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Flaxseed mucilage/HPMC and sodium alginate/polyvinyl alcohol composite bilayer film as a promising drug carrier for periodontal treatment

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Abstract
Purpose: Present study focused on formulation of mucoadhesive bilayer composite films for treatment of periodontitis and evaluation of its physicochemical properties.
Methods: Solvent casting technique was used to prepare films. Primary layer (D) was prepared with flaxseed and HPMC composite to sustain release of doxycycline hyclate. Second layer (S) was comprised of sodium alginate and PVA composite for faster release of clove oil. Both layers were combined to generate bilayer film (B). All formulations were characterized further to get optimized formulation.
Results: ATR-FTIR results showed intactness of drug and clove oil in presence of excipients. pH of films was compatible with periodontal cavity and thickness was suitable to insert into cavity. Immediate release layer showed faster disintegration and swelling. Content of clove oil was above 80%. Rate of swelling of a primary layer was slow and drug content complies with United States Pharmacopoeia. SEM analysis revealed intact, non-porous and smooth films. Films exhibited better mechanical strength and bio-adhesiveness. Clove oil was released from immediate release layer within 10 min and doxycycline hyclate release was retarded minimum of up to 8 h in primary layer as well as bilayer. Formulation also showed a significant effect on both *E-coli* and *S. Aureus*.
Conclusion: In current study bilayer were successfully prepared and characterized. Optimized formulation be effectively used for 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 it is widely spreadable chronic disease around the world. Around 20 to 50% population around globe is suffering from periodontal diseases and tooth loss.[1] It is mainly caused by bacterial attack on tissues that support and surrounds teeth. Space between tooth and gingiva is referred to as periodontal pocket and disease of periodontal pocket is known as periodontitis. Periodontitis is a complex inflammation caused by periodontal microorganisms that induce destruction of periodontal tissue. Predominantly gram-negative, microaerophilic, anaerobic bacteria colonize as biofilm in sub-gingival area that alter connective tissue and bone metabolism leading to periodontal damage. Severity of disease also depends on 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 area. Investigations were carried out to slow progression or to improve periodontal status by systemic administration of antibiotics. A class of tetracycline antibiotics has been studied for treatment of periodontitis.[3] Doxycycline hyclate from this class has been reported to exert an anti-inflammatory and antibacterial effect. 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 supress activity of matrix metalloproteinases that is responsible for destruction of periodontal tissues.[4] Besides, it stimulates formation of bone tissue by instigation of inhibition of bone resorption and osteoblasts. Local administration of antibiotics leads to a reduction in pocket depth and a better effect was achieved by use of doxycycline hyclate.[5] Owing to antimicrobial and non-antimicrobial properties doxycycline hyclate was selected as first model drugs in bilayer film. Clove oil was permitted by USFDA in dentistry by way of a natural analgesic and antiseptic.[6] Eugenol suppresses 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 bacteria and gram-positive bacteria.[8] Antimicrobial activity of eugenol on some bacteria was due to induction of cell lysis via leakage of lipids and protein in cell membrane.[9]

Flax seed was reported to contains protein and a mixture of various carbohydrates mainly rhamnose, galactose, glucose, and arabinose. Acidic polysaccharides galacturonic acid, pectin-like polymers, rhamnogalacturonan and arabinoxylans were also reported by researchers. It also composed of 3 to 9% of water-soluble heteropolysaccharide of total seed content, that 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 was reported to have 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] It was also explored as mucoadhesive and sustained release polymer. HPMC is a hydrophilic polymer that fits to a group of hydroxyethyl ether. It is soluble in both organic and aqueous solvents and forms transparent, flexible films in an aqueous solution. Low toxicity biodegradability and biocompatibility are key properties of HPMC to explore its application in pharmaceutical and biomedical fields. Several researchers combined HPMC with other polymers and lipids to form composites with enhanced characteristics. Sustained release and mucoadhesive properties of HPMC were also reported by scientists.[12] Polysaccharide of alginic acid; sodium alginate is a made up of α-l-guluronic (G) and β-d-mannuronic (M) acids units. It is integral element of cell wall of brown algae and a few bacteria. It is widely available, cheap in cost and biodegradable in nature. It can form transparent, water-insoluble, thermo irreversible gels by crosslinking with di and trivalent ions and hence finds wide application in pharmaceuticals. Sodium alginate has been extensively explored as a film former and drug carrier but is always used in combination with another polymer to form a film.[13] Polyvinyl alcohol is a synthetic polymer made by complete or partial hydrolysis of polyvinyl acetate. It is 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 for enhancing mechanical strength. It undergoes fast swelling and dissolution in water. It exhibits excellent film forming ability and studied by researchers for a variety of targeted applications.[15]

Taking note of above data, current research work was aimed to prepare composite films to treat periodontitis. Composite was prepared in two layers. First layer was aimed at sustained release of doxycycline hyclate and comprises flaxseed and HPMC. Second layer was made to release clove oil immediately and was made up of sodium alginate and PVA composite. Films were evaluated and explored as a carrier for doxycycline hyclate and clove oil.

MATERIAL 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. Hydroxypropyl methylcellulose was purchased from Loba chemie, India. Sodium alginate was purchased from Thermosil fine chem industries, Pune, India. Polyvinyl alcohol was acquired from Research-lab fine chem industries, India. All other reagents used were of analytical grade.

Extraction of flaxseed mucilage

Flaxseeds were purchased from a local store and cleaned. Flaxseeds (30gm) drenched in 900 mL of distilled water at 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. Supernatant solution was then kept for normalizing to ambient conditions (27°C). Mixture was placed into centrifuge test tubes and rotated at 3900 rpm, 15 minutes for separation of mucilage solution from flaxseed. Flaxseed was subsequently filtered with cheesecloth to obtain reaming mucilage that is attached to flaxseed coat. Ethanol was added to filtered extract to precipitate the mucilage. Mucilage that had precipitated was isolated and dehydrated for 5 h in a hot air oven at 50°C. Yield of dried mass was quantified, phytochemical screening was performed and stored in a desiccator.[16]

Preparation of double-layer film

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

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

Preparation of secondary clove oil -loaded film (S)

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

Preparation of Bilayer film (B)

Primary layer was dried thoroughly and 0.5% w/v of a freshly prepared calcium chloride solution was sprinkled over it. Polymeric mixture of sodium alginate and PVA-containing clove oil was casted over primary layer. Bilayer film was further dried in a hot air oven and peeled off (Figure 3). Film was enveloped in an aluminum foil and kept in desiccator.[19] (Table I and II).

Evaluation of films

Surface pH Determination

Agar plates formulated in phosphate buffer pH 6.8, films were permitted to hydrate for 2 h on agar plates. pH meter (Mettler Toledo, India) was positioned in contact with hydrated patch and pH of surface was checked. Average of three measurements was recorded.[20]

ATR-FTIR

By using an attenuated total reflectance (ATR) accessory, Tensor 37 FTIR equipment (Bruker, Germany) spectra of pure drug, physical mixture of drug and excipient, and optimum formulation was recorded. By averaging 10 scans at resolution of 4 cm^{-1}, single spectra in 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 utilized for measurement of thickness at 10 different points. A thickness of films was estimated by digital vernier caliper (Mitutoyo, Japan). 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 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 to a 100 mL volumetric flask, final volume was made with pH 6.8 phosphate buffer. 1 mL of aliquot was removed from solution and diluted to 10 mL with phosphate buffer pH 6.8. Absorbance of resulting solution was measured at 271.3 nm by a UV-visible spectrophotometer (Shimadzu-1800). Similar method was followed for second film loaded with clove oil by recording absorbance at 283 nm. For bilayer film, absorbance was recorded at 271.3 and 283 nm for doxycycline hyclate and clove oil respectively. [23]

Disintegration time

Film was cut into 9.0×9.0 mm^2 and placed in a petri plate containing 5 mL purified water and time needed to disintegration of secondary film completely was recorded. Average of three determinations of results was noted.[24]

Surface morphology

Surface morphology of 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 transactional view of bilayer film with 100x power lenses. For SEM, specimen sample mounted on metal stubs with a double-sided adhesive band, gold was sputtered on specimen to confirm sufficient electrical conductivity. Images were taken using ET detector with 10 kV excitation energy.[25]

Folding endurance

Films was cut into 1.0×1.0 cm^2 and continually creased at same point until disruptions. Number of counts film could be creased without breaking was noted.

Tensile strength and elongation at failure percentage (EF%)

Texture Analyzer (CT-3 Brookfield, USA) was used to investigate tensile strength of film.

Sample of 4 cm^2 was taken and secured between two clamps of probe TA-DGA. Bottom clamp was detained

immobile and film was stretched apart by top clamp at a speed of 2.0 mm/s to a distance of 6 mm with a trigger load of 0.05 N. Force required to break film was recorded. Data assemblage and calculations were accomplished using Texture Pro CT V1.3 Build 14 software. Tensile strength at break rate was calculated using formula:

$$\text{Tensile strength (N/cm2)} = \text{Breaking force (N)} / \text{Cross} - \text{sectional area of sample (cm2)}$$

Elongation at break %, a measure of percentage of a film that has ruptured, it was determined using 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

Bioadhesion force was estimated using a Texture Analyzer (CT-3/100, Brookfield, USA) equipped with a 100 g load cell. Bioadhesive force was recorded by porcine buccal mucosa. Mucosal membrane was cut and underlying connective tissue was separated. It was thoroughly cleaned with pH 6.8 phosphate buffer and secured between two circular discs positioned at bottom perspex support. Mucosal membrane was exposed to probe via top spherical disc with a void of 12.7 mm diameter. Discs were let down into jacketed glass container composed of pH 6.8 phosphate buffer and sustained at 37 ± 1 ˚C. Membrane was equilibrated at this temperature condition for 30 min. Buccal film was firmly secured using thread on bottom side of probe. Circular cavity and probe were brought into line to safeguard film originate in intimate contact with mucosal membrane. Prior to study, buccal film was hydrated with pH 6.8 phosphate buffer. Load of 90 gm was applied and a probe was lowered at a speed of 0.5 mm/s to contact tissue for 120 s. It was removed at 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 strength of bioadhesion of film. Bioadhesion force (N) was calculated using formula:

Bioadhesion force (N) = Bioadhesive strength (g)× 9.8/1000

Swelling studies

Films were weighed individually and initial weight was noted (W1). Films were placed separately in petri-dish enclosing pH 6.8 phosphate buffer. Samples were isolated from petri plate hourly and extra amount of buffer was wiped carefully by using filter paper. Hydrated films were weighed (W2). Swelling index was determined by 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 hold in 50 mL phosphate buffer 6.8 maintained at 37ºC 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 0.5h, 1h, 2h, 3h, 4h, 5h, 6h,7h, 8h to quantify doxycycline hyclate. Sink conditions were maintained by replacing an identical quantity of pre-warmed buffer solution. Samples were investigated using a UV spectrophotometer at 271.1 nm and 283 nm for doxycycline hyclate and clove oil respectively. Drug release experimentation was completed in triplicate and mean was reported. 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 periodontal film

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

RESULTS AND DISCUSSION

Periodontitis results in pain and inflammation surrounding teeth as a result of infection to gingival tissue that emerges a need to counteract pain and eradicate infection. Owing to antimicrobial properties, doxycycline hyclate was selected as model drug. Combination of eugenol with antibiotics was reported to derive synergistic effect.[28] Eugenol is reported to be present in clove oil, hence in current study clove oil was used in combination with doxycycline hyclate. Mucilage from flax seed was isolated by a simple process and characterized. Yield and ash value of mucilage were found to be 6.3% and 4.2% respectively. Isolated sample showed presence of carbohydrates and protein. These results were found in agreement with evaluation of flaxseed mucilage carried out by Kaewmanee *et al.* (2014).[29] First layer of bilayer film was tried to formulate by flaxseed mucilage alone but due to high viscosity of mucilage, obtained film was sticky and difficult to peel off from petri dish. Hence 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 low concentration of HPMC was selected. Earlier studies also suggest that films prepared with a lower concentration of polymer were visually more homogeneous and thinner and drug distribution in film was uniform. Sodium alginate forms a clear transparent, flexible film but brittleness of sodium alginate restricts its use as an excellent film former; hence it was combined with polyvinyl alcohol (PVA) which is highly elastic and biocompatible. Some researchers reported that blends of this polymer were found to enhance mechanical strength of film as well as resulting product is highly hydrophilic. 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 odor of clove oil. In film casting technique, drying was carried out at a gentle rate hence aggregation and creases on film surface were not noticed. Doxycycline hyclate is freely soluble in water and polymers also form clear solutions, hence obtained films were transparent. Flax seed mucilage and sodium alginate had a slightly yellowish to an off-white color, hence final films also exhibited same color (Figure 4b). Incorporation of glycerin as a plasticizer in films resulted in flexible films while films produced without glycerin were brittle. Morphology of film was not affected by addition of glycerin.

Surface pH

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

ATR-FTIR

This test was performed to find out compatibility amongst excipient and drug. Spectra of doxycycline hyclate showed characteristic peaks at 1665.65, 1328.27 cm^{-1} conforming to C=O group, peaks between 3537.02 to 3812.43 cm^{-1} represented C-H, N-H and O-H stretching, peak at 1456.39 cm^{-1} showed presence of C–H and N–H in-plane bend vibrations. 1217.98 cm^{-1} peak was indicative of C-N stretching. These results were found in agreement with research carried out by other researchers.[30] In addition to this USP monograph suggested bands for doxycycline hyclate tablet 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 drug in presence of excipients. Primary layer (D) also resembles peaks of doxycycline hyclate with slight shifting of peaks. Slight shifting of peaks might be attributed to physical interactions due to formation of composite. Spectra of clove oil showed a peak at 3000.25 cm^{-1} and 3634.58 cm^{-1} owing to O-H stretching, 1657.35 cm^{-1} representative of C-H stretching vibration of benzene, eugenol methyl C-H deformation vibration denoted at 1365.87 cm^{-1}, 1758.24 cm^{-1} peak resembled C=O carboxylic acid stretching vibration, phenolic hydroxyl C-O stretching vibration appeared at 1278 cm^{-1}. C-O-C aromatic ether vibration was denoted at 1033. 21cm^{-1}. Spectra were indicative of presence of eugenol and ether group, benzene ring, and phenolic hydroxyl peaks confirmed presence of eugenol. Spectra were found in agreement with spectra reported in other research work. [31] Second layer S3 film also showed presence of similar groups additionally peak of PVA at 2932.57 cm^{-1} was observed. S3 film spectra closely resemble spectra of clove oil indicating intactness of clove oil in film. Various spectra were revealed in Figure 5.

Thickness and weight

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

Drug content

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

Disintegration time

Official guidelines are deficient of determination of disintegration time of films. Pharmacopoeia describes standard disintegration tests for conventional dosage forms, but for films when this method was tried, film was adhered to

wall of tube, also small pieces float inside tube which made visual detection difficult. Due to these practical difficulties erroneous results might be obtained. Generally, for films disintegration test is done by two methods; slide frame method and petri dish method; out of which petridish method was adopted for current study. Disintegration time for primary layer was fund 8 h indicating slower penetration of solvent due to high viscosity of polymers. This was promising property to maintain film at site of administration for prolonged drug release. It was proposed that secondary films should disintegrate faster as compared to primary layer and release clove oil to counteract inflammation and pain. Disintegration time for secondary layer (S) was recorded and it was found in range of 8.46 ± 0.74-11.86 ± 0.08 minutes. Water molecules were rapidly penetrated in films causing dispersion of film into small pieces that ultimately released clove oil at a faster rate as compared to doxycycline hyclate. Rapid penetration of water in secondary film might be result of lyophobic nature of sodium alginate and PVA. From these results, optimized ratio of sodium alginate and PVA was selected as 200:200 and further tests were carried out on optimized bilayer film.

Surface methodology studies

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

Folding endurance

It is an index to investigate mechanical property and flexibility of film. Optimum value of folding endurance eases manufacturing and administration of films. A direct relation exists between folding endurance and mechanical property of film. Folding endurance of primary layer (D) and bilayer was (B) 218 ±16 and 304 ±18 times respectively. Bilayer film exhibit more value of folding endurance indicating more flexibility and mechanical strength. Near about similar values were reported for HPMC films in combination with Eudragit RL 100 and Carbopol-934.[32] Secondary layer (S) showed folding endurance in range of 190 ± 0.15 to 213 ± 0.13 times, which was found higher than 145 and 152 reported in earlier research.[33] All films had a good value of folding endurance, showing films are flexible with good mechanical strength.

Tensile strength and elongation at failure percentage (EF%)

Maximum resistance of film to break under applied load is tensile strength and it is an index to confirm mechanical strength of film. For primary layer (D) and bilayer (B), tensile strength was 4.11±0.04 and 4.16±0.02 N/cm² respectively. Largest shift in film's length before breakage is called elongation at break. Maximum deformation a film can experience before breaking, it symbolizes film's ductility and resistance to distortion. It is defined as maximum distortion a film could undergo before it fails or breaks. EF for primary layer (D) and bilayer (B) was 5.14±1.6 and 6.12±1.5% respectively. Amount of drug in film also affects mechanical properties of film, in current study dose of drug is less, hence mechanical properties of optimized film were found 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 in agreement with research carried out by Mehrsima Ghavami-Lahiji,2019 *et. al.*[34]

In vitro bio adhesion force

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

Swelling index

For natural polymers exhibiting swelling-controlled release, swelling is a crucial component of drug release. Swelling studies play a substantial role in bioadhesive properties as swelling of polymer contributes to polymer chains disentanglement and relaxation that initiates diffusion of polymeric chains into mucus membrane for process of bioadhesion. A swelling study for 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 media, polymer chains undergo relaxation and interpenetration that causing swelling. Further expansion of polymeric matrix may inintialte generation of slippery mucilage, consequents' early release of drug that has been trapped therein. Figure 9 illustrates swelling index of films. Swelling index of bilayer film increased steadily till 25 min and reached equilibrium. At the end of 30 min, swelling index started declining at a very slow rate, these results might be attributed to slow erosion of polymers. Swelling index of a primary layer ranged between 6.5-13.9%, it increased till 30 min and retained equilibrium till 35 min after that it started eroding at a very slow rate. Rate of hydration of bilayer film was found higher than 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 swelling index declined slightly, possibly due to polymeric erosion of both layers. Surface area and rate of solvent diffusion in films might be major contributing factors to initiate swelling of films . These results can be correlated with mucoadhesion study where bilayer film showed slightly higher mucoadhesion as compared to primary layer. More swelling resulted in relaxation of polymeric chain and exposure of polymer at bioadhesive site. A faster swelling bilayer initiated quick formation of adhesive bonds.

In- vitro drug release

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

Near about 33.82% drug was escaped from film primary layer D at inintial 3 h followed by a cumulative 93.71% drug release of up to 8 h. For bilayer film (B), 29.81 % of drug was released at inintial 3 h and 91.58% at the end of 8 h from sustained release layer (Figure 11). Immediate release layer showed 69.25% release of clove oil within 10 min. from bilayer film (B). Clove oil is insoluble in aqueous-based media. It acted as an additional hurdle for diffussion of media in B film and additionally contribute to sustained release of doxycycline hyclate. Obtained results were in consensus with earlier studies about bilayer films, wherein components of another layer (clove oil) had impact on release of actives from primary layer [19]. Flaxseed mucilage and HPMC were reported as sustained release polymer. Both forms very viscous gel upon contact with dissolution media and doxycycline hyclate molecules dissolve in media and then gradually diffuse out. This process of dissolution and diffusion was time-consuming hence, sustained release effect was achieved. 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 model was 0.9821, 0.9924, 0.872 and 0.8406 respectively. Release mechanism of drug was concentration dependant manner and it followed first-order kinetic. Obtained results were found in agreement with research carried out for metronidazole-loaded films for periodontal treatment.[37]

In vitro antimicrobial activity of periodontal film

In vitro antimicrobial activity of periodontal film was tested by Kirby-Bauer disk diffusion method. This procedure is routinely adopted for susceptibility testing of microbial isolates as it gives reliable results comparable to standard Epsilometer test and useful to test clinical efficacy of antibiotics. This test was based on fact that for a given antibiotic, zone of inhibition is related to MIC. To carry out test Mueller Hinton Media (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 nutrients source. Starch is incorporated to trap any of toxic metabolites produced by microbes. Starch hydrolysis generates dextrose, which acts as energy source. Rate of diffusion of antibiotics is enhanced in presence of starch. Agar acts as a solidifying agent. *E. coli* (ATCC25922) and *S. aureus* (ATCC25323) bacteria were used to measure zone of inhibition on both films to determine their antibacterial activity (ATCC25323) (Figure 12).

Zone of inhibition was calculated from four samples, it was found that primary layer (D) exhibited a zone of inhibition of 16.4± 1.25 mm for *E-coil* and 11.5± 2.21 mm for *S-aureus*. Whereas clove oil loaded films (S) showed zone of inhibition of 16.6 ± 1.84 mm and 12.1 ± 2.16 mm on *E-coil* and *S-aureus* species. Zone of inhibition for bilayer film (B) was found to be 22.5 ± 1.28 and 20.8± 2.47 mm for *E-coil* and *S-aureus* respectively. Effect of formulation on gram-negative bacteria was more as compared to positive bacteria. Bilayer film indicated a slightly higher zone of inhibition as compared to primary layer film of doxycycline hyclate. Antimicrobial effect was contributed to combined activity of clove oil and doxycycline hyclate. Periodontitis is mainly caused by *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* infection and it belongs to gram-negative bacteria. Antibacterial studies revealed that bilayer film had a more profound effect on gram-negative bacteria, results were indicative of effectiveness of bilayer film in controlling periodontal infection.

CONCLUSION

Mucoadhesive bilayer films were formulated for twin delivery of clove oil and doxycycline hyclate as immediate and sustained release layers respectively, for treatment of periodontitis. Clove oil layer was formulated to control pain and inflammation and sustained antimicrobial effect was contributed by doxycycline hyclate layer. Primary layer was composed of flaxseed and HPMC and found effective to retard doxycycline hyclate release. Formulation containing an equal amount of sodium alginate and PVA showed better disintegration, hence selected as an

optimized batch for immediate release of clove oil. Bilayer film prepared by casting primary and secondary layer showed promising effects as an antibacterial agent. Hence it can be concluded that bilayer formulation was effective to treat periodontal conditions.

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References

1. Sanz M. European workshop in periodontal health and cardiovascular disease. European Heart Journal Supplements. 2010;12:B2.
2. Yucel-Lindberg T, Båge T. Inflammatory mediators in the pathogenesis of periodontitis. Expert Reviews in Molecular Medicine. 2013;15:e7.
3. Park J-B. Effects of doxycycline, minocycline, and tetracycline on cell proliferation, differentiation, and protein expression in osteoprecursor cells. Journal of Craniofacial Surgery. 2011;22(5):1839-1842.
4. Ioannou I, Dimitriadis N, Papadimitriou K, Vouros I, Sakellari D, Konstantinidis A. The effect of locally delivered doxycycline in the treatment of chronic periodontitis. A clinical and microbiological cohort study. Journal of Oral & Maxillofacial Research. 2010;1(4):e1.
5. Bogren A, Teles RP, Torresyap G, Haffajee AD, Socransky SS, Wennström JL. Locally delivered doxycycline during supportive periodontal therapy: a 3-year study. J Periodontol. 2008;79(5):827-835.
6. Nejad SM, Özgüneş H, Başaran N. Pharmacological and toxicological properties of eugenol. Turk J Pharm Sci. 2017;14(2):201-206.
7. Leem H-H, Kim E-O, Seo M-J, Choi S-W. Antioxidant and anti-inflammatory activities of eugenol and its derivatives from clove (*Eugenia caryophyllata* Thunb.). Journal of the Korean Society of Food Science and Nutrition. 2011;40(10):1361-1370.
8. Leite AM, Lima EdO, Souza ELd, Diniz MdFFM, Trajano VN, Medeiros IAd. Inhibitory effect of beta-pinene, alpha-pinene and eugenol on the growth of potential infectious endocarditis causing gram-positive bacteria. Revista Brasileira de Ciências Farmacêuticas. 2007;43:121-126.
9. Oyedemi S, Okoh A, Mabinya L, Pirochenva G, Afolayan A. The proposed mechanism of bactericidal action of eugenol,∝-terpineol and g-terpinene against Listeria monocytogenes, Streptococcus pyogenes, Proteus vulgaris and Escherichia coli. African Journal of Biotechnology. 2009;8(7):1280-1286.
10. Barbary O, Al-Sohaimy S, El-Saadani M, Zeitoun A. Extraction, composition and physicochemical properties of flaxseed mucilage. J Adv Agric Res. 2009;14(3):605-621.
11. Khalloufi S, Corredig M, Goff HD, Alexander M. Flaxseed gums and their adsorption on whey protein-stabilized oil-in-water emulsions. Food Hydrocolloids. 2009;23(3):611-618.
12. Kelemen A, Katona B, Módra S, Aigner Z, Sebe I, Pintye-Hódi K, et al. Effects of sucrose palmitate on the physico-chemical and mucoadhesive properties of buccal films. Molecules. 2020;25(22):5248.
13. Pamlényi K, Kristó K, Jójárt-Laczkovich O, Regdon G, Jr. Formulation and optimization of sodium alginate polymer film as a buccal mucoadhesive drug delivery system containing cetirizine dihydrochloride. Pharmaceutics. 2021;13(5):619.
14. Nagarkar R, Patel J. Polyvinyl alcohol: A comprehensive study. Acta Scientific Pharmaceutical Sciences. 2019;3(4):34-44.
15. Qureshi D, Sahoo A, Mohanty B, Anis A, Kulikouskaya V, Hileuskaya K, et al. Fabrication and characterization of poly (vinyl alcohol) and chitosan oligosaccharide-based blend films. Gels. 2021;7(2):55.
16. Fekri N, Khayami M, Heidari R, Jamee R. Chemical analysis of flaxseed, sweet basil, dragon head and quince seed mucilages. Res J Biol Sci. 2008;3(2):166-170.
17. Nurdianti L, Rusdiana T, Sopyan I, Putriana NA, Aiman HR, Fajria TR. Characteristic comparison of an intraoral thin film containing astaxanthin nanoemulsion using sodium alginate and gelatin polymers. Turk J Pharm Sci. 2021;18(3):289.
18. İnal Ö, Yapar EA, Baykara T. Physical properties and in vitro release studies on sotalol monolithic films prepared by eudragit polymers. Turk J Pharm Sci. 2010;7(2):151-160.
19. Li A, Khan I, ullah khan I, Yousaf A, Shahzad Y. Gellan gum-based bilayer mucoadhesive films loaded with moxifloxacin hydrochloride and clove oil for possible treatment of periodontitis. Drug Des Devel Ther. 2021;15:3937-3952.

20. Khan G, Yadav SK, Patel RR, Nath G, Bansal M, Mishra B. Development and evaluation of biodegradable chitosan films of Metronidazole and Levofloxacin for the management of periodontitis. AAPS PharmSciTech. 2016;17(6):1312-1325.
21. Marin MM, Albu Kaya MG, Iovu H, Stavarache CE, Chelaru C, Constantinescu RR, et al. Obtaining, evaluation, and optimization of doxycycline-loaded microparticles intended for the local treatment of infectious arthritis. Coatings. 2020;10(10):990.
22. Junmahasathien T, Panraksa P, Protiarn P, Hormdee D, Noisombut R, Kantrong N, et al. Preparation and evaluation of metronidazole-loaded pectin films for potentially targeting a microbial infection associated with periodontal disease. Polymers. 2018;10(9):1021.
23. Biswajit B, Mankad A, Dutta A. Methylphenidate Fast Dissolving Films: Development, Optimization Using Simplex Centroid Design and In Vitro Characterization. Turk J Pharm Sci. 2022;19(3):251.
24. Bhyan B, Jangra S, Kaur M, Singh H. Orally fast dissolving films: innovations in formulation and technology. Int J Pharm Sci Rev Res. 2011;9(2):9-15.
25. Kandekar UY. Exploiting natural polymer reinforced microspheres and investigating its characteristics for sustained release. International Journal of Pharmaceutical Sciences and Research. 2020;11(7):3367-3378.
26. Wong CF, Yuen KH, Peh KK. An *in-vitro* method for buccal adhesion studies: importance of instrument variables. Int J Pharm. 1999;180(1):47-57.
27. Pandit A, Khandagale K, Nakhate V, Dharmadhikari N. Antifungal topical gel of leaf extract of amaranthus viridis l. for treatment of cutaneous candidiasis. Indian Drugs. 2019;56(12):39-44.
28. Hemaiswarya S, Doble M. Synergistic interaction of eugenol with antibiotics against gram negative bacteria. Phytomedicine. 2009;16(11):997-1005.
29. Kaewmanee T, Bagnasco L, Benjakul S, Lanteri S, Morelli CF, Speranza G, et al. Characterisation of mucilages extracted from seven Italian cultivars of flax. Food Chemistry. 2014;148:60-69.
30. Yadav S, Mishra B. Preformulation studies on combination of ornidazole and doxycycline in pharmaceutical dosage forms: infra-red spectroscopy and simultaneous ultra-violet method development. Journal of Chemical and Pharmaceutical Research. 2016;8(8):564-573.
31. Hongfang G, Hui Y, Chuang W. Controllable preparation and mechanism of nano-silver mediated by the microemulsion system of the clove oil. Results in Physics. 2017;7:3130-3136.
32. Semalty M, Semalty A, Kumar G. Formulation and characterization of mucoadhesive buccal films of glipizide. Indian J Pharm Sci. 2008;70(1):43.
33. Lakshmi P, Lavanya D, Ali MH. Effect of synthetic super disintegrants and natural polymers in the preparation of donepezil hydrochloride fast disintegration films. International Current Pharmaceutical Journal. 2014;3(3):243-246.
34. Ghavami-Lahiji M, Shafiei F, Najafi F, Erfan M. Drug-loaded polymeric films as a promising tool for the treatment of periodontitis. J Drug Deliv Sci Technol. 2019;52:122-129.
35. Kandekar UY, Chaudhari PD, Chandrasekhar K, Pujari RR. Exploration of mucoadhesive microparticles by using *Linum usitatissimum* mucilage. Lat Am J Pharm. 2019;38(12):2463-2472.
36. Peh KK, Wong CF. Polymeric films as vehicle for buccal delivery: swelling, mechanical, and bioadhesive properties. Journal of Pharmacy and Pharmaceutical Sciences. 1999;2(2):53-61.
37. Shafiei F, Ghavami-Lahiji M, Jafarzadeh Kashi TS, Najafi F. Drug release kinetics and biological properties of a novel local drug carrier system. Dental Research Journal. 2021;18:94.

Tables

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

Formulation	Sodium alginate (mg)	Polyvinyl Alcohol (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

Table 2. Formulation of 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

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

*(n = 3, mean ± SD) All the readings were taken in triplicate

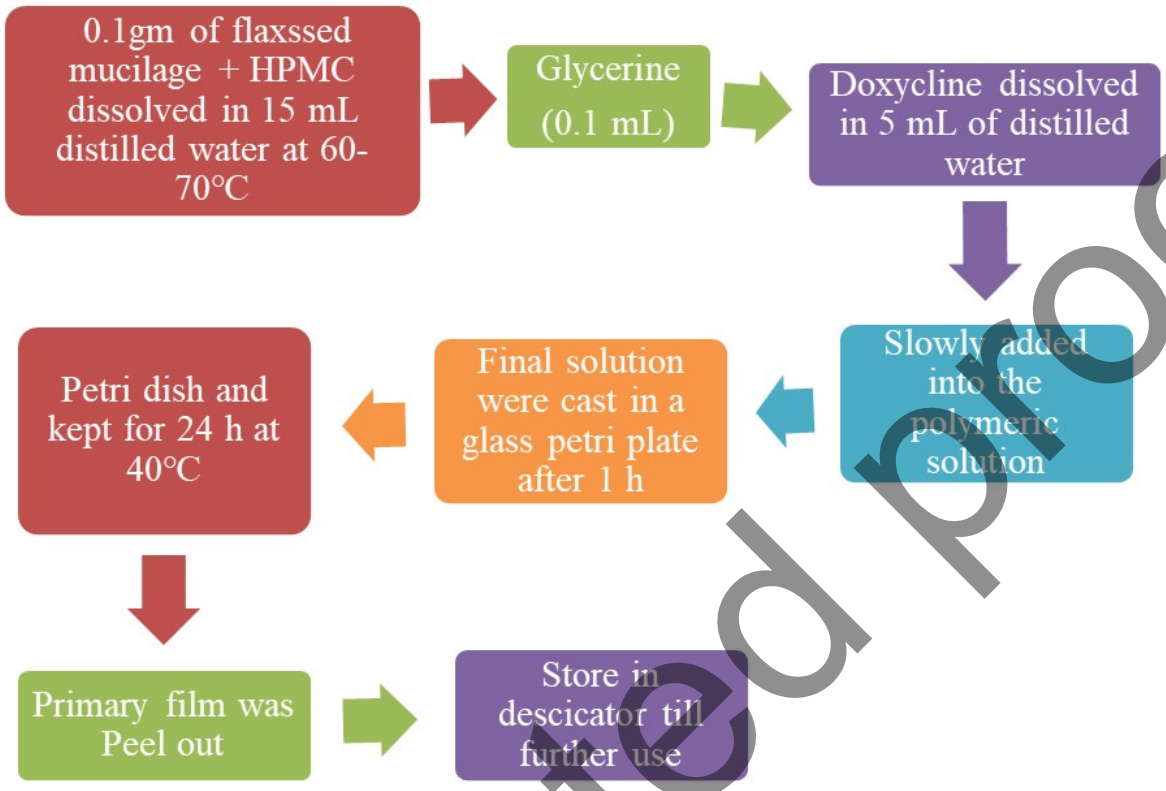

Figure 1 -Schematic representation of the preparation of the primary layer

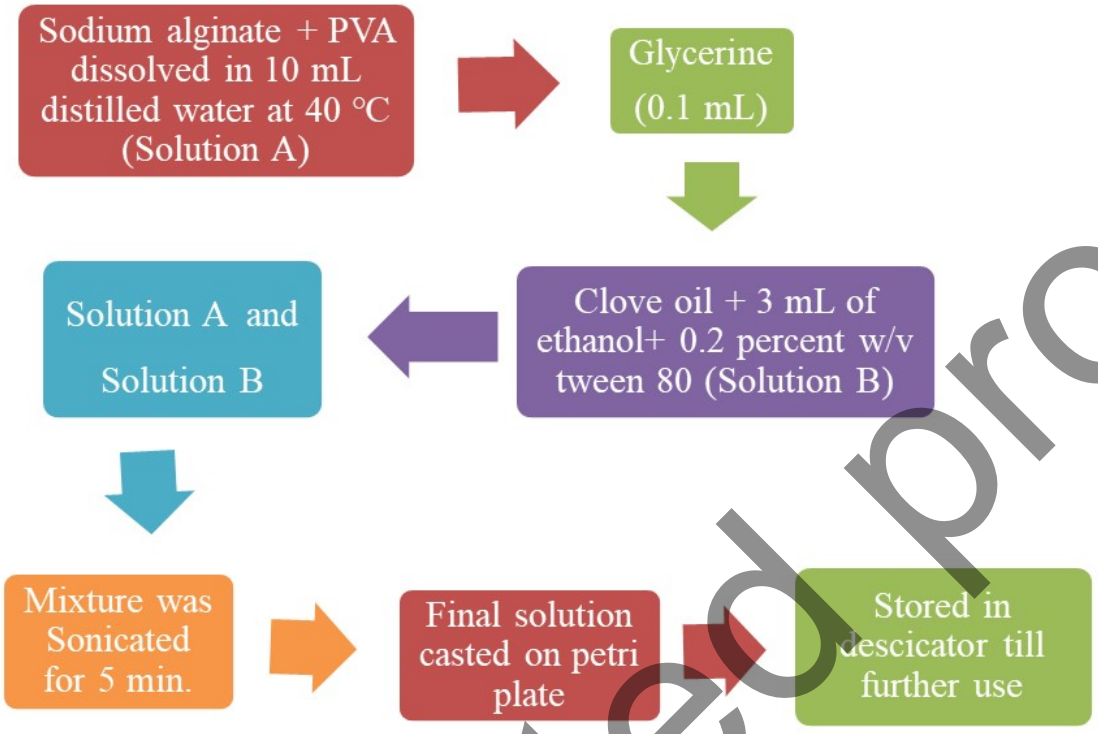

Figure 2 -Schematic representation of the preparation of the secondary layer

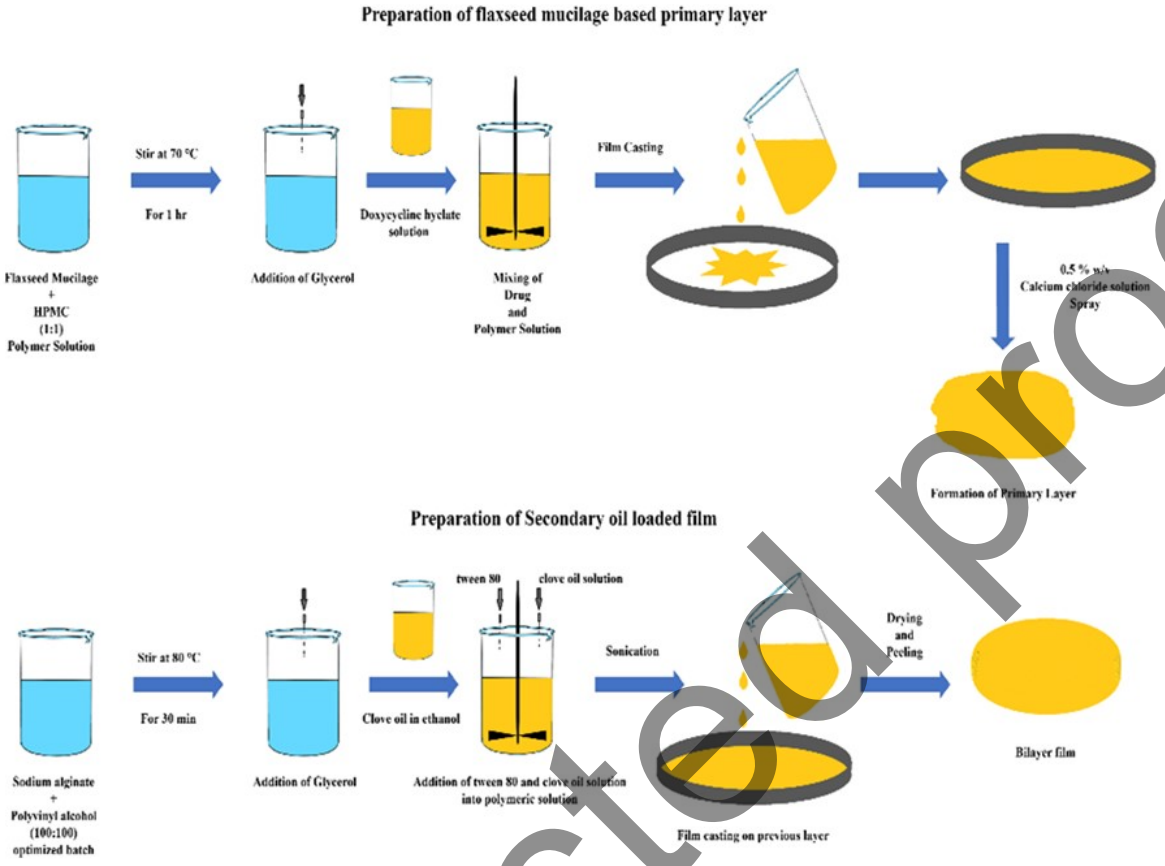

Figure 3. Schematic representation of the preparation of bilayer film

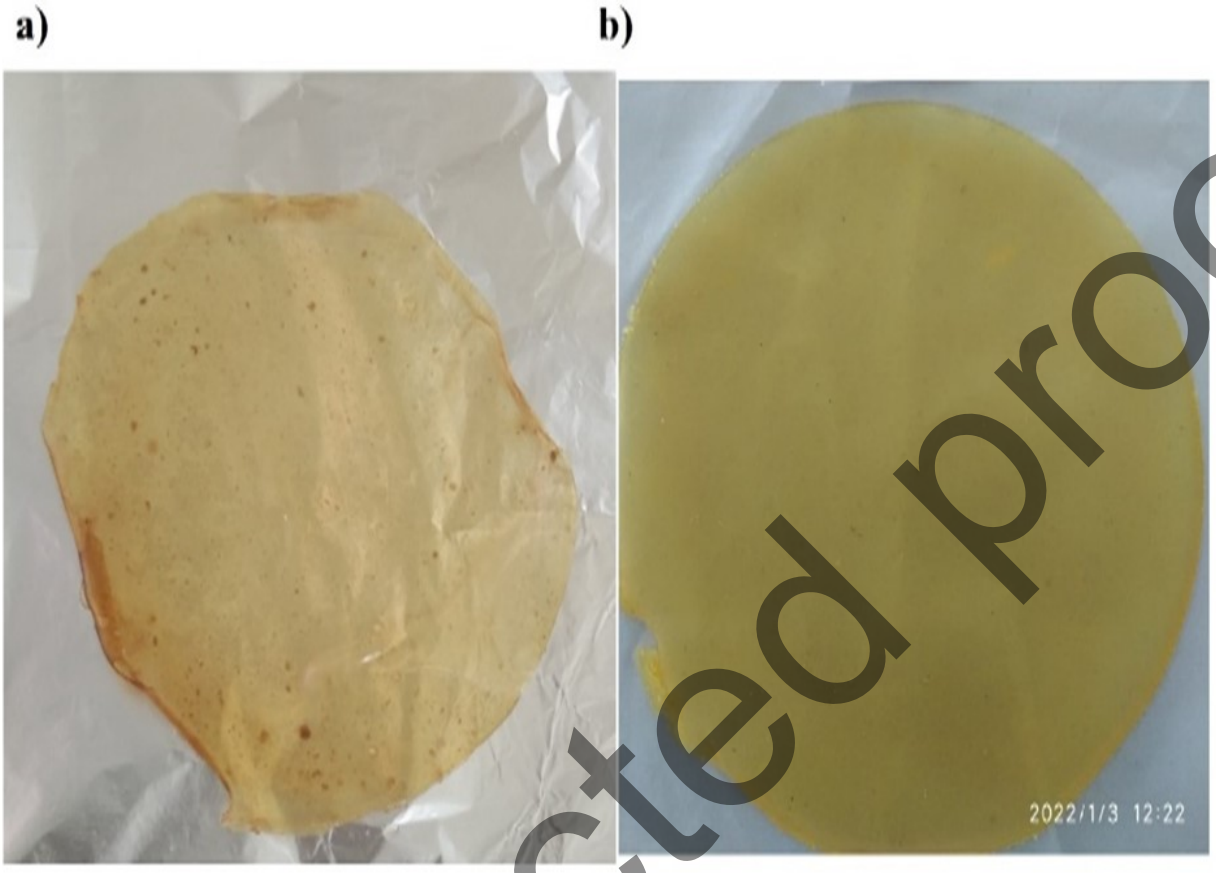

Figure 4 - a) plain flaxseed mucilage-based doxycycline hyclate loaded film (D), b) bilayer film (B) composed of doxycycline hyclate in primary layer and clove oil in secondary layer

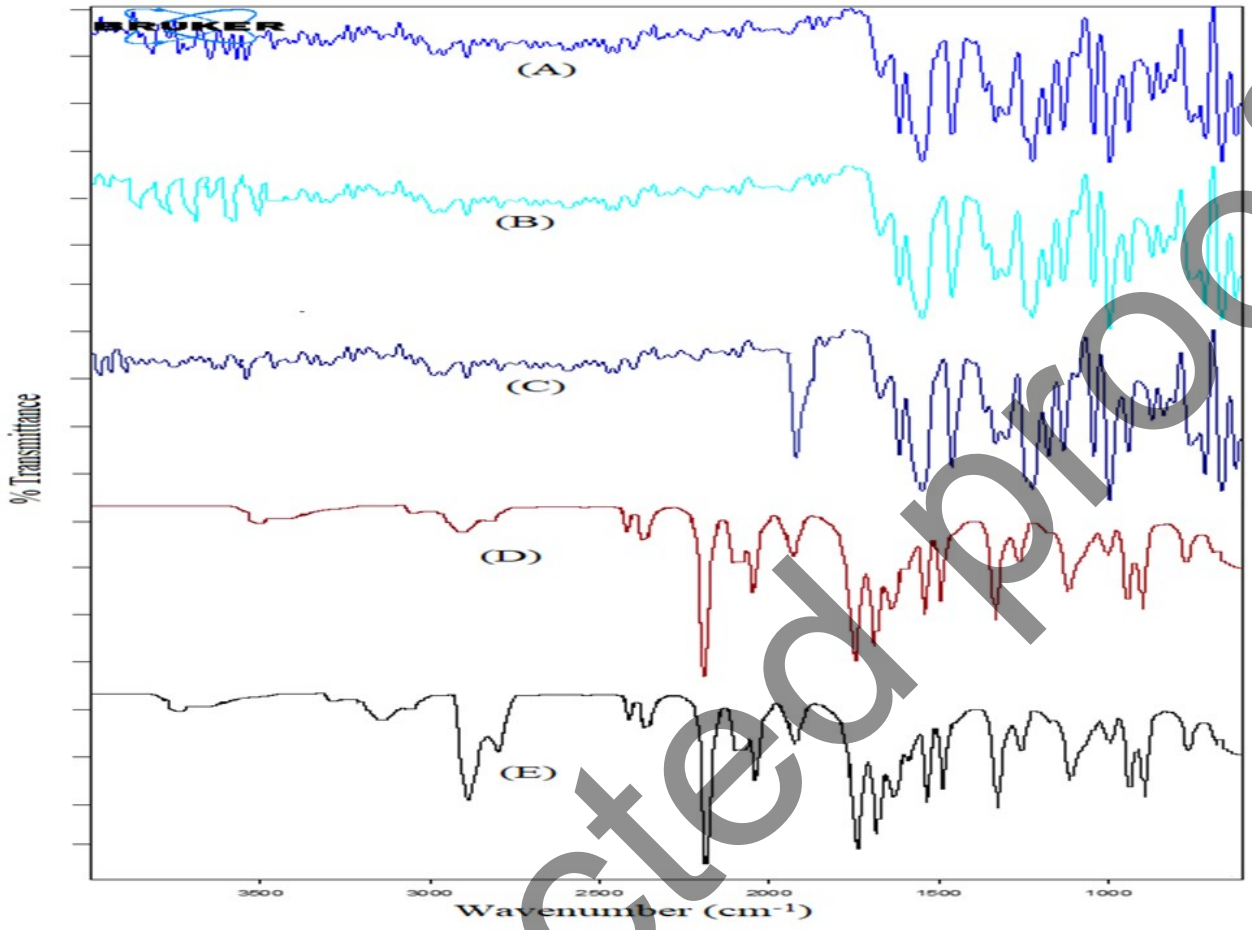

Figure 5. ATR-FTIR spectra of (A) Doxycycline hyclate (B) Physical mixture doxycycline hyclate, HPMC, Flaxseed (C) Formulation of primary layer (D1) (D) Clove oil (E) Formulation S3 layer.

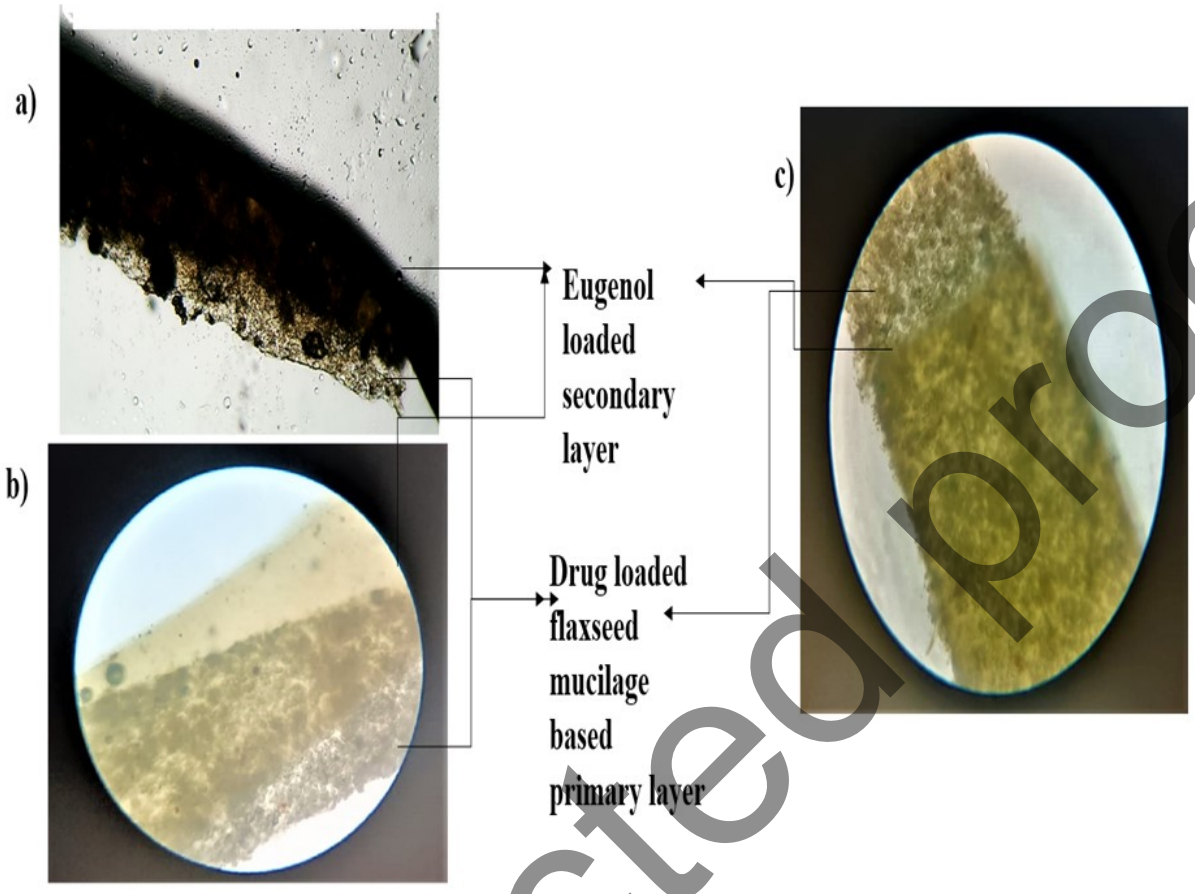

Figure 6. Optical microscopy of bilayer film a),b) and c) showing a transverse sectional view of bilayer film under 100x magnification power

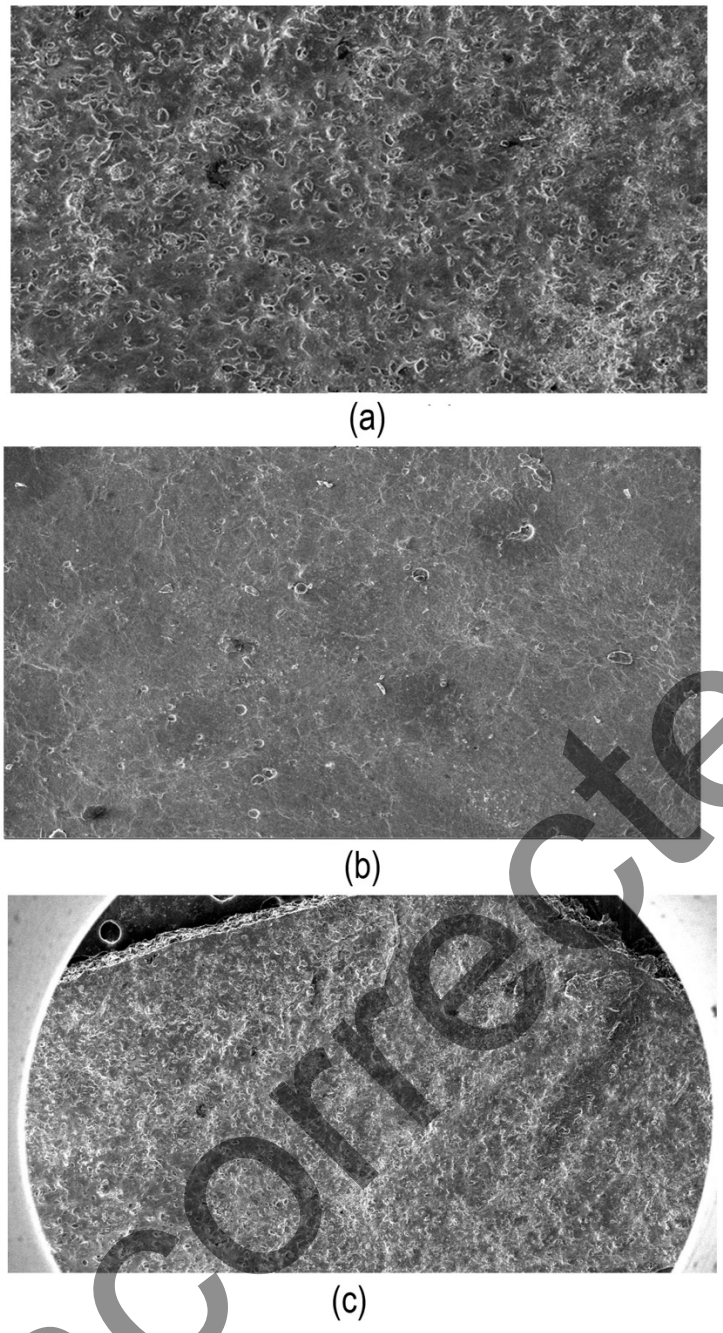

Figure 7 -Scanning electron microscopic images showing the surface morphology of bilayer Film a) Primary layer containing doxycycline hyclate b) Secondary layer containing eugenol c) Bilayer film B

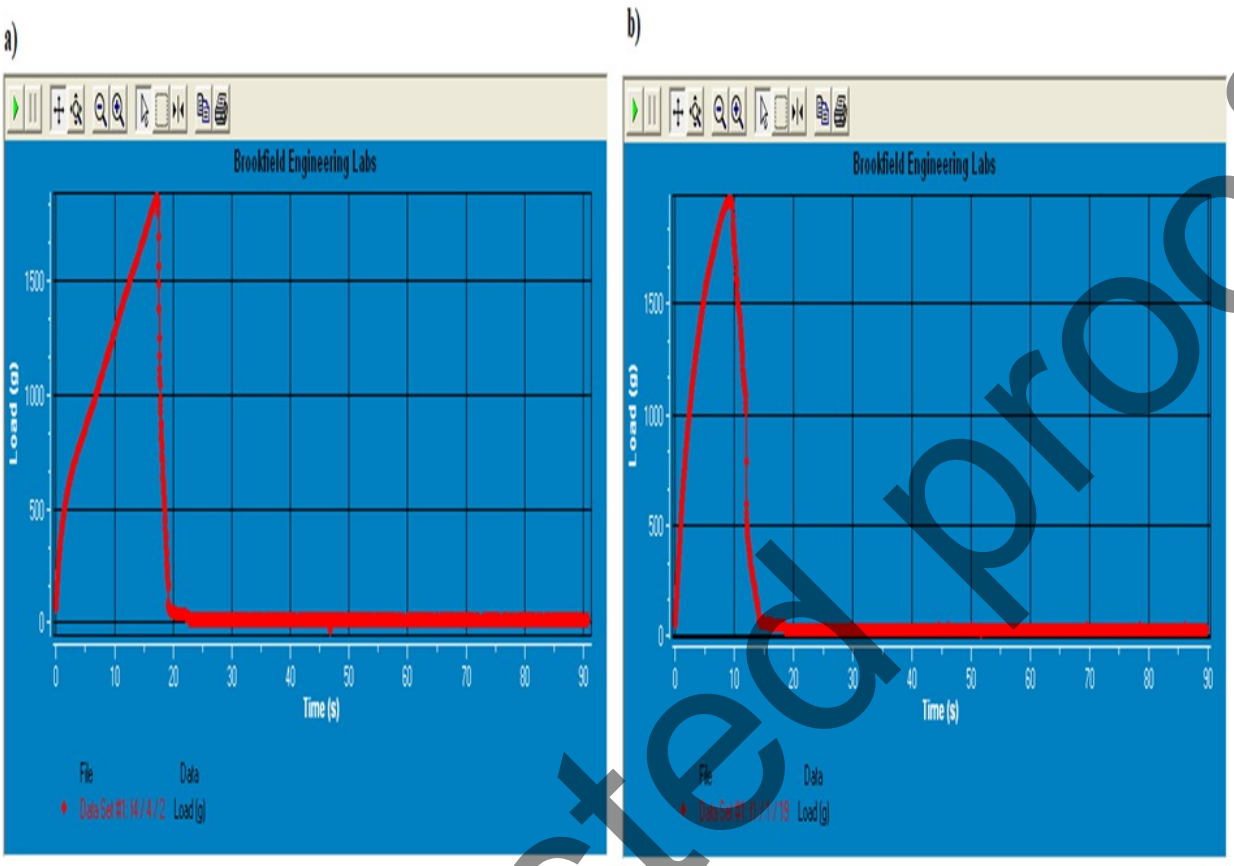

Figure 8. Graph of CT3 texture analyzer of tensile strength were a) Bilayer periodontal film formulation b) Single primary layer film

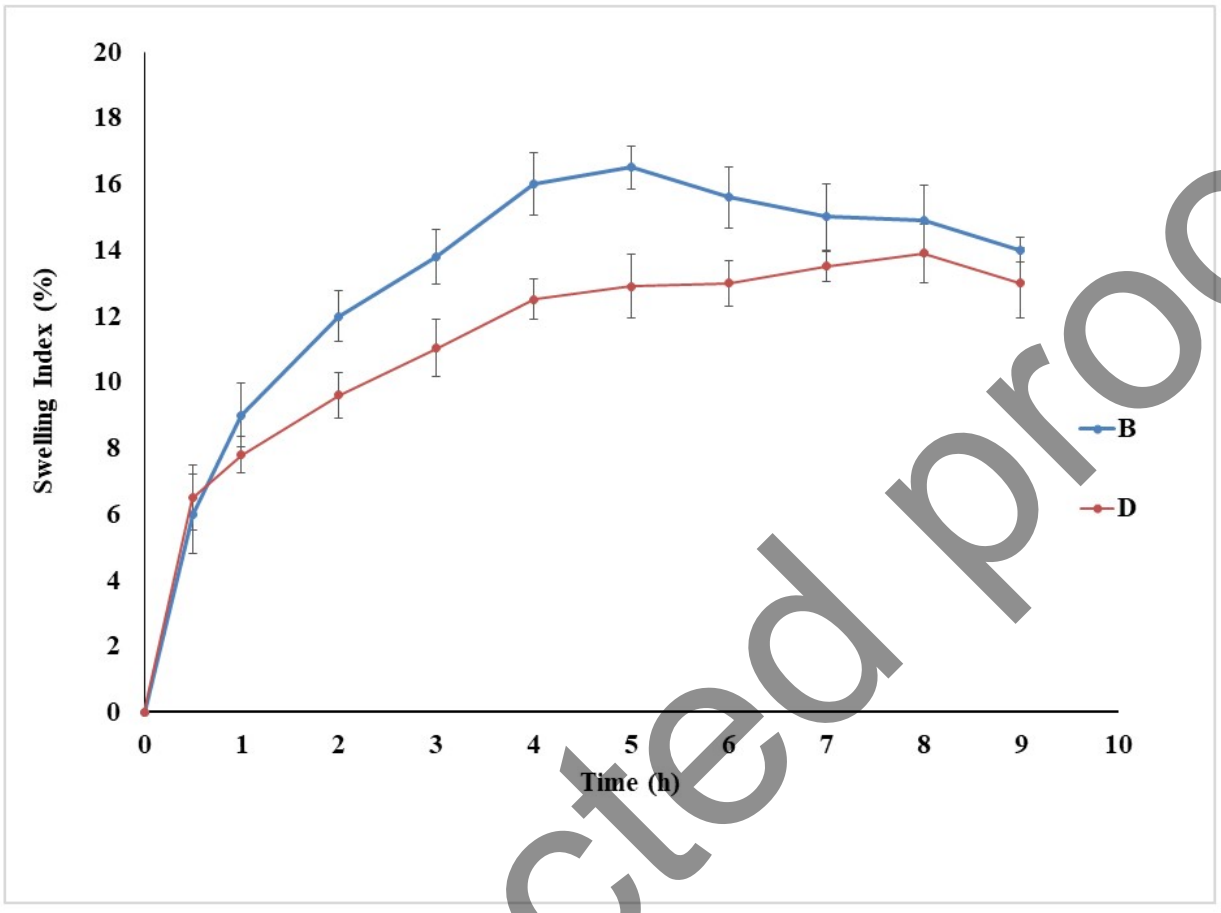

Figure 9 -The swelling ratio of B and D film

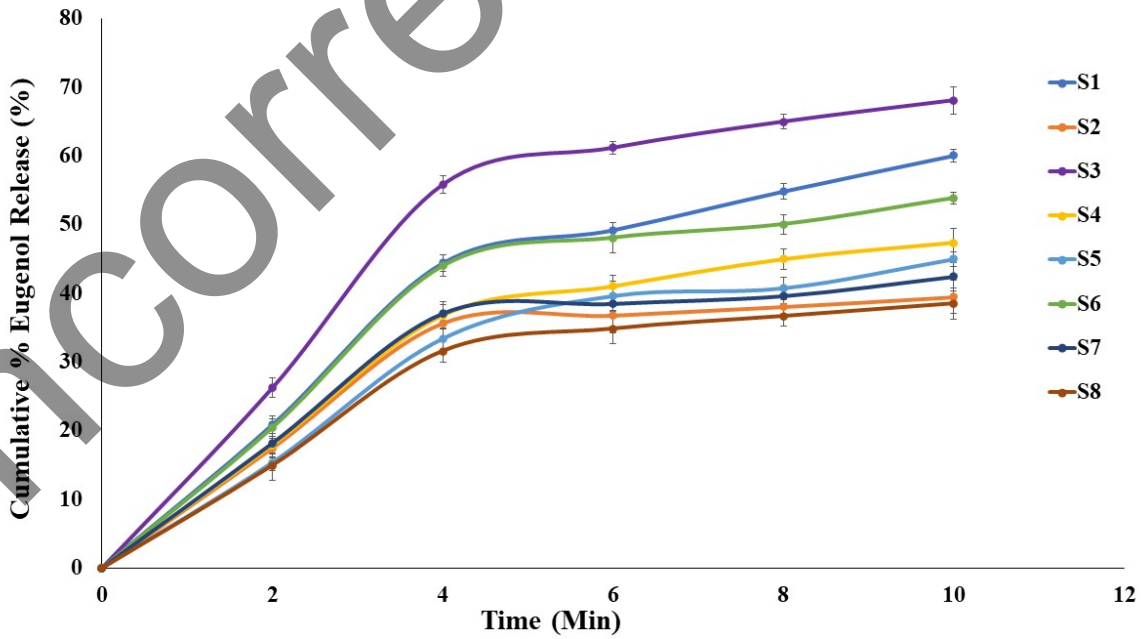

Figure 10. Cumulative _ drug release profile of Immediate release film

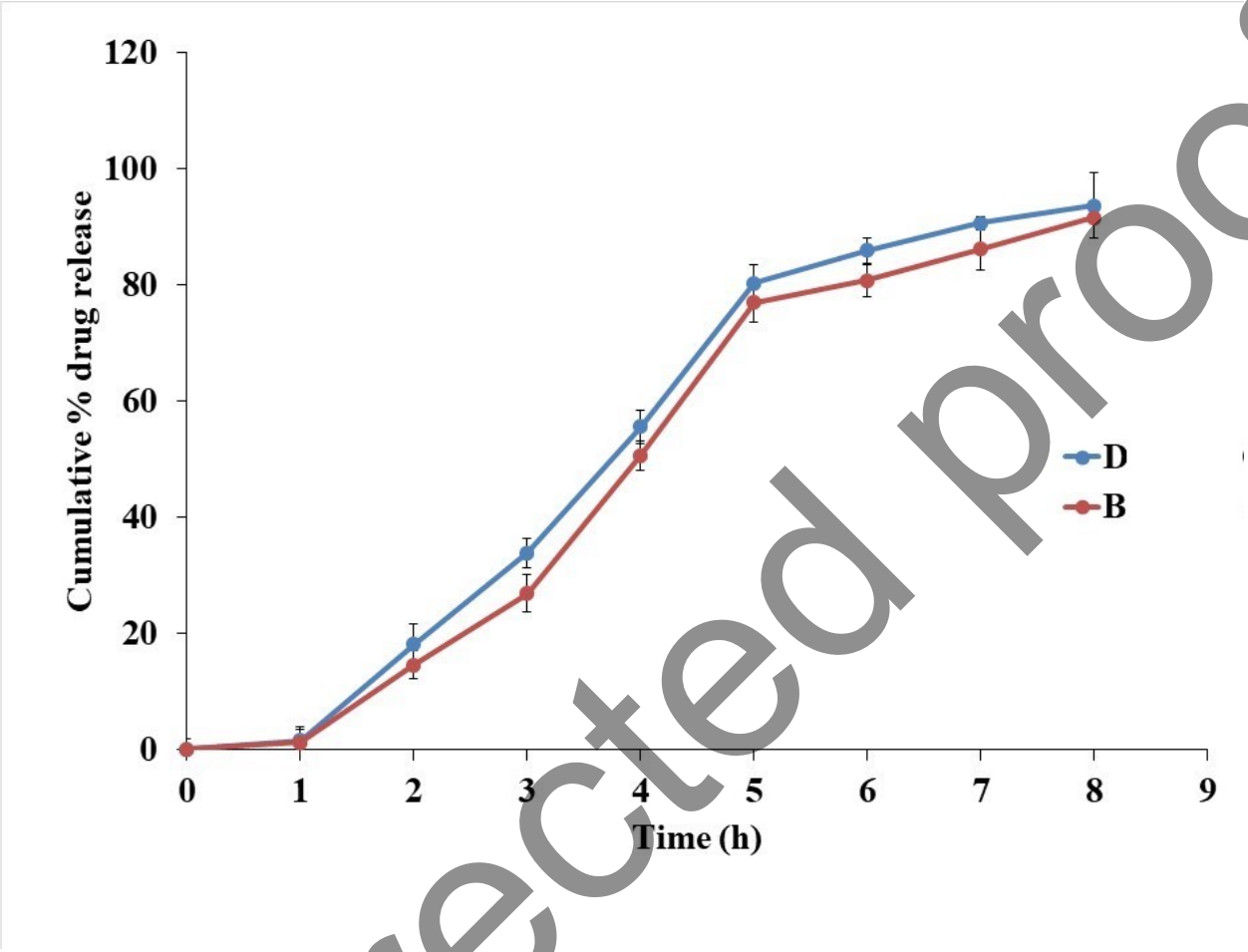

Figure 11 -Cumulative _ drug release profile of B film and D film

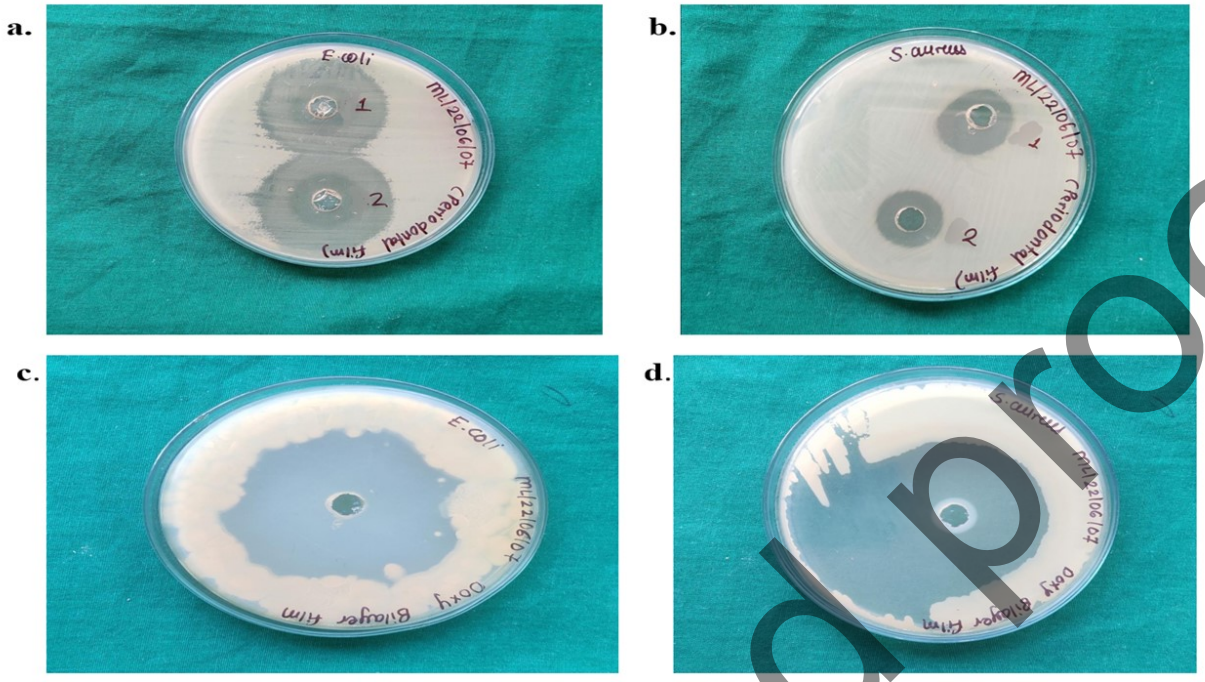

Figure 12- Antimicrobial activity of films: a. Effect of primary layer film containing doxycycline hyclate (1) and clove oil (2) on E-coil, b. Effect of primary layer film containing doxycycline hyclate and clove oil on the zone of S-aureus c. Effect