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ORIGINAL ARTICLE

Flaxseed Mucilage/Hydroxypropyl Methylcellulose and Sodium Alginate/Polyvinyl Alcohol Composite Bilayer Film as a Promising Drug Carrier for Periodontal Treatment

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ABSTRACT

Objectives: The present study focused on the formulation of mucoadhesive bilayer composite films for the treatment of periodontitis and evaluation of their physicochemical properties.

Materials and Methods: The solvent casting technique was used to prepare films. The primary layer (D) was prepared with flaxseed and hydroxypropyl methylcellulose composite to sustain the release of doxycycline hyclate. The second layer (S) comprised sodium alginate and polyvinyl alcohol composite for faster release of clove oil. Both layers were combined to generate the bilayer film (B). All formulations were characterized further to obtain an optimized formulation.

Results: Attenuated total reflection-Fourier transform infrared radiation results showed intactness of drug and clove oil in the presence of excipients. The pH of the films was compatible with the periodontal cavity and the thickness was suitable for inserting into the cavity. The immediate release layer showed faster disintegration and swelling. The content of clove oil was above 80%. The rate of swelling of the primary layer was slow and drug content complied with the United States Pharmacopoeia. Scanning electron microscope analysis revealed intact, non-porous and smooth films. Films exhibited better mechanical strength and bioadhesiveness. Clove oil was released from the immediate release layer within 10 min, and doxycycline hyclate release was retarded to a minimum of up to 8 h in the primary layer as well as the bilayer. Formulation also had a significant effect on both *Escherichia coli* and *Staphylococcus aureus*.

Conclusion: In the current study, bilayers were successfully prepared and characterized. The optimized formulation can be effectively used for the treatment of periodontitis.

Keywords: Periodontitis, flax seed mucilage, HPMC, sodium alginate, polyvinyl alcohol, doxycycline hyclate, clove oil

INTRODUCTION

Periodontal diseases have gained considerable attention as they are a widely spread chronic disease around the world. Around 20 to 50% population around the globe is suffering from periodontal diseases and tooth loss.[1] It is mainly caused by bacterial attack on tissues that support and surround teeth. The space between the tooth and gingiva is referred to as the periodontal pocket, and disease of the periodontal pocket is known as periodontitis. Periodontitis is a complex inflammation caused by periodontal microorganisms that destroy periodontal tissue. Predominantly gram-negative, microaerophilic, anaerobic bacteria colonize as biofilms in the subgingival

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area, altering connective tissue and bone metabolism, leading to periodontal damage. The severity of disease also depends on the host's immune response to bacterial challenges and environmental factors (smoking, chewing tobacco).[2]

Mechanical methods are available to control periodontal infection, but procedures to clean periodontal pockets are tedious due to limited accessibility in the area. Investigations were conducted to slow progression or improve periodontal status by systemic administration of antibiotics. A class of tetracycline antibiotics has been studied for the treatment of periodontitis.[3] Doxycycline hyclate from this class exerts anti-inflammatory and antibacterial effects. The United States Food and Drug Administration (USFDA) authorized doxycycline hyclate 20 mg capsule as an addition to root planning and scaling for periodontitis treatment. It presents an anti-inflammatory effect as an anti-collagenase agent and suppresses the activity of matrix metalloproteinases that are responsible for the destruction of periodontal tissues.[4] In addition, it stimulates the formation of bone tissue by instigating the inhibition of bone resorption and osteoblasts. Local administration of antibiotics reduces pocket depth, and a better effect was achieved by the use of doxycycline hyclate.[5] Owing to its antimicrobial and non-antimicrobial properties, doxycycline hyclate was selected as the first model drug in a bilayer film. Clove oil was permitted by USFDA in dentistry as a natural analgesic and antiseptic.[6] Eugenol suppresses the expression of cyclooxygenase II enzyme and cytokines, thus showing an anti-inflammatory effect.[7] It is also reported to exhibit antibacterial potential in both negative and gram-positive bacteria.[8] The antimicrobial activity of eugenol on some bacteria was due to the induction of cell lysis *via* the leakage of lipids and proteins in the cell membrane.[9]

Flax seed was reported to contain protein and a mixture of various carbohydrates, mainly rhamnose, galactose, glucose, and arabinose. Acidic polysaccharide galacturonic acid, pectin-like polymers, rhamnogalacturonan, and arabinoxylans were also reported. It is also composed of 3 to 9% of water-soluble heteropolysaccharides of total seed content, which is of low molecular weight and possesses a Newtonian flow pattern even at high concentrations. It exhibits shear thinning flow above 1% concentration. Flax seed mucilage has numerous applications in food and pharmaceuticals. It possesses excellent rheological characteristics and water holding capacity.[10] Hence, found application as a thickening agent, emulsifying agent, drug release retardant and mucoadhesive agent, *etc*.[11] hydroxypropyl methylcellulose (HPMC) has also been explored as a mucoadhesive and sustained release polymer. HPMC is a hydrophilic polymer that fits to a group of hydroxyethyl ethers. It is soluble in both organic and aqueous solvents and forms transparent, flexible films in aqueous solution. Low toxicity biodegradability and biocompatibility are key properties of HPMCs, and its application in pharmaceutical and biomedical fieldsis explored. Several researchers have combined HPMC with other polymers and lipids to form composites with enhanced characteristics. Sustained release and mucoadhesive properties of HPMC have also been reported by scientists.[12] Polysaccharide of alginic acid: sodium alginate is made up of α-l-guluronic (G) and β-d-mannuronic (M) acid units. It is an integral element of the cell wall of brown algae and a few bacteria. It is widely available, inexpensive, and biodegradable in nature. It can form transparent, water-insoluble, thermoirreversible gels by crosslinking with di- and trivalent ions and hence has wide application in pharmaceuticals. Sodium alginate has been extensively explored as a film former and drug carrier, but it is always used in combination with another polymer to form a film.[13] Polyvinyl alcohol (PVA) is a synthetic polymer produced by complete or partial hydrolysis of polyvinyl acetate. It is a biodegradable, biocompatible, tasteless, odorless, and translucent granular powder soluble in water. It has been studied for several pharmaceutical applications.[14] It can be blended with natural materials to enhance mechanical strength. It undergoes rapid swelling and dissolution in water. It exhibits excellent film-forming ability and has been studied by researchers for various targeted applications.[15]

Taking note of the above data, the current research work aimed to prepare composite films to treat periodontitis. The composite was prepared in two layers. The first layer was aimed at the sustained release of doxycycline hyclate and comprised flaxseed and HPMC. The second layer was made to release clove oil immediately and comprised sodium alginate and PVA composite. Films were evaluated and explored as carriers of doxycycline hyclate and clove oil.

MATERIALS AND METHODS

Materials

Flaxseeds were purchased from a domestic market in Pune, Maharashtra, India. Doxycycline hyclate was purchased from Swapnaroop Drug Agency, Aurangabad, India. Clove oil was purchased from Aaria Bio-Lifesciences Research, India. HPMC was purchased from Loba Chemie, India. Sodium alginate was purchased from Thermosil Fine Chemical Industries, Pune, India. PVA was acquired from Research-Lab Fine Chemical Industries, India. All other reagents used were of analytical grade.

Extraction of flaxseed mucilage

Flaxseeds were purchased from a local store and cleaned. Flaxseeds (30 g) drenched in 900 mL of distilled water at a ratio of 1:30. To obtain a mucilage solution, soaked flaxseed was stirred at 1000 rpm at 80 °C to 100 °C for at least 3 h using a hot plate magnetic stirrer. The supernatant solution was then kept for normalizing to ambient conditions (27 °C). The mixture was placed into centrifuge test tubes and rotated at 3900 rpm for 15 min to separate the mucilage solution from flaxseed. Flaxseed was subsequently filtered with cheesecloth to obtain reaming mucilage attached to the flaxseed coat. Ethanol was added to the filtered extract to precipitate the mucilage. The precipitated mucilage was isolated and dehydrated for 5 h in a hot air oven at 50 °C. The yield of the dried mass was quantified, phytochemical screening was performed, and the mass was stored in a desiccator.[16]

Preparation of the double-layer film

Preparation of the primary flaxseed mucilage drug-loaded film layer (D)

The primary layer was prepared by solubilizing 0.1 g flaxseed mucilage polymer and 0.1 g HPMC in 15 mL distilled water for 1 hour at 60-70 °C with continual stirring. Doxycycline hyclate was solubilized in 5 mL of purified water and slowly mixed into the polymeric composition while constantly stirring. Glycerin was added as a plasticizer, and the polymeric composition was cast in a petri-plate. The petri plate was then placed in a hot air oven (Biotechniques, India) for 24 h at 40 °C (Figure 1). Film was enveloped in aluminum foil and kept in a desiccator.[17]

Preparation of the secondary clove oil-loaded film (S)

A second polymeric layer was formulated by solubilizing sodium alginate and PVA in 10 mL of purified water with uninterrupted stirring. Glycerin was incorporated as a plasticizer in the polymeric composition. Clove oil was dissolved in 3 mL of ethanol using 0.2% *w/v* tween 80. The resulting solution was loaded dropwise in a polymeric composition of sodium alginate and PVA and sonicated to eliminate entrapped air. The solution was finally spread evenly in a petri dish and dehydrated in an oven for 24 h at 40 °C. The film was enveloped in aluminum foil and kept in a desiccator (Figure 2).[18]

Preparation of the bilayer film (B)

The primary layer was dried thoroughly and 0.5% *w/v* of a freshly prepared calcium chloride solution was sprinkled over it. A polymeric mixture of sodium alginate and PVA-containing clove oil was cast over the primary layer. The bilayer film was further dried in a hot air oven and peeled off (Figure 3). The film was enveloped in aluminum foil and kept in desiccator (Table 1, 2).[19]

Evaluation of the films

Surface pH determination

Agar plates expressed in phosphate buffer (pH 6.8) were allowed to hydrate for 2 h on agar plates. A pH meter (Mettler Toledo, India) was positioned in contact with the hydrated patch, and the pH of the surface was checked. The average of three measurements was recorded.[20]

Attenuated total reflectance-Fourier transform infrared radiation (ATR-FTIR)

ATR accessory, Tensor 37 FTIR equipment (Bruker, Germany) spectra of pure drug, physical mixture of drug and excipient, and optimum formulation were recorded. By averaging 10 scans at a resolution of 4 cm^{-1}, single spectra in the wavelength range of 4000 to 400 cm^{-1} were obtained.[21]

Thickness and weight

Three films selected with a surface area of 9 × 9 mm^2 were used for the measurement of thickness at 10 different points. The thickness of the films was estimated using a digital vernier caliper (Mitutoyo, Japan). The average weight was calculated by weighing 9 × 9 mm^2 films on an analytical balance (Shimadzu, Japan). Both these readings were recorded in triplicate, and the mean was estimated.[22]

Drug content

Primary film equivalent to a surface area of 1 cm^2 was miscibilized in 10 mL phosphate buffer (pH 6.8) and transferred

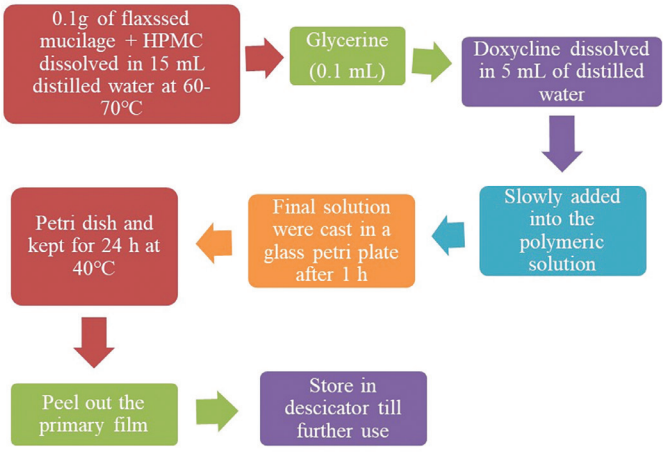

Figure 1. Schematic representation of the preparation of the primary layer

HPMC: Hydroxypropyl methylcellulose

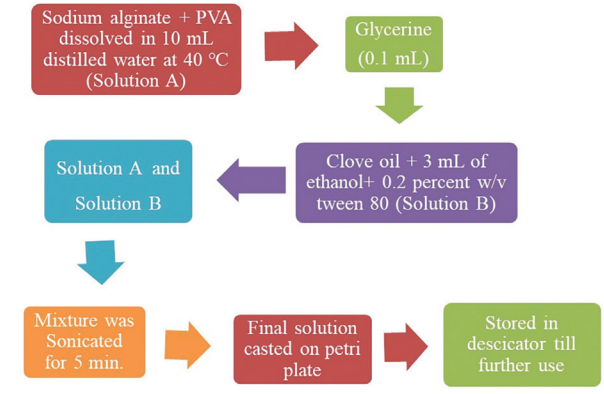

Figure 2. Schematic representation of the preparation of the secondary layer

PVA: Polyvinyl alcohol

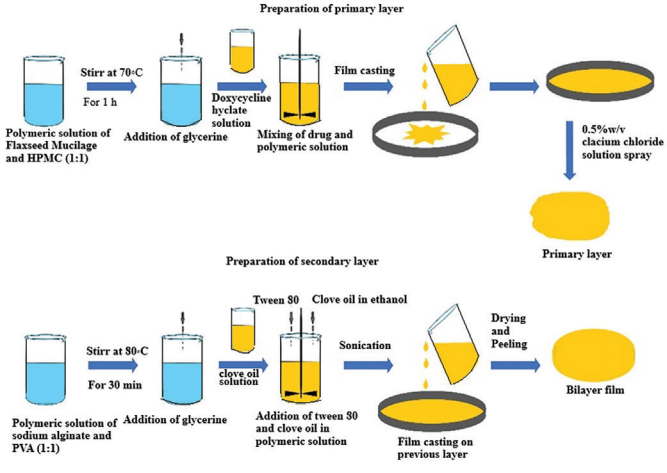

Figure 3. Schematic representation of the preparation of the bilayer film

HPMC: Hydroxypropyl methylcellulose, PVA: Polyvinyl alcohol

Table 1. Formulations of secondary layer oil-loaded films S1-S8

Formulation	Sodium alginate (mg)	PVA (mg)	Glycerin (mL)	Clove oil (mg)	Distilled water (mL)
S1	400	300	0.1	10	10
S2	200	400	0.1	10	10
S3	200	200	0.1	10	10
S4	200	300	0.1	10	10
S5	400	400	0.1	10	10
S6	400	200	0.1	10	10
S7	300	400	0.1	10	10
S8	300	200	0.1	10	10

PVA: Polyvinyl alcohol

Table 2. Formulation of the bilayer film (B)

Formulation	Flaxseed mucilage (mg)	HPMC (mg)	Sodium alginate (mg)	PVA (mg)	Doxycycline hyclate (mg)	Clove oil (mg)	Glycerin (mL)	Distilled water (mL)
Primary layer (D)	100	100	-	-	40	-	0.1	20
Secondary layer (S)	-	-	200	200	-	50	0.1	10

PVA: Polyvinyl alcohol, HPMC: Hydroxypropyl methylcellulose

to a 100 mL volumetric flask; the final volume was made with pH 6.8 phosphate buffer. A 1 mL aliquot was removed from the solution and diluted to 10 mL with phosphate buffer pH 6.8. The absorbance of the resulting solution was measured at 271.3 nm using a ultraviolet (UV)-visible spectrophotometer (Shimadzu-1800). A similar method was followed for the second film loaded with clove oil by recording the absorbance at 283 nm. For the bilayer film, absorbance was recorded at 271.3 and 283 nm for doxycycline hyclate and clove oil, respectively.[23]

Disintegration time

The film was cut into 9.0 × 9.0 mm² and placed in a petri plate containing 5 mL purified water, and the time needed to completely disintegrate the secondary film was recorded. The average of three determinations of results was noted.[24]

Surface morphology

The surface morphology of the film was spotted by optical microscopy (Metzer, India) and scanning electron microscopy (SEM) (JEOL JSM- 6360A scanning microscope, Tokyo, Japan). Optical microscopy was used to observe the transactional view of the bilayer film with 100x power lenses. For SEM, the specimen sample was mounted on metal stubs with a double-sided adhesive band, and gold was sputtered on the specimen to confirm sufficient electrical conductivity. Images were taken using an Everhart-Thornley detector with 10 kV excitation energy.[25]

Folding endurance

Films were cut into 1.0 × 1.0 cm² and continually creased at the same point until disruptions. The number of counts film that could be creased without breaking was noted.

Tensile strength and elongation at failure percentage (EF%)

A texture analyzer (CT-3 Brookfield, USA) was used to investigate the tensile strength of the film. A sample of 4 cm^2 was taken and secured between two clamps of probe texture analyzer-dual grip assembly. The bottom clamp was detained immobile, and the film was stretched apart by the top clamp at a speed of 2.0 mm/s to a distance of 6 mm with a trigger load of 0.05 N. The force required to break the film was recorded. Data assemblage and calculations were performed using Texture Pro CT V1.3 Build 14 software. The tensile strength at break rate was calculated using the formula:

Tensile strength (N/cm^2) = Breaking force (N)/cross-sectional area of sample (cm^2)

Elongation at break %, a measure of the percentage of a film that has ruptured, was determined using the following equation:

$$\text{Elongation at break \%} = \frac{\text{Increase film lengath at break } (\Delta L)}{\text{Inital film lengath } (L)} \times 100$$

In vitro bioadhesion force

The bioadhesion force was estimated using a texture analyzer (CT-3/100, Brookfield, USA) equipped with a 100 g load cell. Bioadhesive force was recorded in porcine buccal mucosa. The mucosal membrane was cut and the underlying connective tissue was separated. It was thoroughly cleaned with pH 6.8 phosphate buffer and secured between two circular disks positioned at the bottom perspex support. The mucosal membrane was exposed to the probe *via* a top spherical disk with a void of 12.7 mm diameter. Discs were placed in jacketed glass containers composed of pH 6.8 phosphate buffer and maintained at 37 ± 1 °C. The membrane was equilibrated at this temperature for 30 min. The buccal film was firmly secured using thread on the bottom side of the probe. The circular

cavity and probe were brought into line to safeguard the film originating in intimate contact with the mucosal membrane. Prior to the study, buccal film was hydrated with pH 6.8 phosphate buffer. A load of 90 g was applied, and the probe was lowered at a speed of 0.5 mm/s to contact tissue for 120 s. It was removed at a speed of 2 mm/s.[26] Data assemblage and calculations were performed using Texture Pro CT V1.3 Build 14 software. Ahesiveness and adhesive force were used to evaluate the strength of bioadhesion of the film. Bioadhesion force (N) was calculated using the following formula:

Bioadhesion force (N) = Bioadhesive strength (g)/1000

Swelling studies

Films were weighed individually, and the initial weight was noted (W1). Films were placed separately in a petri dish enclosing pH 6.8 phosphate buffer. Samples were isolated from petri plates hourly, and extra buffer was wiped carefully using filter paper. Hydrated films were weighed (W2). The swelling index was determined by the following formula:

$$\text{Swelling index (\%)} = \left(\frac{W2-W1}{W1}\right) \times 100$$

In vitro drug release

Film was kept in a dialysis bag filled with 1 mL of pH 6.8 phosphate buffer and held in 50 mL phosphate buffer 6.8 maintained at 37 °C with shaking in a thermostatic horizontal shaker at 75 rpm. Aliquots of 1 mL were removed at time intervals of every 2 min interval for 10 min to analyze clove oil and 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h to quantify doxycycline hyclate. Sink conditions were maintained by replacing an identical quantity of the pre-warmed buffer solution. Samples were investigated using a UV spectrophotometer at 271.1 and 283 nm for doxycycline hyclate and clove oil, respectively. Drug release experimentation was completed in triplicate, and the mean was reported. The release of doxycycline hyclate was fitted in different kinetic models such as first order, zero order, Higuchi and Korsmeyer-Peppas and R^2 value was determined.

In vitro antimicrobial activity of the periodontal film

The drug-loaded film was studied for its antimicrobial activity against *Escherichia coli* (ATCC25922) and *Staphylococcus aureus* (ATCC25323) using the Kirby-Bauer diffusion technique. Concisely, sterile Mueller-Hinton Agar (MHA) was poured into plates up to a depth of 4 mm under sterile conditions using a laminar air flow unit. After solidification, plates were dried for 30 min in incubation to remove excess moisture from the surface. The inoculum of *S. aureus* and *E. coli* was selected and inoculated on surface MHA agar separately with a wire loop and spread with the help of a sterile spreader. After stabilization of culture, wells of each 6 mm diameter were pressed with a sterile cork borer and removed from the petri dish. Disc-shaped polymeric films B and D equivalent to 4 mg/0.5 mL were placed into wells and incubated at 35-37 °C for 24 hous. After 24 h, the zone of inhibition was measured using a zone reader.[27]

RESULTS

Periodontitis results in pain and inflammation surrounding teeth because of infection to gingival tissue, resulting in a need to counteract pain and eradicate infection. Owing to its antimicrobial properties, doxycycline hyclate was selected as the model drug. The combination of eugenol with antibiotics was reported to derive a synergistic effect.[28] Eugenol is reported to be present in clove oil; hence, in the current study, clove oil was used along with doxycycline hyclate. Mucilage from flax seeds was isolated by a simple process and characterized. The yield and ash value of mucilage were found to be 6.3% and 4.2%, respectively. The isolated sample showed the presence of carbohydrates and protein. These results were in agreement with the evaluation of flaxseed mucilage carried out by Kaewmanee et al.[29] The first layer of bilayer film was tried to develop by flax seed mucilage alone, but due to the high viscosity of mucilage, the obtained film was sticky and difficult to peel off from the petri dish. Hence, the first layer of film was prepared by combining flax seed mucilage and HPMC. A film composed of a higher concentration of HPMC was stiffer, less flexible, non-uniform, and might require a longer time to prepare; hence, a low concentration of HPMC was selected. Earlier studies also suggested that films prepared with a lower concentration of polymer were visually more homogeneous and thinner, and drug distribution in the film was uniform. Sodium alginate forms a clear transparent, flexible film, but the brittleness of sodium alginate restricts its use as an excellent film former; hence, it was combined with PVA, which is highly elastic and biocompatible. Some researchers reported that blends of this polymer were found to enhance the mechanical strength of the film and that the resulting product is highly hydrophilic. The formulation of mucoadhesive bilayer films containing doxycycline hyclate and clove oil was carried out (Figure 4a), and further films were evaluated. All films were transparent, free from creases, flexible, and had a characteristic clove oil odor. In the film casting technique, drying was carried out at a gentle rate; hence, aggregation and creases on the film surface were not noticed. Doxycycline hyclate is freely soluble in water and polymers also form clear solutions; hence, the obtained films were transparent. Flax seed mucilage and sodium alginate had a slightly yellowish to off-white color; hence, the final films also exhibited the same color (Figure 4b). Incorporation of glycerin as a plasticizer in films resulted in flexible films, whereas films produced without glycerin were brittle. Morphology of the film was not affected by the addition of glycerin.

Surface pH

Extreme pH can cause local irritation and discomfort in the periodontal cavity. The film pH was 6.4, which was well suited with the oral cavity. Indicating films were inert and compatible with the oral cavity.

ATR-FTIR

This test was performed to determine compatibility between excipient and drug. The spectra of doxycycline hyclate showed characteristic peaks at 1665.65, 1328.27 cm^{-1} conforming

to the C=O group; peaks between 3537.02 and 3812.43 cm^{-1} represented C-H, N-H, and O-H stretching; and a peak at 1456.39 cm^{-1} showed presence of C-H and N-H in-plane bend vibrations. 1217.98 cm^{-1} peak indicated C-N stretching. These results were found to be in agreement with research carried out by other researchers.[30] In addition to this United States Pharmacopeia (USP) monograph suggesting bands for doxycycline hyclate tablets at 935, and 659 cm^{-1}, drug sample showed bands at 990.61 and 659.62 cm^{-1}. Physical mixture of flaxseed, HPMC, and doxycycline hyclate shows characteristic peaks of doxycycline hyclate, indicating intactness of the drug in the presence of excipients. The primary layer (D) also resembles peaks of doxycycline hyclate with slight shifting of peaks. Slight shifting of the peaks might be attributed to physical interactions due to the formation of the composite. The spectra of clove oil showed peaks at 3000.25 cm^{-1} and 3634.58 cm^{-1} owing to O-H stretching, 1657.35 cm^{-1} representative of the C-H stretching vibration of benzene, eugenol methyl C-H deformation vibration denoted at 1365.87 cm^{-1}, 1758.24 cm^{-1} peak resembled the C=O carboxylic acid stretching vibration, and phenolic hydroxyl C-O stretching vibration appeared at 1278 cm^{-1}. The C-O-C aromatic ether vibration was denoted at 1033.21 cm^{-1}. The spectra indicated the presence of eugenol and ether groups, and the benzene ring and phenolic hydroxyl peaks confirmed the presence of eugenol. Spectra were found in agreement with spectra reported in other research work.[31] Second layer S3 film also showed the presence of similar groups, and an additional peak of PVA at 2932.57 cm^{-1} was observed. S3 film spectra closely resemble the spectra of clove oil, indicating the intactness of clove oil in the film. Various spectra are shown in Figure 5.

Thickness and weight

The thickness and weight of the film were determined to confirm the uniformity of the film and to ensure the even distribution of the polymeric solution throughout the petridish. Film thickness is an important physical parameter that potentially influences the feeling of comfort in the periodontal cavity, barrier properties, dose accuracy, disintegration, and dissolution. The average film thickness and weight of the primary layer (D) were 0.21 ± 0.06 mm and 0.143 ± 0.07 g, respectively. For the second layer (S), the thickness and weight were found to be between 0.21 and 0.28 mm and 0.123-0.189 g, respectively (Table 3). PVA shows a highly ordered crystalline structure and was responsible for producing soft, thin films with high flexibility. The thickness and weight of bilayer film (B) were 0.34 ± 0.062 mm and 0.143 ± 0.07 g, respectively. The thickness of the bilayer film was suitable to insert into a periodontal cavity having a width smaller than 0.5-3 mm.

Drug content

The drug content of the primary layer was 98% and doxycycline hyclate found in the bilayer film was 97%. For the doxycycline hyclate tablet, USP had a specified limit of 90 to 120%. Clove oil content in the film was found to be in the range of 80%-92% (Table 3). The film containing the lowest amount of polymer showed the maximum amount of clove oil entrapment as the polymeric solution was less viscous. Oil has been miscibilized easily in the polymer and homogeneously mixed throughout the blend. Surprisingly, a higher concentration of polymeric solution showed less clove oil; these results might be attributed to the uneven distribution of the drug in the viscous polymeric solution. Nevertheless, all films showed more than 80% clove oil entrapment.

Disintegration time

Official guidelines are deficient in determining the disintegration time of films. Pharmacopoeia describes standard disintegration tests for conventional dosage forms, but for films when this method was tried, the film adhered to the wall of the tube, and small pieces float inside the tube, which made visual detection difficult. Due to these practical difficulties, erroneous results might be obtained. Generally, for films, the disintegration test is done by two methods: the slide frame method and the petri dish

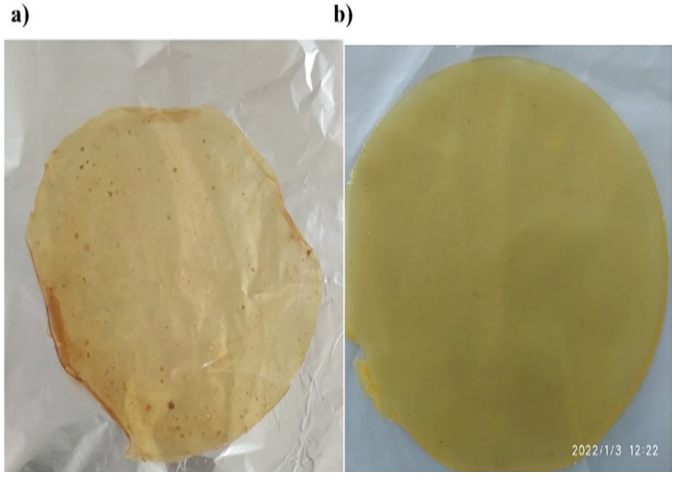

Figure 4. a) Plain flaxseed mucilage-based doxycycline hyclate loaded film (D), b) Bilayer film (B) Composed of doxycycline hyclate in the primary layer and clove oil in the secondary layer

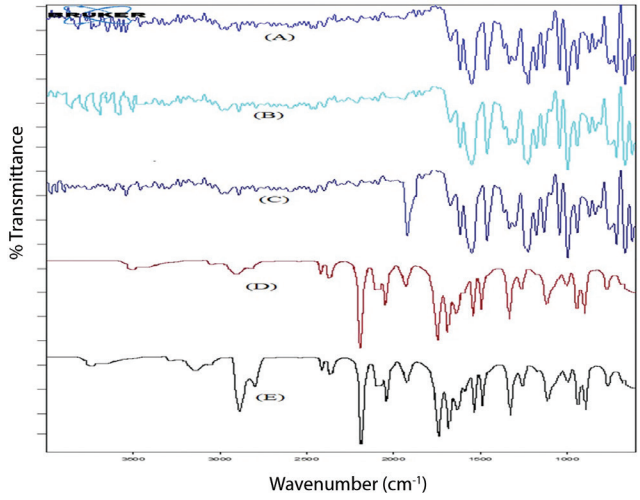

Figure 5. ATR-FTIR spectra of (A) doxycycline hyclate, (B) physical mixture of doxycycline hyclate, HPMC, and flaxseed, (C) formulation of primary layer (D1), (D) clove oil, and (E) formulation of S3 layer

ATR: Attenuated total reflection, FTIR: Fourier transform infrared radiation, HPMC: Hydroxypropyl methylcellulose

method, out of which the petri dish method was adopted for the current study. The disintegration time for the primary layer was 8 h, indicating slower penetration of the solvent due to the high viscosity of the polymers. This was a promising property to maintain film at the site of administration for prolonged drug release. It was proposed that secondary films should disintegrate faster than the primary layer and release clove oil to counteract inflammation and pain. The disintegration time for the secondary layer (S) was recorded and found to be in the range of 8.46 ± 0.74-11.86 ± 0.08 minutes. Water molecules rapidly penetrated the films, causing dispersion of the film into small pieces that ultimately released clove oil at a faster rate than doxycycline hyclate. Rapid penetration of water into the secondary film might be the result of the lyophobic nature of sodium alginate and PVA. From these results, the optimized ratio of sodium alginate and PVA was selected as 200:200, and further tests were carried out on the optimized bilayer film.

Surface methodology studies

Because this property could not be identified directly, optical microscopy was used to confirm the creation of two distinct layers in the bilayer films. The bilayer films had two different layers, as illustrated in Figure 6. SEM analysis (Figure 7) revealed a distinct structure of the film with a smooth matrix and good integrity without any pores or cracks.

Folding endurance

It is an index to investigate the mechanical properties and flexibility of a film. The optimal value of folding endurance eases the manufacturing and administration of films. A direct relation exists between the folding endurance and mechanical properties of the film. The folding endurance of the primary layer (D) and bilayer was (B) 218 ± 16 and 304 ± 18 times, respectively. Bilayer films exhibit higher folding endurance, indicating more flexibility and mechanical strength.

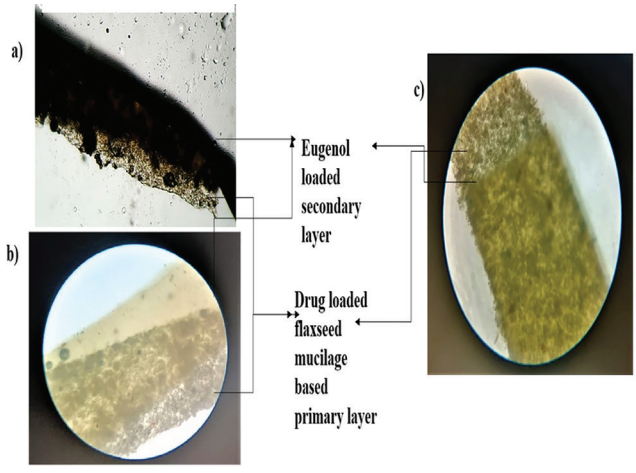

Figure 6. Optical microscopy of bilayer film a, b and c showing a transverse sectional view of the bilayer film under 100x magnification power

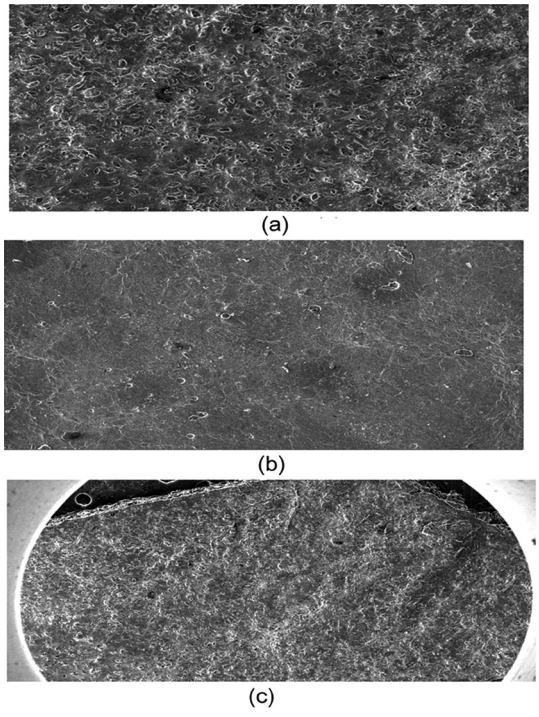

Figure 7. Scanning electron microscopic images showing the surface morphology of bilayer film a) primary layer containing doxycycline hyclate, b) secondary layer containing eugenol, c) bilayer film B

Table 3. Properties of single-layer oil-loaded films: Clove oil content (%), folding endurance, film thickness (mm), % clove oil release after 10 min, weight uniformity (g), disintegration time (min.)

Formulation code	Clove oil content (%)	Folding endurance	Film thickness (mm)	% Clove oil release after 10 min.	Weight uniformity (g)	Disintegration time (min.)
S1	80 ± 1.25	196 ± 0.18	0.22 ± 0.06	60 ± 1.29	0.123 ± 0.03	10.13 ± 0.98
S2	85 ± 1.36	201 ± 0.12	0.25 ± 0.06	39.43 ± 1.36	0.145 ± 0.01	8.46 ± 0.74
S3	92 ± 1.54	213 ± 0.15	0.28 ± 0.06	68.028 ± 1.02	0.167 ± 0.06	7.89 ± 0.45
S4	81 ± 2.61	210 ± 0.11	0.21 ± 0.06	47.32 ± 2.24	0.189 ± 0.04	11.84 ± 1.26
S5	83 ± 1.52	205 ± 0.13	0.24 ± 0.06	44.92 ± 1.21	0.132 ± 0.09	8.95 ± 0.39
S6	80 ± 2.21	209 ± 0.10	0.27 ± 0.07	53.8 ± 1.29	0.165 ± 0.07	9.36 ± 0.06
S7	82 ± 1.24	200 ± 0.14	0.23 ± 0.04	42.39 ± 1.27	0.149 ± 0.02	11.86 ± 0.08
S8	87 ± 1.87	204 ± 0.16	0.26 ± 0.08	38.45 ± 1.32	0.187 ± 0.05	10.12 ± 0.47

All the readings were taken in triplicate, (n= 3, mean ± SD), SD: Standard deviation, min: Minute

Approximately similar values were reported for HPMC films along with Eudragit RL 100 and Carbopol-934.[32] The secondary layer (S) showed folding endurance in the range of 190 ± 0.15 to 213 ± 0.13 times, which was found to be higher than the 145 and 152 reported in earlier research.[33] All films had a good value of folding endurance, showing that the films are flexible with good mechanical strength.

Tensile strength and EF%

The maximum resistance of the film to break under an applied load is the tensile strength, which is an index to confirm the mechanical strength of the film. For the primary layer (D) and bilayer (B), the tensile strength was 4.11 ± 0.04 and 4.16 ± 0.02 N/cm^2, respectively. The largest shift in the film length before breakage is called elongation at break. Maximum deformation a film can experience before breaking, which symbolizes the film's ductility and resistance to distortion. It is defined as the maximum distortion a film could undergo before it fails or breaks. EF for the primary layer (D) and bilayer (B) were 5.14 ± 1.6 and 6.12 ± 1.5% respectively. The amount of drug in the film also affects the mechanical properties of the film; in the current study, the dose of drug is less; hence, the mechanical properties of the optimized film were found to be good. Bilayer film has excellent mechanical properties than primary layer. Figure 8 shows that time required for bilayer film for tensile strength was more as compared to primary layer of film. These results were found to be in agreement with research carried out Ghavami-Lahiji et al.[34]

In vitro bioadhesion bio adhesion force

In our earlier studies, we reported flax seed as a mucoadhesive polymer.[35] Other researchers have also reported that flaxseed mucilage has mucoadhesive potential. HPMC has been widely explored as a mucoadhesive agent. Mucoadhesive drug delivery methods for periodontal disease would have significant advantages such as simplicity of entry into the periodontal pocket and good retention within it. For primary (D) and bilayer films (B), the bioadhesive force was found to be 4.24 ± 0.04 N and 4.58 ± 0.06 N, respectively. This value was found to be slightly better than studies carried out for HPMC/PEG 400/CP.[36]

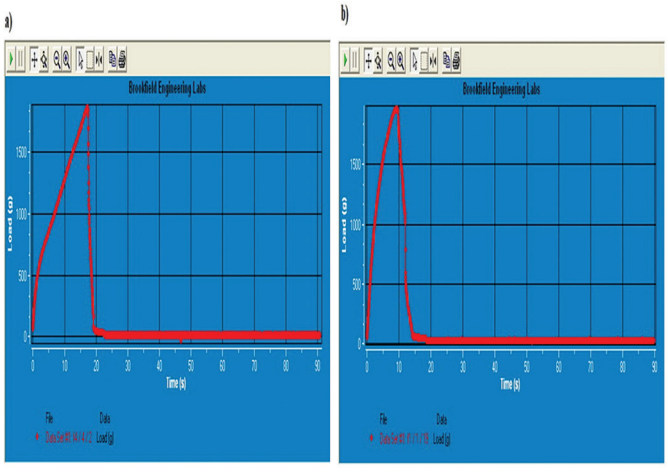

Figure 8. Graph of CT3 texture analyzer of tensile strength: a) bilayer periodontal film formulation and b) single primary layer film

Swelling index

For natural polymers that exhibit swelling-controlled release, swelling is a crucial component of drug release. Swelling studies play a substantial role in bioadhesive properties because swelling of polymers contributes to polymer chain disentanglement and relaxation, which initiates the diffusion of polymeric chains into the mucus membrane for the process of bioadhesion. A swelling study for the primary layer and bilayer film was carried out and varied from 6.65 to 13.9 and 6 to 16.5%, respectively. When flaxseed polymer comes in contact with aqueous medium, polymer chains undergo relaxation and interpenetration, causing swelling. Further expansion of the polymeric matrix may initiate the generation of slippery mucilage, consequently leading to the early release of the drug that has been trapped therein. Figure 9 illustrates the swelling index of films. The swelling index of the bilayer film increased steadily until 25 min and reached equilibrium. At the end of 30 min, the swelling index started declining at a very slow rate. These results might be attributed to the slow erosion of polymers. The swelling index of the primary layer ranged between 6.5% and 13.9%, increased until 30 min and retained equilibrium until 35 min after which it started eroding at a very slow rate. The rate of hydration of the bilayer film was found to be higher than that of the primary layer, as in addition to flaxseed and HPMC, it was also composed of highly swellable sodium alginate. Both B and D films had a good swelling index up to 3 h, after which the swelling index declined slightly, possibly due to polymeric erosion of both layers. The surface area and rate of solvent diffusion in films might be major contributing factors for initiating swelling of films. These results can be correlated with the mucoadhesion study where the bilayer film showed slightly higher mucoadhesion compared with the primary layer. Increased swelling resulted in relaxation of the polymeric chain and exposure of the polymer at the bioadhesive site. A faster swelling bilayer initiated the rapid formation of adhesive bonds.

In vitro drug release

Release of clove oil from the immediate release layer was evaluated for 10 min. Release of clove oil was found in range of 38.45 to 60% and found to be dependent on the concentration

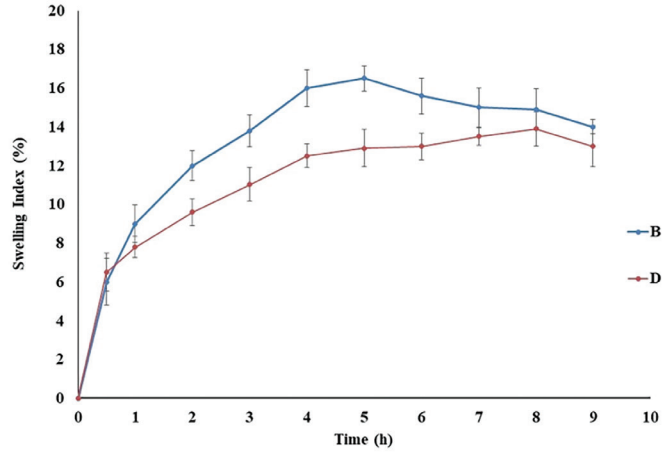

Figure 9. Swelling ratio of B and D films

of polymers. The highest concentration of sodium alginate and PVA (400:400) released only 44% of clove oil. Formulation containing an equal amount of sodium alginate and PVA at the lowest concentration (200:200) released 68% clove oil. *In vitro* drug release from different batches is depicted in Figure 10. Hence, formulation S3 containing a lower number of both polymers was selected as an optimized formulation to prepare the bilayer. Both sodium alginate and PVA are hydrophilic. They undergo swelling at a rapid rate in contact with phosphate buffer 6.8 and release clove oil. The current study also expects a faster release of clove oil to counteract pain and inflammation.

Approximately 33.82% of the drug escaped from film primary layer D at initial 3 h followed by a cumulative 93.71% drug release of up to 8 h. For the bilayer film (B), 29.81% of the drug was released at the initial 3 h and 91.58% at the end of 8 h from the sustained release layer (Figure 11). Immediate release layer showed 69.25% release of clove oil within 10 min from the bilayer film (B). Clove oil is insoluble in aqueous medium. It acted as an additional hurdle for diffusion of media in the B film and additionally contributed to the sustained release of doxycycline hyclate. The obtained results were in agreement with earlier studies on bilayer films, where components of another layer (clove oil) had an impact on the release of actives from the primary layer.[19] Flaxseed mucilage and HPMC have been reported as sustained release polymers. Both forms a very viscous gel upon contact with the dissolution media, and the doxycycline hyclate molecules dissolve in the media and then gradually diffuse out. This process of dissolution and diffusion was time-consuming; hence, a sustained release effect was achieved. The release of doxycycline hyclate at a slower rate was desired to provide a continuous antibacterial shield. Drug release was fitted in different kinetic models such as first order, zero order, Korsmeyer-Peppas, and Higuchi. R^2 value for different kinetic models were 0.9821, 0.9924, 0.872, and 0.8406, respectively. The release mechanism of the drug was in a concentration-dependent manner and it followed first-order kinetics. The obtained results were found to be in agreement with research carried out for metronidazole-loaded films for periodontal treatment.[37]

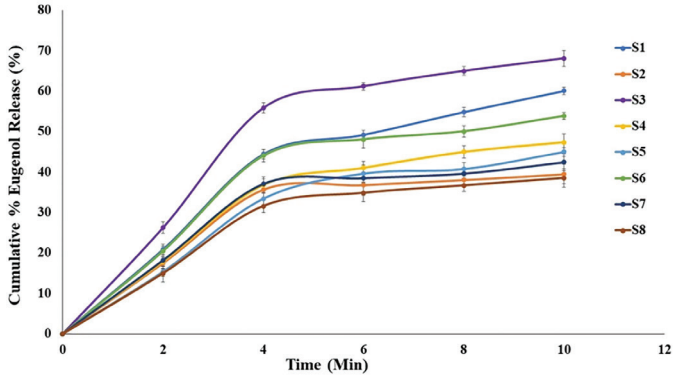

Figure 10. Cumulative drug release profile of the immediate release film

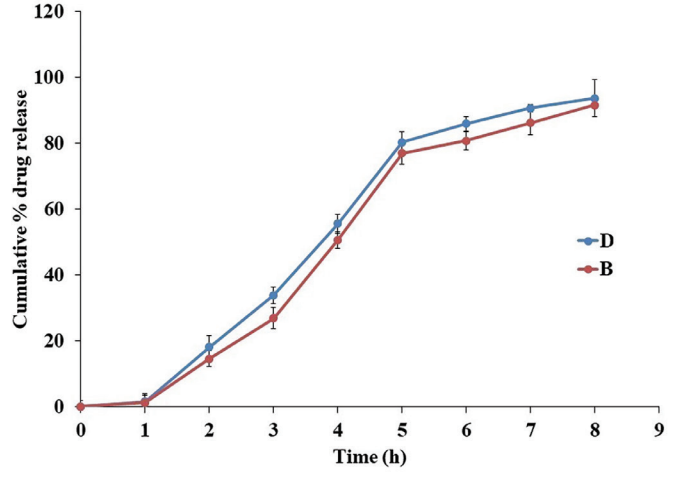

Figure 11. Cumulative drug release profile of the B and D films

In vitro antimicrobial activity of the periodontal film

The *in vitro* antimicrobial activity of the periodontal film was tested using the Kirby-Bauer disk diffusion method. This procedure is routinely adopted for the susceptibility testing of microbial isolates because it gives reliable results comparable to those of the standard epsilometer test and is useful to test the clinical efficacy of antibiotics. This test was based on the fact that for a given antibiotic, the zone of inhibition is related to minimum inhibitory concentration. To conduct the test, MHA was used. MHA is a non-differential and non-selective medium. It is composed of acid hydrolysate and beef extract, which acts as a nutrient source. Starch is incorporated to trap any toxic metabolites produced by microbes. Starch hydrolysis generates dextrose, which acts as an energy source. The rate of diffusion of antibiotics is enhanced in the presence of starch. Agar acts as a solidifying agent. *E. coli* (ATCC25922) and *S. aureus* (ATCC25323) bacteria were used to measure the zone of inhibition on both films to determine their antibacterial activity (ATCC25323) (Figure 12).

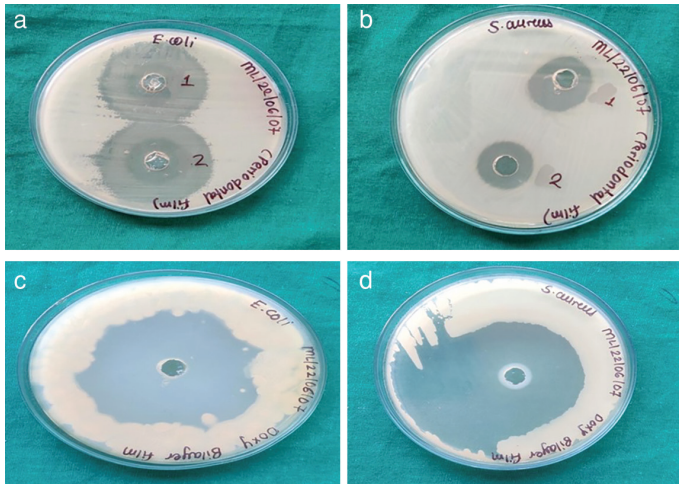

Figure 12. Antimicrobial activity of films: a. effect of primary layer film containing doxycycline hyclate (1) and clove oil (2) on *Escherichia coli*, b. effect of primary layer film containing doxycycline hyclate and clove oil on the zone of *Staphylococcus aureus*, c. effect

The zone of inhibition was calculated from four samples, and it was found that the primary layer (D) exhibited a zone of inhibition of 16.4 ± 1.25 mm for *the E. coli* and 11.5 ± 2.21 mm for the *S. aureus*. In contrast, clove oil-loaded films (S) showed zones of inhibition of 16.6 ± 1.84 mm and 12.1 ± 2.16 mm on *E. coli* and *S. aureus* species. The zone of inhibition for bilayer film (B) was found to be 22.5 ± 1.28 and 20.8 ± 2.47 mm for *E. coli* and *S. aureus*, respectively. The effect of the formulation on gram-negative bacteria was greater than that on positive bacteria. The bilayer film indicated a slightly higher zone of inhibition compared with the primary layer film of doxycycline hyclate. The antimicrobial effect contributed to the combined activity of clove oil and doxycycline hyclate. Periodontitis is mainly caused by *Aggregatibacter, Actinomycetemcomitans,* and *Porphyromonas gingivalis* infection and is a gram-negative bacterium. Antibacterial studies revealed that the bilayer film had a more profound effect on gram-negative bacteria, indicating the effectiveness of the bilayer film in controlling periodontal infection.

DISCUSSION

The diamentions, pH and mechanical strength of bilayer film was suitable to insert in periodontal pocket. All the films exhibited good drug entrapment. Disintegration time for immediate release layer was sufficient to exert preliminary therapeutic effect and treatment goal will be effectively achieved by sustained release layer. Primary layer and bilayer films showed good mucoadhesive strength and swelling owing to polymer properties. Antimicrobial studies were suggestive of microbicidal activity of films on gram-positive and gram-negative bacteria which could be beneficial to counteract periodontal infection.

CONCLUSION

Mucoadhesive bilayer films were developed for the twin delivery of clove oil and doxycycline hyclate as immediate and sustained release layers, respectively, for the treatment of periodontitis. Clove oil layer was developed to control pain and inflammation, and sustained antimicrobial effect was contributed by the doxycycline hyclate layer. The primary layer was composed of flax seed and HPMC and was found to be effective in retarding doxycycline hyclate release. The formulation containing an equal amount of sodium alginate and PVA showed better disintegration, and hence, it was selected as an optimized batch for the immediate release of clove oil. Bilayer films prepared by casting primary and secondary layers showed promising effects as antibacterial agents. Hence, it can be concluded that the bilayer formulation was effective in treating periodontal conditions.

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Ethics

Ethics Committee Approval: There is no requirement for ethical approval as animals were not used in current study.

Informed Consent: There is no requirement for informed consent as study was not conducted on helthy human volunteers/patients.

Authorship Contributions

Concept: U.Y.K., C.R.G., A.P.P., N.P.P., Design: U.Y.K., C.R.G., N.P.P., N.M.M., Data Collection or Processing: U.Y.K., C.R.G., A.P.P., N.P.P., A.P.P., Analysis or Interpretation: U.Y.K., A.P.P., K.R.K., P.D.C., N.M.M., Literature Search: U.Y.K., C.R.G., N.P.P., Writing: U.Y.K., C.R.G., A.P.P., N.P.P.

Conflict of Interest: The authors have no relevant financial or non-financial interests to disclose.

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