vigorously stirred to form the w/o/w double emulsion. To harden the microspheres, the solution was swirled for 30 min at room temperature. After that, DCM was removed using a water suction method, and the mixture was centrifuged to separate the solid microspheres (Figure 1). The microspheres obtained underwent three rounds of washing with distilled water, freeze-dried, and their average size was determined using a Beckman Coulter LS 230 Laser Diffraction Particle Size Analyser.

Topography Analysis

Omega-3 fatty acid microspheres were analyzed for their morphological features using Scanning Electron Microscope (SEM). To prepare the samples for imaging, a sputter-coater was employed to apply a thin layer of gold at room temperature. Following this gold coating process, the microspheres were subjected to SEM analysis using a field-emission scanning electron microscope (JEOL JSM-IT 800; JEOL USA, Peabody, MA) at specifically 190X and 200X magnifications, to assess their overall structure.

Cytotoxic Assay

Cytotoxicity was evaluated using the brine shrimp lethality assay. Brine shrimp eggs were incubated in artificial sea water, prepared with sea salt, dry yeast, and oxygen supplied by an aquarium pump. After 2 days of incubation at 22-29°C, a light source was used to attract the nauplii to one side of the tank, and they were collected with a Pasteur pipette. The nauplii were separated from the eggs by pipetting them into small beakers filled with seawater. Ten nauplii were placed in each well containing a Sodium Chloride (NaCl) solution. Different volumes of omega-3 fatty acid microspheres (25, 50, 100 µg/mL) were added to the wells. A control well, containing only nauplii and NaCl solution, was included for comparison. The wells were left undisturbed for one day, after which the number of nauplii in each well was counted and recorded to assess the lethality of the extract.

Anti-inflammatory Activity

The anti-inflammatory property of the synthesized microspheres was evaluated using a protein denaturation assay with Bovine Serum Albumin (BSA). BSA, which constitutes approximately 60% of the total protein in animal serum, denatures upon heating, exposing antigens. In this experiment, varying concentrations of the microspheres (25, 50, and 100 µg/mL) were mixed with 2 mL of a 1% BSA solution. The pH of the reaction mixture was adjusted to 6.8 using 1N hydrochloric acid. The samples were then incubated at room temperature for 20 min in a water bath. After cooling to room temperature, the absorbance was measured at 660 nm. Diclofenac sodium, a known anti-inflammatory agent, was used as a control at various concentrations. The percentage of inhibition was calculated using the formula:

$$\% \text{ inhibition} = [(\text{Absorbance of control} - \text{Absorbance of sample}) / \text{Absorbance of control}] \times 100.$$

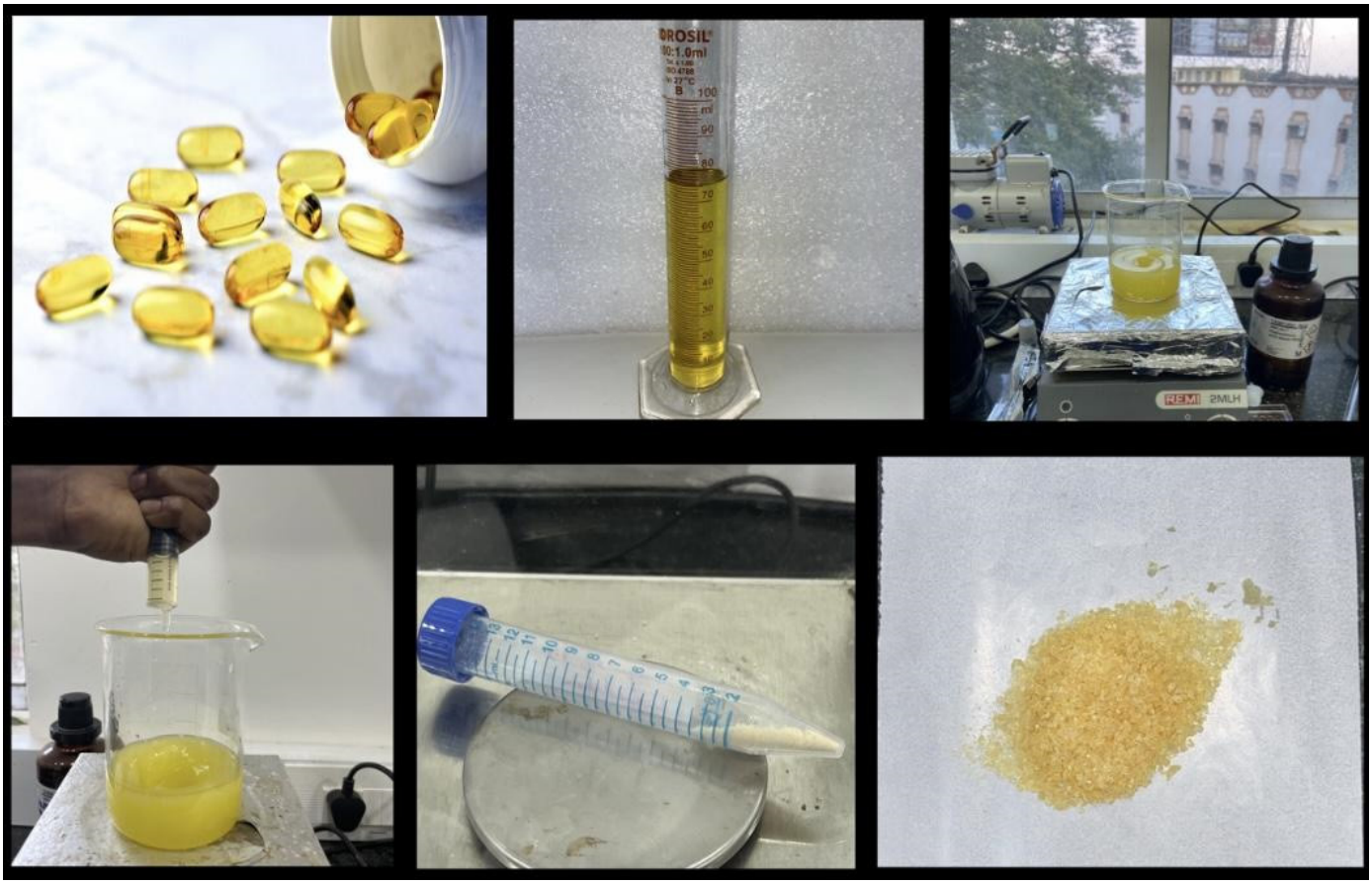

Figure 1: Preparation of omega 3 fatty acid microspheres..

Antioxidant Activity

To assess the antioxidant potential, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was employed. Microspheres at varying concentrations (25, 50, and 100 µg/mL) were combined with 1 mL of 0.1 mM DPPH dissolved in methanol and 450 µL of 50 mM Tris HCl buffer, maintaining a pH of 7.4. The mixture was incubated for 30 min. Following this, the reduction in DPPH free radicals was determined by measuring the absorbance at 517 nm. Butylated Hydroxytoluene (BHT) served as the control. The percentage of radical inhibition was calculated using the formula:

$$\% \text{ inhibition} = [(\text{Absorbance of control} - \text{Absorbance of sample}) / \text{Absorbance of control}] \times 100.$$

RESULTS

Particle Size and Topography Analysis

The data from the particle size analysis revealed that over 90% of the produced microspheres ranged between 32 and 55 µm in size, with a mean diameter of 39±13 µm. The microspheres presented moderately rough and consistent surface, suggesting that the microspheres might not be detrimental to the tissue and might be suitable for local drug delivery (Figure 2).

Brine Shrimp Lethality Assay

Figure 3 illustrates the lethality of nauplii at various extract concentrations (25 µg/mL, 50 µg/mL, and 100 µg/mL) over two days. At a concentration of 25 µL, no nauplii mortality was observed on either day. However, on day 2, the mortality rate was 20% at both 50 µg/mL and 100 µg/mL concentrations.

Protein Denaturation Assay

Figure 4 demonstrates the significant anti-inflammatory effects of omega-3 fatty acid microspheres. The inhibition percentages for the microspheres at concentrations of 25 µg/mL, 50 µg/mL, and 100 µg/mL were 50%, 60%, and 70%, respectively, closely matching the control's inhibition rates of 55%, 65%, and 75%. The anti-inflammatory activity of the microspheres increased with higher concentrations, from 25 µg/mL to 100 µg/mL.

DPPH Assay

According to the DPPH assay, the microspheres were initially tested at a concentration of 25 µg/mL, then subsequently increased to 50 µg/mL and 100 µg/mL. The antioxidant activity of the microspheres increased with the rise in concentration from 25 µg/mL to 100 µg/mL (Figure 5).

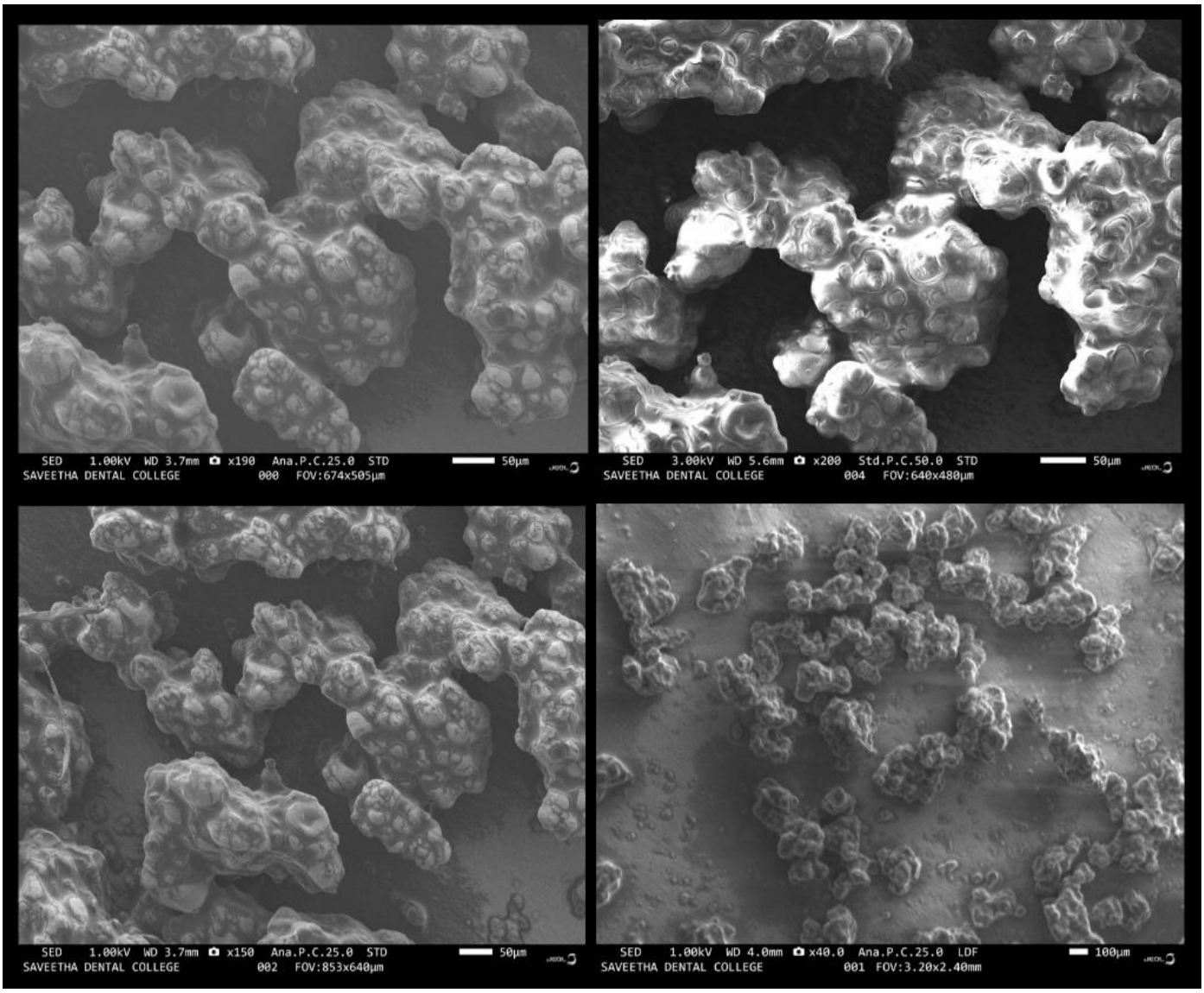

Figure 2: SEM image of omega-3 fatty acid microspheres.

DISCUSSION

The creation of microspheres infused with omega-3 fatty acids heralds a remarkable breakthrough in medical research, especially in the realm of anti-inflammatory and antioxidant treatments. This study adeptly engineered and characterized these omega-3 fatty acid microspheres, revealing their profound non-cytotoxic, anti-inflammatory and antioxidant properties. Additionally, the study meticulously examined their surface characteristics, highlighting the potential of these microspheres to transform therapeutic approaches in medicine.

In this study, SEM was employed to investigate the surface morphology and structural attributes of the synthesized microspheres. SEM, known for its high-resolution, three-dimensional imaging capabilities, enables detailed characterization at both the micro- and nano-levels. The SEM analysis revealed several noteworthy features of the microspheres. Notably, the surfaces exhibited a subtle roughness, a characteristic that can positively influence interactions with gingival tissues and improve biocompatibility.[14] Additionally, the SEM images demonstrated a uniform size distribution among the microspheres, with a majority measuring between 32 and 55 µm in diameter. This size range is essential for local drug delivery systems aimed at gingival tissues, as it supports controlled release.[15] The consistent size of the microspheres highlights the precision and reproducibility of the fabrication process used in this study. Moreover, SEM analysis underscored the spherical shape of the microspheres, confirming their structural integrity and making them well-suited for oral or mucosal administration.

The anti-inflammatory activity of omega-3 fatty acids is a fascinating and complex subject, involving multiple biochemical pathways and cellular mechanisms that work in concert to reduce inflammation in the body. Omega-3 fatty acids are essential polyunsaturated fats that play a critical role in maintaining

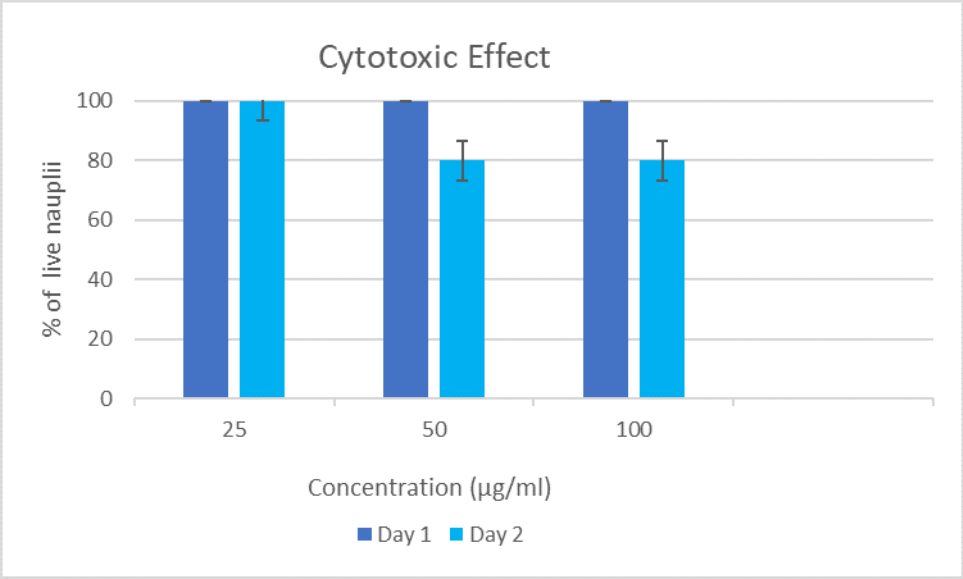

Figure 3: Lethality of the nauplii at different concentrations of microspheres.

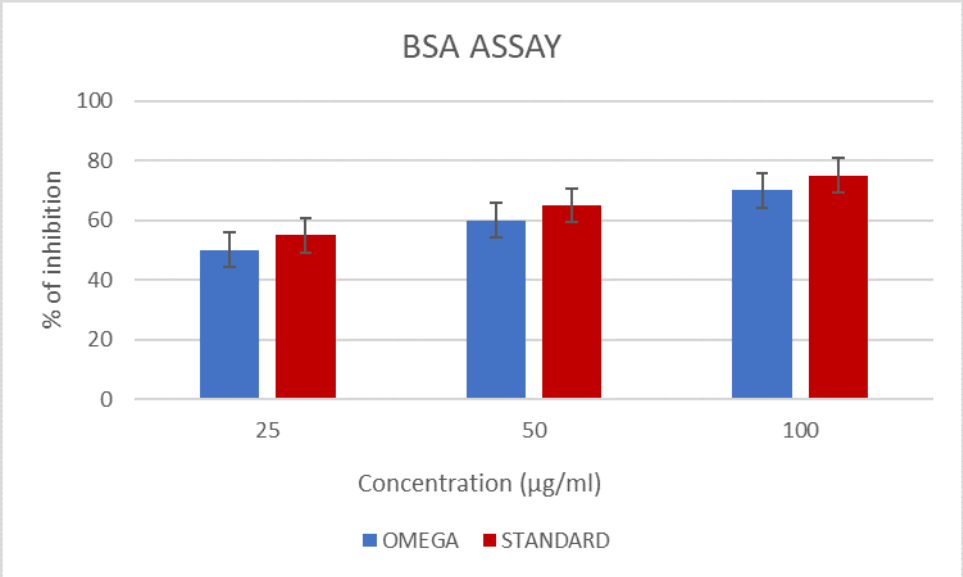

Figure 4: Graphical representation of the anti-inflammatory activity of omega-3 fatty acid microspheres.

human health. Among the various types of omega-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid are the most studied for their anti-inflammatory effects.[16] Omega-3 fatty acids influence the eicosanoid pathway by competing with arachidonic acid for the same enzymes, cyclooxygenase and lipoxygenase. This competition results in the production of less inflammatory eicosanoids, such as prostaglandins and leukotrienes derived from eicosapentaenoic acid, which are generally less potent than those derived from arachidonic acid. By reducing the production of pro-inflammatory eicosanoids, omega-3 fatty acids help mitigate inflammation.[17] Furthermore, omega-3 fatty acids are precursors to pro-resolving mediators, such as resolvins, protectins, and maresins.[18] These molecules actively participate in resolving inflammation by promoting the clearance of inflammatory cells and mediators, thereby restoring tissue homeostasis.

Omega-3 fatty acids have shown potential as an alternative treatment for patients suffering from discogenic and arthritic spine pain, who are already using analgesics for pain management.[19] Recent study has also emphasized the ability of omega-3 metabolites to lower triglyceride-rich lipoproteins and their anti-inflammatory properties, highlighting their cardioprotective benefits.[20] Furthermore, omega-3 polyunsaturated fatty acids have been found to alleviate depressive symptoms and act as potent anti-inflammatory agents, likely through the production of pro-resolving mediators. This makes them useful in treating neurodegenerative and neurological conditions.[21] Moreover, the

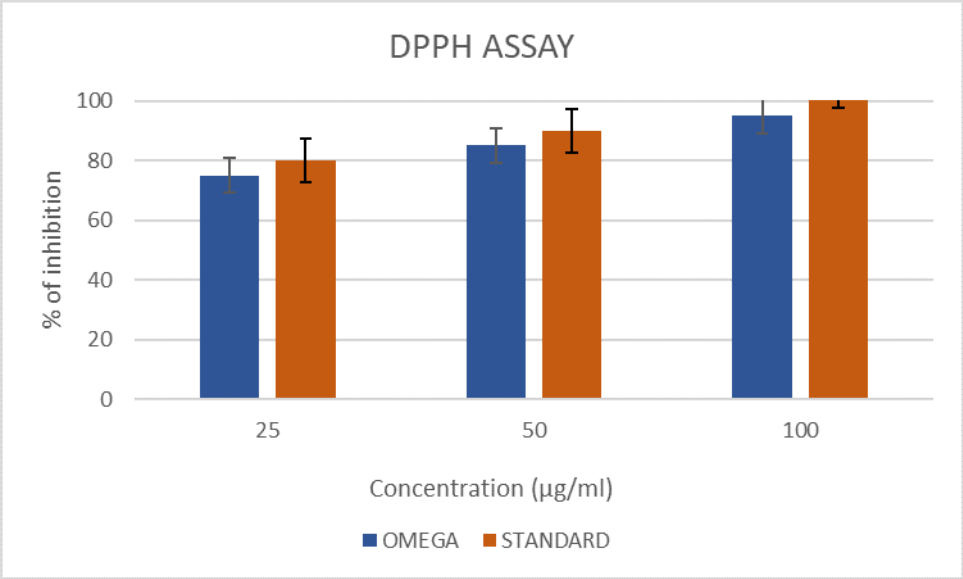

Figure 5: Graphical representation of the antioxidant activity of omega-3 fatty acid microspheres.

anti-inflammatory properties of omega-3 fatty acids have been shown to be effective in managing autoimmune diseases.[22] In the present study, the anti-inflammatory properties of omega-3 fatty acid microspheres were evaluated using a denaturation inhibition assay, which demonstrated a dose-dependent increase in inhibition. The inhibition percentages were 50%, 60%, and 70%, at concentrations of 25 µg/mL, 50 µg/mL, and 100 µg/mL, respectively. These results suggest that higher concentrations of the microspheres are more effective in reducing protein denaturation, a critical indicator of inflammation. Our findings also highlight the anti-inflammatory activity of omega-3 fatty acids.

Additionally, in the current study, different concentrations of omega-3 fatty acid microspheres were compared to butylated hydroxytoluene to assess antioxidant activity. The assay evaluated the ability of the microspheres to neutralize or inhibit the DPPH radical, thereby indicating their antioxidant efficacy. The results showed a dose-dependent response, with higher concentrations of microspheres exhibiting greater free radical scavenging activity. Furthermore, the study results revealed that the prepared formulations of omega-3 fatty acid microspheres were non-cytotoxic. Both *in vitro*[23,24] and *in vivo*[25,26] data strongly indicate that omega-3 fatty acids may function as antioxidants, rather than pro-oxidants, in various cell types, including vascular cells. This action helps to reduce inflammation and oxidative stress, thereby lowering the risk of atherosclerosis and degenerative disorders like cardiovascular disease.

The present study reveals that omega-3 fatty acid microspheres exhibit promising biological properties that suggest their potential for mitigating oxidative stress and reducing inflammation, particularly in the context of periodontal diseases. Their demonstrated non-cytotoxic, antioxidant and anti-inflammatory effects highlight their capability to protect periodontal tissues from oxidative damage and manage inflammation more effectively. This makes them a potentially valuable local drug delivery system, capable of providing targeted and sustained therapeutic benefits. However, to fully harness their potential, extensive clinical trials are necessary to determine the optimal dosage, refine delivery methods, and develop effective treatment regimens. Such research is crucial to validate their efficacy and establish practical guidelines for their use in clinical settings, ultimately enhancing periodontal disease management and patient outcomes.

CONCLUSION

In conclusion, the development of omega-3 fatty acid microspheres described in this study offers a promising and adaptable tool for biomedical research, providing a sustainable and efficient method for therapeutic delivery. These microspheres demonstrated non-cytotoxicity with exceptional anti-inflammatory and antioxidant properties, coupled with commendable surface characteristics. Further research is essential to explore and maximize the clinical potential of omega-3 fatty acid microspheres, aiming to enhance patient outcomes and drive advancements in healthcare innovation.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL STATEMENT

The study protocol was approved by Institutional Ethical Committee, Saveetha Dental College and Hospitals, Chennai.

ABBREVIATIONS

SEM: Scanning Electron Microscopy; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **DCM:** Dichloromethane; **BSA:** Bovine serum albumin; **BHT:** Butylated hydroxytoluene.

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