THYMOL AND EUGENOL LOADED CHITOSAN DENTAL FILM FOR TREATMENT OF PERIODONTITIS

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ABSTRACT

The main objective of this study was to formulate an intrapocket chitosan (CS) dental film loaded with thymol (TH) and eugenol (EU) for the treatment of periodontal diseases. The antibacterial and antifungal efficacy of TH (5μg/mL) measured in terms of zone of inhibition against *S. mutans* was 10±2mm and against *C. albicans* was 10±4mm. The zone of inhibition measured for EU (5μg/mL) was 2.1±3mm against *S.mutans* and 3±2mm for *C. albicans.* The minimum inhibitory concentration of TH and EU was found to be in the range of 100-150μg/mL against both *S. mutans* and *C. albicans*. For the local delivery of TH and EU, the CS films were prepared by solvent casting method and evaluated for various physicochemical parameters and *in vitro* antibacterial activity. The film showed good physicochemical properties. The study suggests that CS dental film containing TH and EU is a potential drug delivery device for the topical treatment of periodontal disease.

Keywords: Dental film; Periodontal disease; TH; EU; *S. mutans; C. albicans.*

INTRODUCTION

The occurrence of oral pathogens is associated with periodontal disease and dental caries. Dental caries or tooth decay is the among the most prevalent human diseases, as common as common cold[1]. Caries is characterized by localized and irreversible destruction of the tooth disease that progresses slowly[2]. Periodontal disease refers to any disorder of the tissues surrounding and supporting the teeth, i. e. the periodontium. India suffers a lot of disparities in terms of oral health care. About 95% of the Indian population suffers from periodontal disease[3]. Considering the recent scenario there is the urgent need for action, to be taken to promote sound oral health, prevent dental caries and periodontal diseases, and give importance to activities that promote oral health[4].

Studies have demonstrated that the initiation and progression of the oral disease is primarily due to the increased proliferation of opportunistic microorganisms[5]. A large variety of microorganisms are associated with the oral disease. The clinical efficacy of systemic antibiotic therapy is well established in the management of dental problems but the high oral dose required to achieve the effective concentration in the gingival cervical fluid limits its use. Also, repeated long term use of systemic antibiotics is associated with potential adverse effects, namely gastrointestinal disorders, development of bacterial resistance, superimposed infections and patient non-compliance[6-7-8]. Topical agents like mouthwashes, gels, pastes and dentifrices can help in controlling the microbial plaque and mucosal infections but cannot penetrate into the deep periodontal pockets. Also, the application of topical agents depends on first order kinetics which requires high initial concentration and multiple applications to maintain their sufficient level in GCF for sustained effectiveness[9]. To overcome all these limitations, controlled local delivery within the periodontal pocket can effectively target the microbes in the periodontal pockets as well as maintain therapeutic concentration within the crevicular fluid for a longer period of time. To achieve intrapocket- localized drug delivery, a mucoadhesive, biodegradable, biocompatible, and non-toxic polymer providing controlled drug delivery for prolonged time is desirable. Chitosan (S) is a natural polymer consisting of (1,4)-linked 2-amino-deoxy-b-D-glucan, deacetylated derivative of chitin, obtained from crustacean shell and exhibits all the above features. In addition to this, it has antimicrobial and antiplaque activity against oral pathogens[10-11-12]. Also, CS has filmogenic properties and can form transparent films with good mechanical properties[13]. The delivery system has the advantage of dissolving within the pocket itself without the need for removal. The intrapocket localized drug delivery minimizes

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the systemic uptake of antibiotics, thereby reducing their side effects. During the last few decades, there has been an increasing interest in the study of medicinal plants and their traditional use in dentistry[14]. Plants not only provide safe and cost effective remedies, they are also available and accessible at affordable prices with fewer side effects and no known resistance to microorganisms. Early studies have clearly established that a number of natural plant substances had potential to be utilized in the dental industry, given their activity against cariogenic bacteria and those bacteria associated with periodontal diseases[15]. Thymol (TH) and Eugenol (EU) are the natural compounds derived from the plant essential oils having a wide spectrum of antibacterial, anti-inflammatory, and antifungal activity[16]. PC Braga *et al* reported that the combination of TH and EU showed synergistic action in potentiating the inhibition of C.*albicans* colonization and infectiousness[17]. Pei RS *et al E.Coli* reported that the antibacterial activity of TH and EU in combination against revealed synergistic effect also their MIC decreased from 400mg/L to 100mg/L[18]. Also, our studies revealed that therapeutic concentration of TH and EU is attained earlier as both have lower minimum inhibitory concentration (MIC) which is required for the treatment and management of periodontitis. Furthermore Rui-song pei *et al* reported that combination of TH and EU reduces the negative impact of unpleasant smell of essential oils[19]. So, in view of this, a study was undertaken, first to determine *in vitro* antimicrobial activity and minimum inhibitory concentration of EU and TH against *S. mutans* and *C. albicans* and secondly to formulate CS dental film containing TH and EU for the local delivery in the treatment of periodontitis.

MATERIALS AND METHODS

EU (99.9%) was purchased from Otto Chemie Pvt. Ltd, India. TH (99.9%) and CS were purchased from Loba Chemie Pvt. Ltd, India. All the other chemicals used were of analytical grade.

Inoculation of plates

Muller Hinton Agar plates were prepared by autoclaving the agar plate at 121°C for 15 min. The medium was stored in sterile petri plates under aseptic conditions and then allowed to solidify at room temperature. Inoculation of plates was done by the modified method of Acar and Goldstein using flood –inoculation technique [20]. A small single well isolated colony was emulsified in 2mL sterile saline in test tubes and turbidity of bacterial suspension was adjusted equivalent to 0.5McFarland and 2mL of this was transferred onto the Muller Hinton agar plate and distributed gently over the surface of medium with sterile glass spreaders to obtain uniform inoculums. Polysorbate 80 (0.05%) was added to the agar base. The plates were dried for 5 min.

Determination of antimicrobial activity

Agar disc diffusion method was used for screening of antimicrobial activity of TH and EU against *S. mutans* and *C. albicans*, respectively [21]. The sterile filter paper discs of diameter 6mm were impregnated with the test material (5uL of TH and EU) individually as well as in combination and placed aseptically on the inoculated plates. The plates were left at ambient temperature for 30 minutes to allow pre diffusion prior to incubation at 37° C for 24 h. The broad spectrum antibiotic chlorhexidine was used as positive control for obtaining comparative results. Plates were observed after 24-48h incubation around the discs. Antibacterial activity was evaluated by measuring the diameter of zone of inhibition of bacterial growth[22].

Determination of minimum inhibitory concentration

The minimum inhibitory concentration was determined by broth dilution method[23]. An inoculum (1mL) of each strains of *S. mutans* and *C. albicans* (containing0.05% Polysorbate 80) was inoculated separately into each dilution tubes containing TH and EU, respectively, in incrementally (usually geometrically) increasing proportion 50ug/mL, 100ug/mL, 150ug/mL, 200ug/mL and 250ug/mL and incubated for 48 hours at 30-35°C. After incubation, the MIC was identified as the lowest concentration of the chemical agent, which resulted in the confirmed inhibition of the growth of tested micro-organisms.

Fourier Transform Infrared Spectroscopic (FTIR) Studies

To investigate any possible interaction between drug and excipients, Fourier transform infrared (FTIR) spectra of samples were taken on Shimadzu (model 8400S, Tokyo, Japan). FTIR spectra of pure drugs and polymer and the physical mixture of drugs and polymer and films were scanned in the range between 4000 and 400 cm^{-1}.

Fabrication of dental films

Solvent casting method was used to prepare TH and EU loaded CS dental films[19]. Accurately weighed quantity of CS was dissolved in a mixture of 40mL of 0.4% acetic acid aqueous solution and glycerol 0.5-1.0% w/V (Loba Cheim Pvt. Ltd India).Glycerol was used as plasticizer. The solution was stirred for 24 h for complete solubilization of CS in acetic acid. Weighed amounts of TH and EU

in DMSO were incorporated in polymer solution with stirring. The solution was stirred for a further 2 h, and then allowed to stand overnight before casting into thin films in the petri dish and dried at room temperature, as shown in Table I.

Physical Characterization of Dental Films Thickness of the films

The thicknesses of the films having surface area 1 cm^2 were measured using a screw gauge and the average thickness was calculated[24].

Uniformity of weight of the films

Uniformity of the weight of the film was carried out by weighing 10 films which were cut from different places of each formulation separately; on the electronic balance and the average weight was calculated [25].

Surface pH

The surface pH of periodontal films were determined by allowing the films to swell for 2 h on the surface of agar plates prepared by dissolving 2% (w/V) agar in warmed distilled water under stirring and then pouring the solution into the petri dish for gelling or to solidify at room temperature. The surface pH was then determined by using pH paper (S. D. Fine Chemicals, India) placed on the surface of the wetted film[26].

Folding endurance

Folding endurance of the films was determined by repeatedly folding the film at the same place till it broke or folded up to 300 times without breaking. The film that could be folded a number of times at the same place without breaking is considered as folding endurance[27].

Swelling Index

The film samples 1 × 1 cm were weighed (*Wo*) and allowed to swell in petri dish (9 cm diameter) containing 5 ml phosphate buffer (pH 6.8). At defined time intervals up to 24 h, the swollen films were reweighed (*Wt*) after removing the excess moisture carefully from the surface of films using filter paper[28]. The swelling index of each system was calculated using equation (1):

$$\text{Swelling Index}(\%) = \frac{W_t - W_o}{W_o} \times 100 \quad (1)$$

Where W_t is the weight of film at time t, and W_o is the weight of the film at time 0.

Uniformity of Drug Content

The uniformity of the drug content in the films was determined by dissolving individually weighed films (n = 3) in a volumetric flask containing 5 mL of glacial acetic acid. After gentle stirring for an hour, the volume was made up to 100 mL with phosphate buffer pH 6.8. The resultant solution was filtered and the filtrate was analyzed for TH and EU contents using simultaneous equation method by doing adequate dilutions. The following simultaneous equation method was used as shown in equations (2) and (3):

$$CT = \frac{\text{A1ay2-A2ay1}}{ax1ay2\text{-}ax2ay1} \quad (2)$$

$$CE = \frac{A1ax2\text{-}A2ax1}{ax1ax2\text{-}ax2ay1} \quad (3)$$

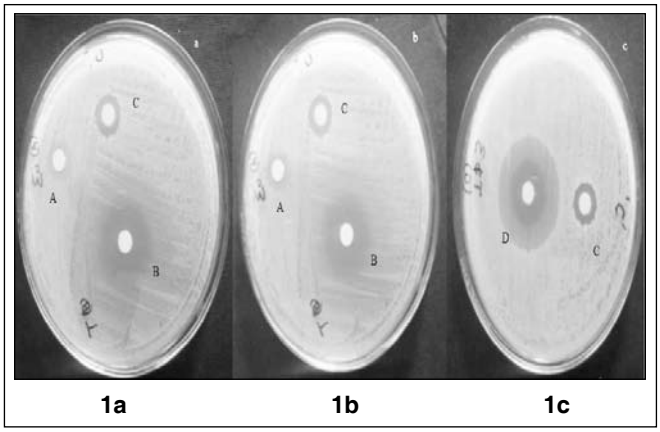
The λ_{max} for TH and EU was obtained at 274 and 283 nm, respectively, using UV spectrophotometer (Shimadzu-1700, Japan). In the above equations, A1 and A2 are the absorbance of samples at 274 and 283 nm respectively, ax1 and ax2 are absorptivities of TH at λ1 and λ2, respectively, and ay1 and ay2 are absorptivities of EU at λ1 and λ2, respectively. CT and CE are the concentrations of TH and EU respectively[19].

Tensile strength

A Texture Analyzer CT3 (Brookfield, USA) equipped with a 4.5 kg load cell and Texture Pro CT software was used to determine the tensile strength of the prepared films. Rectangular samples (3 × 3 cm) were held between two clamps of probe positioned at a distance of 2 cm. The lower clamp was held stationary and the dental film were stretched by the upper clamp moving at a rate of 0.5 mm/sec until the strip tore. The tensile work done during this process and the tensile deformation/elongation of the film at the moment of tearing were also measured. The measurement was repeated three times for each film sample[29].

In vitro release study

In vitro release of drug from the films were studied by simulating stagnant intrapocket condition. The films were cut into circular shape with diameter of 5 mm and placed in 5 mL vials containing 2 mL phosphate buffer pH 6.8, previously warmed at 37°C. At predetermined time intervals, the whole release medium was withdrawn at different time intervals over a period of 8 h and replaced with an equal volume of previously warmed fresh buffer solution. The samples withdrawn were analyzed for TH



1a 1b 1c

Fig. 1a Antimicrobial activity of EU (A), TH (B), Chlorhexidine (C) against *S. mutans*

Fig. 1b Antimicrobial activity of EU (A), TH (B), Chlorhexidine (C) against *C. albicans*

Fig. 1c Antimicrobial activity of Mix TH&EU(1:1), (D) Chlorhexidine (C) against *S. mutans*

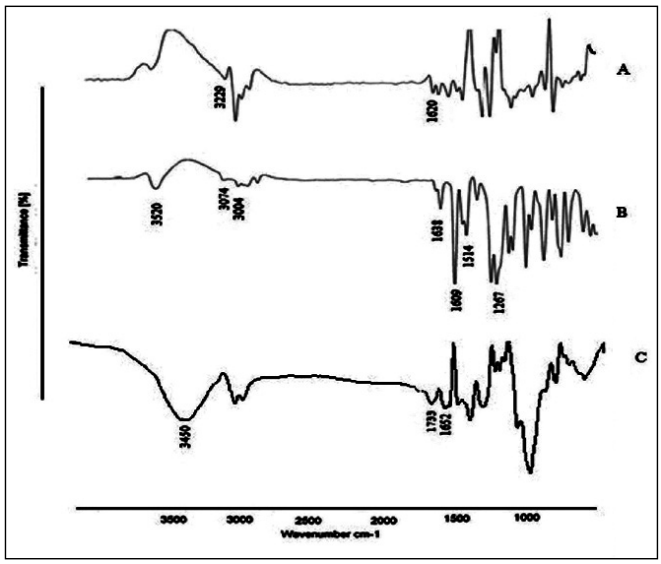


Fig. 2: FTIR spectra of (A) Pure TH, (B) Pure EU (C) TH- EU-CS physical mixture.

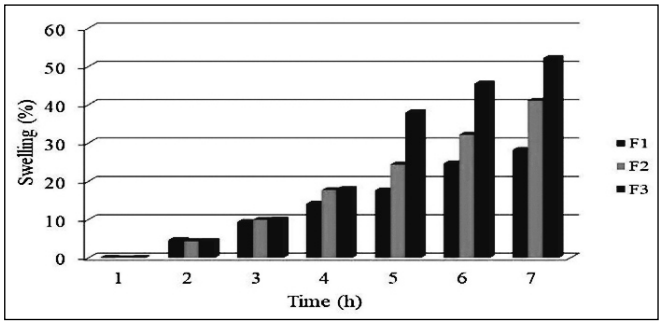


Fig. 3: Histograms representing percent swelling index of selected batches

Table I: Composition of Different Batches of Film

Batch	CS (g)	Glycerol (%)	TH (mg)	Drugs	EU (mg)
F1	0.8	0.5%	10		10
F2	0.8	0.75%	10		10
F3	0.8	1%	10		10

Table II: Antimicrobial activity of TH and EU against *S. mutans* and *C. albicans*

Samples	Concentration in ug/mL	Name of organism	Zone of inhibition in mm
EU	5	*S. mutans*	2.1±0.9
TH	5	*S. mutans*	10±1.2
Chlorhexidine	5	*S. mutans*	2.1±0.9
EU	5	*C. albicans*	3±0.26
TH	5	*C. albicans*	10±0.6.
Chlorhexidine	5	*C. albicans*	3.2±0.7

and EU contents by simultaneous equation method as described earlier. Release media of placebo film was used as blank to circumvent any interference from polymer used.

In vitro antibacterial activity of dental films

The optimized dental films loaded with TH and EU were tested for their antibacterial activity against *S. mutans* and *C. albicans* using disc diffusion method. Muller Hinton agar (MHA) plates were used for screening the antimicrobial efficacy. The plates were prepared by pouring 15 mL of molten media into sterile petri plates. Then 1.0 mL of 18-h cultured bacteria adjusted to 0.5 McFarland standards in sterile saline to achieve concentration of 107 $CFUmL^{-1}$ was spread on the surface of MHA agar plates with the help of sterile swab sticks. The disc-shaped polymer film of 5-mm diameter was then placed on the surface of the medium and incubated at 37°C for 24h. At the end of incubation, the zones of inhibitions were examined around the polymer disc film.

RESULTS AND DISCUSSION

Antimicrobial activity assay of TH and EU

The antimicrobial activity of TH and EU against *S. mutans* and *C. albicans* was calculated in terms of zone

Table III: Physicochemical Characteristics of Different Batches

Batch	Weight	Thickness	Total drug content (%)	Folding endurance	Tensile strength	Surface pH
F1	0.17±0.2	0.31±0.2	92.5±0.36	200	524±0.2	6.5±0.2
F2	0.18±0.6	0.30±0.3	96.35±0.8	289	494±0.9	6.7±0.6
F3	0.16±0.4	0.32±0.6	94.58±0.5	300	260±0.5	6.6±0.5

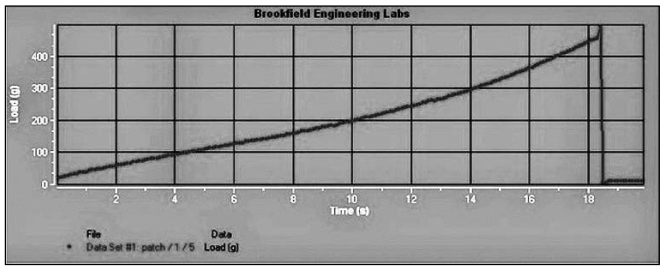


Fig. 4: Tensile strength and elongation of film of batch F2

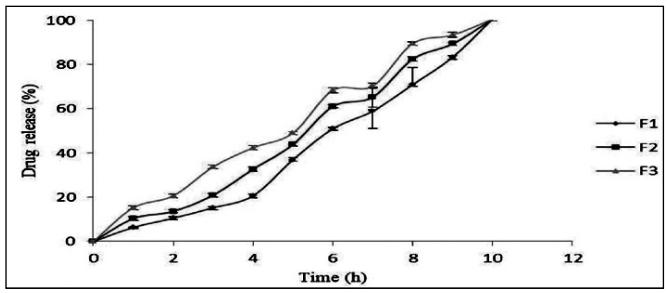
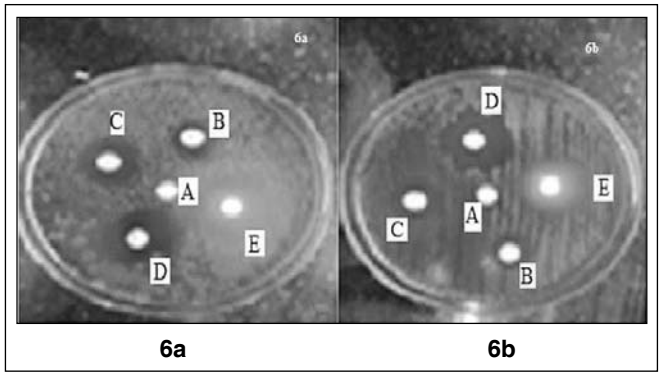


Fig.5: Cumulative percentage release of TH and EU from films in phosphate buffer pH 6.8 (vertical bars represent mean±SD, n=3)



6a **6b**

Fig. 6a: Zone of inhibition of A-pure CS, B-CS film, C- pure EU, D- pure TH, E- TH-EU film against *Streptococcus mutans.*

Fig. 6b: Zone of inhibition of A-pure CS, B-CS film, C- pure EU, D- pure TH, E- TH-EU film against *Candida albicans.*

of inhibition (mm) as shown in Fig. 1a, 1b, and 1c and Table II.

Minimum inhibitory concentration of TH and EU

Minimum inhibitory concentration of TH and EU against *S. mutans* and *C. albicans* was 100ug/mL and 150ug/mL, respectively. The combination of TH and EU provided better antibacterial activity in comparison to single drug. These findings were in line with the studies shown by Braga and Pei[17-18]. Hence, combinations of both drugs were used to formulate the dental film using natural polymer CS.

FTIR

The samples were scanned in the region of 4000–400 cm^{-1} for FTIR studies (Fig.3). Pure TH showed characteristic peaks at 3229 cm^{-1} corresponding to phenolic-OH stretching involving hydrogen bonding. Aromatic character of TH was exhibited by C = C stretching of benzene ring at 1620 cm^{-1}, respectively (Fig. 2A). EU (4-allyl-2- methoxyphenol) demonstrated signature characteristics peaks at wave numbers 3520, 3074, 3004 and 1638 cm^{-1}, corresponding to O-H stretching, Ar-H stretching, and vinyl (C ~ C) stretching. In addition, sharp peaks at 1,609 and 1, 514 cm^{-1} were also found in EU, which can be due to C ~ C stretching of the aromatic moiety, and at 1,267 cm^{-1}, which corresponds to C-O stretching (Fig.2B) FTIR spectra of CS available from the literature was used for the study. It shows characteristic band at 3478 cm^{-1} which is due to–NH2 and–OH groups' stretching vibration, and the peak at 1571 cm^{-1} indicates the presence of amide group. Further, carbonyl stretching of CS can be seen at 1656 cm^{-1}. Infrared spectra of physical mixture of TH, EU and CS showed characteristic peaks of all the excipients and no new bands or shifts in characteristic peaks appeared in any sample, indicating the absence of any possible interaction between the drug and polymer (Fig. 2C). Therefore, it can be speculated that the drugs and polymers are compatible and can be formulated into films.

Thickness of the film and Weight variation

Thickness of each film was measured at different points, and the average thickness was calculated. The average thickness of all prepared periodontal films F-1 to F-3 ranged from 0.31±0.05mm to 0.32±0.08mm Table.3 indicating that there was not much difference in the thickness of the formulated films. Films of all batches were found to have uniform weight with the values varying between 0.16±0.05 g and 0.18±0.08g (n=3). Table III.

The obtained values were within the limits indicating that films of uniform weight and thickness could be obtained using solvent casting method.

Surface pH

To overcome irritation to the oral mucosal membrane due to too much acidic or basic pH, the surface pH of the film plays an important role and Formulations with pH close to neutrality are preferred to avoid periodontal pocket irritation. The surface pH for films F-1 to F-3 ranged from 6 to 7, indicating no periodontal pocket irritation as shown in Table III.

Folding endurance

Folding endurance of the film increased with the increase in the plasticizer concentration, as shown in Table III. Flexibility was imparted to the film due to plasticizer which aids in easy insertion of film into dental pocket. Plasticizers act by reducing the intermolecular forces, softening the rigidity of the film structure by increasing the mobility of the biopolymeric chains, thereby improving the mechanical properties[30]. Thus plasticizers permit flexibility of films by allowing more chain mobility resulting into increased folding endurance. However, in batch F-1, the folding endurance was reduced as the concentration of glycerol was low which hindered the free movement of molecules[24].

Swelling index of film

Release of drug from the film of natural polymer is dependent on swelling of polymers. An optimum swelling is required to achieve desired release of drug from the polymer matrix as well as mucoadhesion and retention of the film in the periodontal pocket. Excessive swelling causes early release of entrapped drug, thereby decreasing mucoadhesion. The swelled films may also ooze out from the pockets causing discomfort to patients. Enhanced swelling occurs due to increase in the hydrophilic character of film owing to the presence of increased amount of plasticizers. Plasticizers being hydrophilic in nature, on contact with water they get dissolved creating porous films which aids in drug dissolution. On the contrary, less amount of plasticizer acts as barrier for polymer swelling reducing the degree of swelling thereby reducing drug dissolution. F-1 showed minimum swelling due to the presence of less amount (0.5%) of plasticizer (Fig. 3). The results from the physicochemical characteristics of films revealed that batch F2 showed drug content of 96.35%, its swelling index was 28.25% at the end of 6h.

Tensile strength

The peak load for the batch was 494g and deformation at the peak load was 9.26mm (Fig. 4). As the concentration of glycerol increases, the mechanical strength of the film decreases and elongation of the film increases. Flexible films are required to aid in easy insertion of film into the periodontal pocket. The flexibility of the film should be optimum. If the film becomes more flexible it loses its mechanical strength and can break. If the films are less flexible, the insertion into the periodontal pocket becomes difficult.

In vitro release study

The release of drug from the films was evaluated in phosphate buffer pH 6.8 for about 8 h by static method as described earlier. The drug release from the periodontal films was in a biphasic manner with an initial burst release followed by sustained release up to 8 h (Fig. 5). The free drug particles present on the surface of the film were readily available to the dissolution media contributing to the immediate burst release of the drug. The immediate burst release of drug from the film is effective in reducing the bacterial load in the pockets and to achieve MIC. The drug release depends on the composition of films. More than 80% of drug release within 8h was observed for all the batches. Also, the release studies have showed that the concentration of plasticizer has a significant effect on the cumulative percent release of both the drugs. Further, release studies showed that plasticizer concentration has a significant effect on cumulative percent release of both the drugs. It was observed that with the increase in plasticizer concentration, the drug release was faster. The change in the release rate was possibly due to the alteration in membrane permeability caused by the modification of film hydrophilicity by the plasticizer[31]. On the basis of release profile, physicochemical properties such as swelling index and burst release, F-2 showed minimum burst release and minimum swelling in the buffer. Burst release is desirable but excess of burst release above the MIC of the drug is undesirable leading to the wastage of drug. Moreover, minimum swelling of films is required so that they maintain their shape and integrity within the periodontal pocket, which is desirable for the treatment of periodontitis.

In vitro antibacterial activity of films

The antibacterial activity of the films was estimated by measuring zone of inhibition against *S. mutans* and *C. albicans*. Zone of inhibition was calculated for optimized polymeric film (batch F2) containing TH+EU film, placebo film, TH film and EU film (Fig.6). TH + EU

film showed higher mean inhibition zone of 38.26±0.40 mm and 35.76±1.10 mm for *S. mutans* and *C. albicans* respectively.TH film exhibited zone of inhibition against *S. mutans* with a value of 26.29±0.5mm and against *C. albicans* with a value of 22.25±0.7mm.The EU film exhibited zone of inhibition against *S. mutans* with a value of 18.36±0.2 whereas lesser activity for *C. albicans* with a value of 14.23±0.6mm. Also CS films exhibited mild activity against *S. mutans* and *C. albicans* with zones of inhibition of 9.23±0.1mm and 7.26±0.36 mm, respectively. Thus, it can be concluded that the combination of the drugs TH and EU provides better activity in comparison to single drug film. Further, the films maintained their antibacterial effect, and loading of TH and EU into polymer matrix has not affected the antibacterial activity of individual drugs. In addition to drugs, slight antimicrobial activity can also be attributed to presence of CS.

CONCLUSION

The antibacterial activity of TH and EU when used in combination proved to be beneficial than individual one. The amount of plasticizer affected the physical characteristics and the drug release of the film. Thus, thymol and eugenol loaded chitosan dental film, due to its mucoadhesivity and antimicrobial effectiveness, could be a potential candidate for the treatment of periodontitis.

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