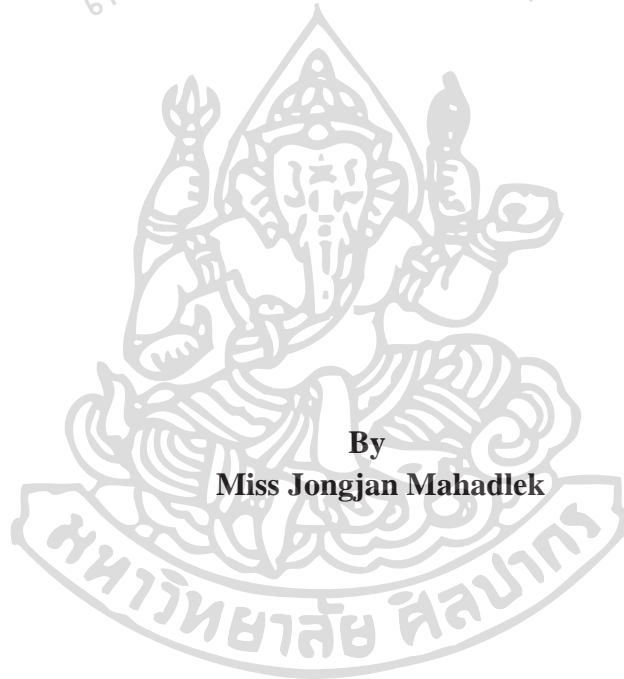




**PREPARATION OF *IN SITU* FORMING GEL SYSTEMS FOR DELIVERY
OF ANTIMICROBIAL AGENTS FOR PERIODONTITIS TREATMENT**

สำนักวิทยาสัมถกิจกลาง



By
Miss Jongjan Mahadlek

**A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree
Doctor of Philosophy Program in Pharmaceutical Technology
Graduate School, Silpakorn University
Academic Year 2012
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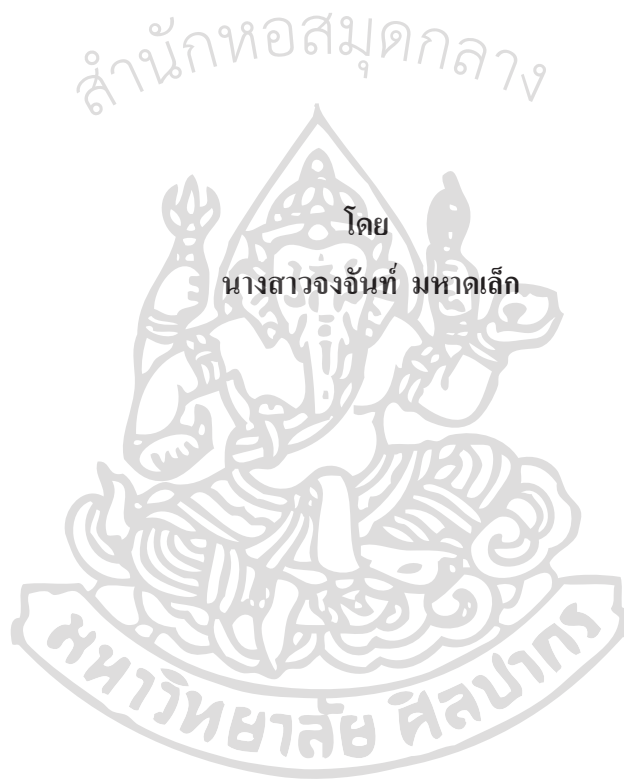
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การเตรียมระบบเจลก่อดตัวเองสำหรับนำส่งยาที่ยับยั้งเชื้อโรคสำหรับการรักษาโรคปริทันต์อักเสบ



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The Graduate School, Silpakorn University has approved and accredited the Thesis title of “Preparation of *in situ* forming gel systems for delivery of antimicrobial agents for periodontitis treatment” submitted by MISS Jongjan Mahadlek as a partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Technology

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Solvent exchanged *in situ* forming gel system is drug delivery system that is in sol form before administration, when it contacts with the body fluid and then the water miscible organic solvent dissipates and water penetrates in the system, leading the polymer precipitation as *in situ* gel at the site of injection. The objective of this research was to study the parameters affecting the gel properties, drug release and antimicrobial activities of *in situ* forming gel systems to deliver antimicrobial agents in periodontitis treatment. The effect of variable parameters *i.e.* types and amount of polymer, drugs and hydrophilic/hydrophobic plasticizers on the gel properties were performed. The gel appearance, pH, viscosity, rheology, syringeability, gel formation, rate of water diffusion into the gels, degradation, drug release behavior and antimicrobial activities against *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, *Streptococcus mutans* and *Porphyromonas gingivalis* were determined. An *in situ* forming gel system was prepared using *N*-methyl-2-pyrrolidone (NMP) as solvent. Each polymers, Ethocel, bleached shellac and Eudragit RS in different amounts were used to formulate the *in situ* forming gel systems. Increasing the amount of each polymer increased the viscosity and the work of syringeability. All *in situ* gel systems showed the Newtonian behavior. The gel formation of Ethocel and Eudragit RS systems after injected into PBS pH 6.8 showed the rigid gel, whereas the bleached shellac was elastic gel. *In situ* forming gel systems containing 5%w/w antimicrobial agents, doxycycline hyclate, metronidazole or benzoyl peroxide showed the antimicrobial activity against *P. gingivalis*. Increasing the amount of each polymer decreased the releasing of doxycycline hyclate. All systems showed the drug release by Fickian diffusion mechanism. PEG1500 and peppermint oil affected the viscosity and syringeability of *in situ* gel systems. Eudragit RS systems (35%w/w) containing peppermint oil could form the elastic gel. Increasing peppermint amount significantly decreased the releasing of doxycycline hyclate ($p < 0.05$). For stability studies, the *in situ* gel system should be kept at 4°C since the gel appearance, gel properties and antimicrobial activity were unchanged as comparing to those of the initial. In the addition, a synergistic effect between doxycycline hyclate and metronidazole was obtained against *P. gingivalis*.

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LIST OF ABBREVIATIONS

% w/w	percent weight by weight
°C	degree Celsius
µg	microgram(s)
µL	microliter(s)
µm	micrometer(s)
cfu/mL	colony forming unit/milliliter
cm	centimeter(s)
Conc.	concentration
D/cm ²	Dyne/square centimeter
e.g.	(Latin); for example
Eq.	equation
<i>et al.</i>	and others
etc.	et cetera (Latin); and other things/ and so forth
G	(needle) gauge
g	gram(s)
h	hour(s)
L	liter(s)
mg	milligram(s)
min	minute(s)
mL	milliliter(s)
msc	model selection criterion
MW	molecular weight
N	newton
nm	nanometer(s)
PBS	phosphate buffer solution
pH	potentia hydrogenii (Latin); power of hydrogen
R ²	coefficient of determination
rpm	revolutions per minute
s	sec(s)
SEM	scanning electron microscope
S.D.	standard deviation

CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the research problem

Periodontitis is an inflammatory disease resulting in the destruction of the periodontium that support the teeth (gums, periodontal ligaments, alveolar bone and dental cementum). It results in the formation of periodontal pockets between the gum and the tooth, which can cause in tooth loss (Becker *et al.*, 1979; Haffajee and Socransky, 1986; Ji *et al.*, 2010). Periodontitis caused by bacterial infection (Vyas *et al.*, 2005). *Porphyromonas gingivalis* is the major pathogen among anaerobic gram-negative bacteria involved in periodontitis (Haffajee and Socransky, 1994; Nishihara and Koseki, 2004; Valenze *et al.*, 2009). The goal of periodontal treatment is to remove the bacterial plaque. Thus, the antimicrobial agents have been used as adjuncts. The bacterial infections are eliminated by antibiotics systemically administered but the multiple doses and long term use of systemic antibiotics have shown several drawbacks including low antibiotic concentration at periodontal pockets (Pitcher *et al.*, 1980), microbial resistance and high peak plasma antibiotic concentrations which may be associated with side effects (Slots and Rams, 1990; Schwach *et al.*, 2000). Several antimicrobial agents have been used for periodontal treatment including tetracycline (Seymour and Heasman, 1995), doxycycline (Schwach *et al.*, 2000), metronidazole (Noyan *et al.*, 1997), ciprofloxacin (Tezel *et al.*, 2005), clarithromycin (Pradeep and Kathariya, 2011). Hence, a more gratifying approach to administering antimicrobial drugs directly into the pocket involves the use of a controlled release device. Intra-pocket drug delivery systems are greatly attractive, which can deliver the drug to its target site, with little or no systemic uptake, so a much smaller dose is required for effective therapy and harmful side effects can be reduced or eliminated. Many intra-pocket drug delivery systems have been proposed for periodontal treatment, including fibers, strips, films and injectable gels (Jain *et al.*, 2008).

In situ gel forming systems are drug delivery systems that are in sol form before administration in the body, but once administered, undergo gelation *in situ*, to form a gel (Jigar, 2011). The gel formation depends on various factors like temperature modulation, pH change, presence of ions and ultra violet irradiation, electrical sensitivity, enzyme sensitive and solvent exchange which the drug gets released in a sustained and controlled manner (Jigar, 2011). Injectable systems are particularly attractive for the delivery of drugs into the periodontal pocket. This application can be easily and rapidly carried out, without pain, by using a syringe (Xiong *et al.*, 2011). Many studies have been developed in *in situ* gel forming drug delivery for periodontal treatment. Chitosan gels (1%w/w) with metronidazole (15%w/w) demonstrated the effectiveness in the periodontitis treatment (Aknabay *et al.*, 2007). Tetracycline-loaded bioadhesive semisolid, polymeric system based upon hydroxyethylcellulose and polyvinylpyrrolidone (Jones *et al.*, 1996) and metronidazole-loaded systems based upon Carbopol 974P, hydroxyethylcellulose and polycarbophil have been mentioned (Jones *et al.*, 1997). Tetracycline-serratiopeptidase containing pluronic gel were designed (Maheshwari *et al.*, 2006). Doxycycline hydrochloride and/or secnidazole loaded into biodegradable polymers, poly (lactide) and poly (lactide-co-glycolide) has been reported (Gad *et al.*, 2008). *In situ* gels of moxifloxacin using gellan gum and sodium alginate based on the concept of ion activated systems was developed for the periodontitis treatment (Kunche *et al.*, 2012). Secnidazole-serratiopeptidase was formulated as a pH-sensitive *in situ* gelling periodontal formulation using alginate with HPMC (Priyanka *et al.*, 2011). *In situ* gel implants of ornidazole with mucoadhesive polymers-Pluronic F-127, HPMC K-100, Carbopol 934P and PVP-K-30 were formulated for periodontal treatment (Rawat *et al.*, 2010). The Atrigel® is injectable biodegradable delivery system containing 10% doxycycline hyclate. This system is based on poly(DL-lactide) dissolved in a biocompatible solvent *N*-methyl-2-pyrrolidone (NMP) (Schwach *et al.*, 2000).

Several polymers were used as *in situ* gelling agents such as gellan gum (Kunche *et al.*, 2012), pluronic F127 (Bruschi *et al.*, 2007), chitosan (Aknabay *et al.*, 2007), carbomer (Bruschi *et al.*, 2007), hydroxyethyl cellulose (Jones *et al.*, 1996), Poly (DL-lactide) (Polson *et al.*, 1997) and PLGA (Maze *et al.*, 1996). In this study, three polymers were investigated such as Ethocel, bleached shellac and Eudragit RS. Ethylcellulose or Ethocel

is a well-known water-insoluble polymer and dissolved in an organic solvent or solvent mixture that can be used to produce water-insoluble films. It has been used as a hydrophobic coating agent in oral formulations for controlled drug release, moisture protection and taste masking (Bodmeier *et al.*, 1994; Dressman *et al.*, 1995; Narisawa *et al.*, 1994; Sadeghi, 2001). Shellac is a natural resin and tasteless and faint odor (Buchbauer *et al.*, 1993). Bleached shellac is supplied as a coarse-off white powder. Shellac is widely used as a moisture barrier coating for tablets and pellets due to its low water vapor and oxygen permeability (Pearnchop *et al.*, 2003). Eudragit RS is commonly used to form water-insoluble film coat for the enteric coating of tablets and sustained-release products. It exhibits a very low permeability and swells in aqueous media independently of pH without dissolving. It is widely used as film-coating materials in oral pharmaceutical formulations and also used in topical formulations. It is generally regarded as nontoxic and nonirritant materials (Rowe *et al.*, 2009). All polymers used in this study were water insoluble polymers thus they were dissolved in organic solvent for preparation of gels.

The mechanism for the formation of *in situ* gel forming systems is precipitation of a polymer by solvent exchange. The polymer and drug are initially dissolved in a water-miscible organic solvent with low toxicity, such as *N*-methyl-2-pyrrolidone (NMP) or dimethyl sulfoxide (DMSO). The diffusion of solvent from polymer solution into surrounding environment resulted in precipitation or solidification of polymer matrix (Nirmal *et al.*, 2010). *N*-methyl-2-pyrrolidone (NMP) is used as solvent in this study. It is thermally stable and can be used as an attractive solubilizer in the pharmaceutical field (Sanghvi *et al.*, 2008). NMP increased the skin permeation of estradiol (Koizumi *et al.*, 2004). However, drug delivery applications of solvent exchange systems have been limited due to high risks of burst release. Plasticizers are additives which were used to control the drug release in pharmaceutical applications (Feldstein *et al.*, 2007; Bodmeier *et al.*, 1997). Thus, the addition of plasticizer should be improved the limitation of burst release. Plasticizers increase the elongation and flexibility, and decrease the tensile strength, Young's modulus and the glass transition temperature (Rahman and Brazel, 2004). There are two plasticizers used in this study including hydrophilic (polyethylene glycol 1500) and hydrophobic (peppermint oil) substances. Polyethylene glycol 1500 is

hydrophilic substance that is essentially nonirritant to the skin. It has been used verified in biodegradable polymeric matrices used in controlled-release systems (Mohl *et al.*, 2004). It is useful as plasticizers in microencapsulated products to avoid rupture of the coating film when the microcapsules are compressed into tablets. Peppermint oil is an essential oil derived from leaves of *Mentha piperita*. It has been used to extend the shelf life of food, showing inhibition against bacteria, fungi and yeast (Jeyakumar *et al.*, 2011). It is used for flavoring pharmaceuticals and oral preparations, such as toothpastes, dental creams, and mouth washes. It is found to be strongly effective against *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Escherichia coli* (Sartoratto *et al.*, 2004) and has also the virucidal activity against herpes simplex virus (Schuhmacher *et al.*, 2003; Mohsenzadeh *et al.*, 2007) and fungicidal activity (Barrera-Necha *et al.*, 2009).

The problem in antimicrobial therapy is the increasing occurrence of resistance to antibiotics, which leads to the insufficiency of antimicrobial treatment. Thus, the combination therapy is used to coverage in the treatment and can reduce side effects. The interesting drugs were used in antimicrobial combination studies including doxycycline hyclate, metronidazole and ciprofloxacin. Doxycycline hyclate is bacteriostatic antibiotics and also inhibits tissue collagenase activity (Yu, 1993; Levy, 1984; Seymour and Heasman, 1995). It is against many bacterial species including *Streptococcus pyogenes*, enterococci, *Staphylococcus aureus* and various anaerobes. This drug is used for periodontal therapy because of interfere with bacterial protein synthesis and inhibit tissue collagenase activity (Choi *et al.*, 2004; Gapski *et al.*, 2004). Metronidazole is bactericidal against anaerobic bacteria. It has been proposed that metronidazole is intracellularly activated by reduction and the toxic effect of reduced intermediates bind to DNA leading to loss of helical structure, strand breakage and impairment of DNA function (Rizzo *et al.*, 2010; Cavalcanti *et al.*, 2004; Oliveira *et al.*, 2009). Metronidazole has been used in the field of periodontal therapy (Rizzo *et al.*, 2010; Haffajee *et al.*, 2003; Noyan *et al.*, 1997). Ciprofloxacin is a wide spectrum, second generation fluoroquinolones. It has been studied for periodontal treatment (Tezel *et al.*, 2005; Tözüm *et al.*, 2004). Ciprofloxacin inhibits the enzyme Topoisomerase II (Aithal *et al.*, 1995; Ahmed *et al.*, 2009). Benzoyl peroxide is an organic compound in the peroxide family. It consists of two benzoyl

peroxide groups bridged by a peroxide link. It is one of the most widely prescribed drugs in acne therapy. It is an anti-bacterial agent which releases free radical oxygen species capable of oxidizing bacterial proteins. Additionally, it also has a mild keratolytic effect (Matsui *et al.*, 1995; Waller *et al.*, 2005; Mahadlek and Phaechamud, 2011). The benzoyl peroxide in Eudragit RS systems containing peppermint oil was studied for periodontitis treatment (Mahadlek *et al.*, 2013).

The aim of this study was to develop the *in situ* gel forming systems of antimicrobial agents for periodontitis treatment. The *in situ* gel formulations containing polymers (Ethocel, bleached shellac and Eudragit RS), drugs (doxycycline hyclate, metronidazole and benzoyl peroxide), solvent (NMP) and plasticizers (PEG1500 and peppermint oil) were prepared to investigate their effects on gel properties such as appearance, pH, viscosity, rheology, syringeability, gel formation, rate of water diffusion into the gels, degradation and antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, *Streptococcus mutans* and *Porphyromonas gingivalis*. Effect of types and amounts of polymer on gel properties were investigated. Effect of drugs on gel properties, release behavior of the systems and antimicrobial activities were evaluated. Effect of hydrophilic and hydrophobic plasticizers on gel properties were investigated.

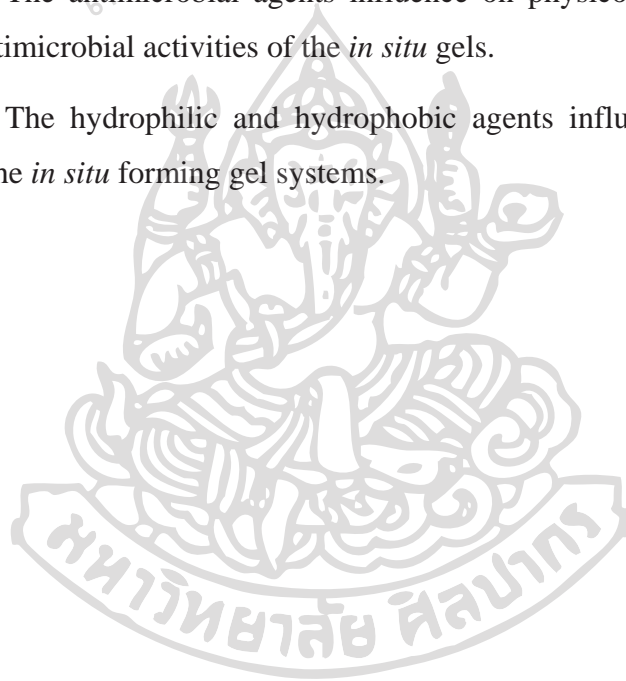
1.2 Objective of this research

- 1) To prepare *in situ* forming gels of antimicrobial drugs for periodontitis treatment.
- 2) To evaluate the influence of formulation factors such as type and amount of polymer on physicochemical properties of gel base.
- 3) To evaluate the influence of antimicrobial agents on physicochemical properties, drug release and antimicrobial activities of the *in situ* gels.
- 4) To investigate the influence of hydrophilic and hydrophobic agents on physicochemical properties of the *in situ* forming gel systems.

5) To apply the pharmaceutical technology in the development of pharmaceutical and dental products.

1.3 The research hypothesis

- 1) *In situ* systems prepared from these polymers (Ethocel, bleached shellac and Eudragit RS) can form gel in the periodontal pocket and control the drug release.
- 2) The formulation factors such as type and amount of polymer influence on physicochemical properties of gel base.
- 3) The antimicrobial agents influence on physicochemical properties, drug release and antimicrobial activities of the *in situ* gels.
- 4) The hydrophilic and hydrophobic agents influence on physicochemical properties of the *in situ* forming gel systems.



CHAPTER 2

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2.1 Periodontal diseases

2.1.1 Periodontal introduction

Periodontal disease is infections caused by accumulation of bacterial in periodontal pocket which produces localized inflammation of the periodontium (Haffajee and Socransky, 1986; Ji *et al.*, 2010). It is a group of conditions, including gingivitis and periodontitis, which affect the supporting structures of the teeth such as gums, periodontal ligaments, alveolar bone and dental cementum (Listgarten *et al.*, 1987; Vyas *et al.*, 2005). More than 700 species of aerobic and anaerobic bacteria have been identified in the oral cavity (Cobb, 2008). The bacteria are the primary cause and initiate damage directly or indirectly by triggering host-mediated responses that lead to self-injury (Vyas *et al.*, 2005). The gingivitis is the early stage of periodontal diseases, which the gingival inflamed and enlarged to deeper tissues leading to gingival swelling and bleeding. Periodontitis is the late stage of this diseases, the supporting collagen is degenerated, alveolar bone begins to resorb and the migration of the gingival epithelium along the tooth surface and then forming a 'periodontal pocket' (Jain *et al.*, 2008; Iqbal *et al.*, 2008). The depth of gap between the gingival and the tooth is normally between 1-3 mm, whereas the pocket depth during periodontitis usually exceeds 5 mm (Friedman and Steinberg, 1990). Periodontal pocket provides ideal conditions for the proliferation of microbes. Important periodontal pathogens are *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Bacteroides forsythus*, *Treponema denticola*, others gram negative anaerobic rods, some gram positive bacteria and even enteric rods/pseudomonas may also play roles in the pathogenesis of periodontitis (Slot, 2002; Jain *et al.*, 2008). The bacteria accumulate in the periodontal pocket allowed to progress the disease, a potential for a further destructive phase exists which the teeth may be lost.

2.1.2 Periodontal treatment

The main aim of periodontal treatment is to control the infection. Thus, the microbial elimination from periodontal pockets is considered a priority in the periodontitis treatment. Systemically applied antimicrobials have been recommended

for the periodontitis treatment. However, multiple systemic doses of antibiotics have shown several drawbacks including inadequate antibiotic concentration at the site of the periodontal pocket (Pitcher *et al.*, 1980), a rapid decline of the plasma antibiotic concentration to subtherapeutic levels (Gates *et al.*, 1994), development of microbial resistance and high peak-plasma antibiotic concentrations, which may be associated with side effects (Slots and Rams, 1990; Schwach *et al.*, 2000). Thus, the local drug delivery systems for the periodontal treatment have developed for improvement in these disadvantages.

2.1.2.1 *Antimicrobial agents*

The selection of the antimicrobial agents in periodontal diseases must be based on the bacterial etiology of the infection (Noyan *et al.*, 1997). Several antibiotics have been studied for their clinical and microbiological efficacy in periodontal diseases as shown in Table 1.

- *Chlorhexidine*

Chlorhexidine is antifungal and antibacterial agent in dentistry is well documented (Leung *et al.*, 2005). It has been used for the control of dental plaque and also effective in periodontitis (Noguchi *et al.*, 1984; Friedman and Golomb, 1982). Chlorhexidine is highly cationic which its mechanism of action relates to reduction in pellicle formation, alteration of bacterial adherence to teeth and alteration of bacterial cell walls causing lysis (Schwach *et al.*, 2000; Fiorellini and Paquette, 1992). Its antibacterial action is due to an increase of the cellular membrane permeability, followed by the coagulation of intracellular cytoplasmic macromolecule (Goffin, 1998).

- *Tetracyclines*

The tetracyclines are a group of broad spectrum antimicrobial agents. Tetracycline, doxycycline and minocycline are used extensively in the management of periodontal diseases. They are bacteriostatic antibiotics which interfere with bacterial protein synthesis and also inhibit tissue collagenase activity (Seymour and Heasman, 1995). They have a broad spectrum of activity inhibiting both Gram negative and Gram positive organisms, including the beta-lactamase

producing strains which occur in approximately 50% of 6-7 mm deep periodontal pockets and against which penicillins are ineffective (Schwach *et al.*, 2000).

Doxycycline is the most potent tetracycline for collagenase inhibition. It has been suggested that such activity relates to the drug's ability to bind with calcium and zinc ions. A further mechanism may be associated with the ability of tetracyclines to scavenge reactive oxygen radicals produced by neutrophils. The inhibitory effect of tetracycline on oxygen radicals may also prevent a wider spectrum of tissue destruction. Thus, tetracyclines may have general antiproteolytic properties (Seymour and Heasman, 1995).

- ***Metronidazole***

Metronidazole has often been chosen for periodontal treatment because of its selective efficacy against obligate anaerobes (Noyan *et al.*, 1997). Metronidazole acts by inhibiting DNA synthesis. It is known to convert into a reactive reduced form and affects specifically anaerobic rods and spirochetes in the subgingival microflora. Other studies reported that adjunctive metronidazole therapy was more effective in adults with deep pockets than with less advanced periodontitis (Fiorellini and Paquette, 1992).

- ***Clindamycin***

Clindamycin has been investigated for treatment of periodontal disease (Higashi *et al.*, 1991; Sauvetre *et al.*, 1993). Systemic clindamycin therapy, as an adjunct to scaling, decreased the incidence of periodontal disease (Gordon *et al.*, 1990). However, clindamycin did not permanently suppress subgingival *Porphyromonas gingivalis* (Eick, *et al.*, 1999), which may explain the recurrence of disease activity in some patients.

- ***Ofloxacin***

Ofloxacin is a newly developed synthetic pyridonecarboxylic acid (PCA) derivative. Although the earlier PCA derivatives were not active against Gram positive bacteria and anaerobes, ofloxacin can kill Gram positive bacteria and anaerobic bacteria (Kimura *et al.*, 1991).

- ***Ciprofloxacin***

Ciprofloxacin is a wide spectrum, second generation fluoroquinolones. It has been studied for periodontal treatment (Tezel *et al.*, 2005; Tözüm *et al.*, 2004). Ciprofloxacin inhibits the enzyme Topoisomerase II. A bacterial cell that has ciprofloxacin in it can no longer uncoil its DNA in order to undergo bacterial DNA synthesis and the cells will die because basic functions like storage, replication, unwinding, repair, and transcription can no longer occur. A second mechanism of action includes inhibiting the enzyme Topoisomerase IV which is used to separate strands after replication and during the process of cell division. It depends on the specific bacterial species as to which enzyme is inhibited but inhibiting either enzyme will be bactericidal. Gram positive bacteria seem to have Topoisomerase IV inhibited more often than Gram negative bacteria which experience more inhibition of DNA gyrase. Quinolones only act on bacteria as they do not bind to human Topoisomerases (Aithal *et al.*, 1995; Ahmed *et al.*, 2009).

- ***Moxifloxacin***

Moxifloxacin is a fourth generation fluoroquinolone with a broad antibacterial activity against Gram positive and Gram negative bacteria. It interferes with bacterial survival by binding to DNA gyrase topoisomerase II and IV (Kunche *et al.*, 2012; Schwach *et al.*, 2000).

2.1.2.2 Guidelines in the selection of systemic antibiotics in periodontal therapy (Serio and Hawley, 2002)

For combinations of anaerobic and facultative periodontal pathogens:

1st choice: Metronidazole and amoxicillin or Augmentin[®] (250 mg each TID for 5-7 days, or metronidazole 500 mg and Augmentin[®] 875 mg each BID for 5-7 days)

2nd choice: Metronidazole and ciprofloxacin (500 mg each BID for 5-7 days)

For anaerobic pathogens (*Porphyromonas gingivalis*, *Prevotella intermedia*, *Bacteroides forsythus*, *Fusobacterium* species, and *Campylocacter* species):

1st choice: Metronidazole and amoxicillin or Augmentin[®] (250 mg each TID for 5-7 days, or metronidazole 500 mg and Augmentin[®] 875 mg BID for 5-7 days)

2nd choice: Augmentin[®] (250-500 mg TID, or 875 mg BID for 7 days)

3rd choice: Metronidazole (500 mg BID for 7 days)

4th choice: Clindamycin (150 mg TID for 5-7 days)

5th choice: Azithromycin (500mg OD for 3-5 days)

6th choice: Doxycycline (100 mg BID for 14-21 days)

2.1.2.3 Periodontal local delivery devices

The periodontal pocket provides a reservoir, which is easily accessible for the insertion of a delivery device. The gingival crevicular fluid (GCF) is medium for the release drug from the dosage form and for the distribute drug whole of the pocket. The GCF in healthy sites has small volumes (0.04 μ L) and low flow rates (0.03 μ L) (Medlicott, 1994; Jain *et al.*, 2008). The treating periodontitis by the intra-pocket drug delivery systems is interesting due to the prospects of maintaining effective high levels of drug in the gingival crevicular fluid for a prolonged period of time to produce the desirable clinical benefits. Intra-pocket drug delivery systems are greatly desirable since the potentially lower incidence of undesirable side effects, improved efficacy and enhanced patient compliance (Jain *et al.*, 2008). Numerous reports of local drug delivering devices have been studied including fibers, strips, films, gels are listed in Table 1. Various polymers and antimicrobial agents have been used in drug delivery devices. Intra-pocket delivery devices in the periodontal pocket had various types such as fibers (Goodson *et al.*, 1979; Tonetti *et al.*, 1990), strips (Addy and Langeroudi, 1984; Addy *et al.*, 1988; Noguchi *et al.*, 1984; Maze *et al.*, 1995; Friedman and Golomb, 1982), films (Golomb *et al.*, 1984; Azoury *et al.*, 1988; El-Kamel *et al.*, 2007; Higashi *et al.*, 1990) and injectable gels (Akncbay *et al.*, 2007; Jones *et al.*, 1996; Bruschi *et al.*, 2007; Polson *et al.*, 1997; Noyan *et al.*, 1997; Maze *et al.*, 1996; Priyanka and Meenakshi, 2011; Kunche *et al.*, 2012). However, the injectable systems are the best interesting systems for the delivery antimicrobial agents into the periodontal pocket. Since the injectable delivery system can be easily

prepared and administered into the pockets. A mucoadhesive gels based on hydroxyethylcellulose, carbopol 974P and polycarbophil containing metronidazole have been used for periodontal treatment (Jone *et al.*, 1997). The semisolid systems based on water free mixtures of lipids (such as glycerol monooleate (monoglyceride) and sesame oil (triglyceride)) is characterized by sol-gel transition and become semisolid when contact with gingival fluid in the periodontal pockets (Noyan *et al.*, 1997). A mucoadhesive gel formulation based on carbopol (4%) containing clindamycin hydrochloride (1%) was evaluated *in vivo* on microbial flora of periodontal pockets deeper than 5 mm that it could reduce the microbial content in the periodontal pockets (Sauvetre *et al.*, 1993). A gel formulation based on hydroxypropylmethylcellulose (2.5%) containing histatin (0.125%) was studied *in vivo* in beagle dogs that beagles treated with active gel demonstrated significantly lower plaque index scores (Paquette *et al.*, 1997). The tetracycline formulations based on poloxamer 407 (20%) containing PEG 2000 (0.5%) and octyl cyanoacrylate (1%) was easy to deliver to the pockets with application times of less than 1 min (Kelly *et al.*, 2004). Release of tetracycline was sustained as the concentration of Aerosil in tetracycline-serratiopeptidase formulation increased (Maheshwari *et al.*, 2006). The commercial injectable biodegradable gels, Atrigel[®], based on poly(DL-lactide) dissolved in a biocompatible solvent *N*-methyl-2-pyrrolidone (NMP) was widely studied (Polson *et al.*, 1997; Jain *et al.*, 2008). The Atrigel[®] loaded with 10% doxycycline hyclate showed high levels of doxycycline (250 µg/mL) in the GCF for a period of 7 days and levels of 10-20 µg/mL were still present for 3 to 5 days after the polymer had been removed (Polson *et al.*, 1997; Jain *et al.*, 2008).

Table 1 Summary of some investigated intra-pocket delivery systems for antimicrobial agents

System	Polymer matrix	Drug incorporated	References
Fibers	Cellulose acetate	Tetracycline HCl	Goodson <i>et al.</i> , 1979
	Ethylene vinyl acetate	Tetracycline HCl	Tonetti <i>et al.</i> , 1990
	Poly(caprolactone)(PCL)	Tetracycline HCl	Tonetti <i>et al.</i> , 1990
Strips	Polyethylemetha acrylate	Tetracycline HCl	Addy and Langeroudi, 1984
		Metronidazole	Addy <i>et al.</i> , 1988
	Hydroxypropyl cellulose	Chlorhexidine	Noguchi <i>et al.</i> , 1984
	Poly(lactide-co-glycolic acid) (PLGA)	Tetracycline HCl	Maze <i>et al.</i> , 1995
	Ethyl cellulose	Chlorhexidine	Friedman and Golomb, 1982
Films	Ethyl cellulose	Metronidazole	Golomb <i>et al.</i> , 1984
		Tetracycline HCl	Azoury <i>et al.</i> , 1988
	Chitosan + PCL	Metronidazole	El-Kamel <i>et al.</i> , 2007
	Eudragit L and Eudragit S	Clindamycin	Higashi <i>et al.</i> , 1990
Gels	Chitosan	Metronidazole	Akncbay <i>et al.</i> , 2007
	Hydroxyethyl cellulose + polyvinylpyrrolidone	Tetracycline	Jones <i>et al.</i> , 1996
	Poloxamer407 + Carbopol 934P	Propolis	Bruschi <i>et al.</i> , 2007
	Poly (DL-lactide) + NMP	Doxycycline hyclate	Polson <i>et al.</i> , 1997
	Glycerol monooleate + sesame oil	Metronidazole	Noyan <i>et al.</i> , 1997
	PLGA	Tetracycline	Maze <i>et al.</i> , 1996
	Aliginat + HPMC	Secnidazole -Serratiopeptidase	Priyanka and Meenakshi, 2011
	Gellan gum + sodium alginate	Moxifloxacin	Kunche <i>et al.</i> , 2012

2.2 *In situ* forming gel systems

In situ forming gel is drug delivery systems, which is in sol form before the administration in the body, when administered it alters to a gel form (Jigar, 2011). Nowadays, *in situ* forming gel systems are gaining importance in drug delivery, since the advantages of *in situ* forming gel systems possess over the conventional formulations, which like sustained and prolonged action, ease of administration, localized delivery, reduced dose and frequency of administration, improved patient compliance and comfort (Hatefi and Amsden, 2002; Sharma *et al.*, 2007; Nirmal *et al.*, 2010). *In situ* gel formulations offers an interesting alternative for achieving systemic drug effects of parenteral routes, which can be inconvenient for oral route, which can result in unacceptably low bioavailability and passes the hepatic first-pass metabolism,

in particular of proteins and peptides (Nirmal *et al.*, 2010). *In situ* forming gel systems have been reported in literature for various biomaterial applications, drug delivery, cell encapsulation and tissue repair (Quaglia, 2008). Various natural and synthetic polymers are used for formulation development of *in situ* forming drug delivery systems (Madan *et al.*, 2009).

2.2.1 Classification of *in situ* gel forming systems

In situ gel forming systems have been classified in two categories as below

2.2.1.1 Based on mechanism of gelation

There are certain broadly defined mechanisms used for triggering the *in situ* gel formation of biomaterials including physiological stimuli (temperature and pH), physical changes in biomaterials (solvent exchange and swelling) and chemical reactions (enzymatic, chemical and photo-initiated) (Nirmal *et al.*, 2010). It was focused on *in situ* formation based on the polymer precipitation mechanism. In this system, a water insoluble polymer and drug were mixed with a biocompatible solvent to form a homogeneous solution or suspension. When this solution was injected into the aqueous medium, the water miscible organic solvent dissipated into the surrounding environment while water migrated into the polymer matrix, leading to the formation of a solid or semisolid form at the injection site due to polymer precipitation, followed by sustained release of the incorporated drug (Lie and Venkatraman, 2012). *N*-methyl pyrrolidone (NMP) has been reported to be useful solvent for this system (Nirmal *et al.*, 2010; Lie and Venkatraman, 2012).

2.2.1.2 Based on route of administration

Various natural and synthetic polymers are used for formulation development of *in situ* forming drug delivery systems. Depending on the route of administration, these *in situ* gel forming systems can be classified as illustrated as follow. Routes of administration are oral, ocular, rectal, vaginal, intraperitoneal and periodontal pocket.

2.2.2 Solvent exchange precipitating materials

The mechanism for the formation of *in situ* gel forming systems is precipitation of a polymer by solvent exchange. The polymer and drug are initially dissolved in a water-miscible organic solvent with low toxicity, such as *N*-methyl-2-pyrrolidone (NMP) or dimethyl sulfoxide (DMSO). Usually such polymers are hydrophobic in nature. Upon injection of the polymer and drug solution, the organic solvent is gradually replaced by water, causing precipitation of the polymer, which forms a solid gel and entraps the drug. However, the drug delivery applications of these systems have been limited due to high risks of burst release and solvent toxicity. Burst release occurs from solvent exchange systems because there is a significant amount of time required for the polymer to precipitate *in situ*. Several factors are known to affect the amount of burst release, including the polymer concentration and molecular weight, solvent used and inclusion of a surfactant (Vernon, 2011). Higher concentrations of polymer will typically precipitate more quickly, which can reduce burst release. However, increasing the polymer concentration is commonly used methods but limited because of the low viscosity requirement of the polymer solution during injection.

2.2.3 Commercial products of *in situ* gel forming systems

Atrigel[®] Technology was patented in 1990 (Dunn *et al.*, 1990; Warren *et al.*, 2009). It is based on the administration of solutions of biodegradable polymers into the soft tissue. After administration, the water-soluble solvent is distributed into the surrounding tissue and the polymer is precipitated due to a backflow of aqueous solutions from the biological environment. The release of the active ingredient from implants has been demonstrated to be influenced not only by the composition of the surrounding pathologically changed tissue, but also by the mechanical conditions in it (Patel *et al.*, 2010), and the process of precipitation of the polymer is considerably influenced by its molecular mass (Solorio *et al.*, 2010). The problem of the system Atrigel[®] Technology is the toxicity of solvents and a sudden initial release of a substantial part of the total doses of the contained active ingredient. Arestin[®] (minocycline HCl 1 mg) microspheres, as an adjunct to scaling and root planing, is a

locally administered antibiotic treatment for severe chronic periodontitis. The unique delivery process involves a powdery substance known as microspheres. Microspheres encapsulate a low dose (1 mg) of the antibiotic minocycline. After it is placed, the Arestin microspheres immediately adhere to the periodontal pocket where the minocycline is released up to 21 days. The microspheres are then completely bioresorbed into the gingiva. Alzamer[®] Depot technology (Alza Corporation) are intended for subcutaneous administration. The incorporating of hydrophobic plasticizers in the systems possess a lower speed of degradation with a smaller burst and a slower release of the active ingredient (Matschke *et al.*, 2002). Regel is one of the Macromed's proprietary drug delivery system and based on triblock copolymer, composed of poly (lactide-co-glycolide)-poly (ethylene glycol)-poly (lactide-co-glycolide). Oncogel[®] is a frozen formulation of paclitaxel in Regel. It is a free flowing liquid below room temperature which upon injection forms a gel *in situ* in response to body temperature. Following injection, the physical properties of polymer undergo a reversible phase change resulting in formation of a water insoluble, biodegradable gel depot (Ramesh *et al.*, 2001). Cytoryn is one of the Macromed's products, which is a novel, peritumoral, injectable depot formulation of interleukin-2 (IL-2) for cancer immunotherapy using Regel drug delivery system.

2.3 Formulation parameters

2.3.1 Polymers

2.3.1.1 Ethocel

Ethylcellulose or Ethocel (EC) is a well-known water-insoluble polymer. It is a semi-synthetic polymer manufactured from cellulose and transferred with sodium hydroxide to alkali cellulose (Figure 1) (Rekhi and Jambhekar, 1995). It is widely used in oral and topical pharmaceutical formulations, since it is nontoxic, non-allergenic and non-irritant. It has been used as a hydrophobic coating agent in oral formulations for controlled drug release, moisture protection and taste masking (Bodmeier *et al.*, 1994; Dressman *et al.*, 1995; Narisawa *et al.*, 1994). Ethylcellulose coating is used to modify the release of a drug (Sadeghi, 2001). It dissolved in an organic solvent or solvent mixture that can be used to produce water-insoluble films.

Higher viscosity ethylcellulose grades tend to produce stronger and more durable films. Drug release through ethylcellulose coated dosage forms can be controlled by diffusion through the film coating.

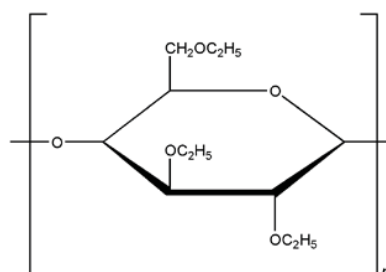


Figure 1 Chemical structure of ethylcellulose

2.3.1.2 Bleached shellac

Shellac is a natural resin that may be obtained in a variety of colors ranging from light yellow to dark red in the form of hard, brittle flakes with or without wax, depending on the refining process. The flakes may be crushed or milled to a coarse or fine powder. Bleached shellac is supplied as a coarse-off white powder. Shellac is tasteless and faint odor (Buchbauer *et al.*, 1993). Shellac is widely used as a moisture barrier coating for tablets and pellets due to its low water vapor and oxygen permeability. Recent research results indicate good application properties and chemical stability of shellac films from aqueous shellac solutions (Pearnchop *et al.*, 2003).

2.3.1.3 Eudragit RS

Eudragit RS is commonly used to form water-insoluble film coat for the enteric coating of tablets and sustained-release products. Eudragit RS is composed of poly(ethyl acrylate, methyl methacrylate, trimethylammonioethyl methacrylate chloride) (1:2:0.1). It exhibits a very low permeability and swells in aqueous media independently of pH without dissolving. Eudragit RS PO is fine, white powders with a slight amine-like odor (Rowe *et al.*, 2009). The chemical structure of Eudragit RS is shown in Figure 2. Polymethacrylate copolymers are widely used as film-coating materials in oral pharmaceutical formulations. They are also used in topical

formulations and are generally regarded as nontoxic and nonirritant materials (Rowe *et al.*, 2009).

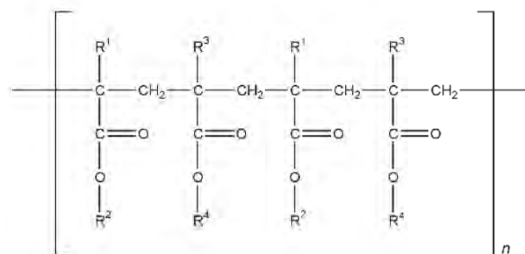


Figure 2 Chemical structure of Eudragit RS; $R^1 = \text{CH}_3$; $R^2 = \text{C}_2\text{H}_5$ and $R^3 = \text{CH}_3$ (Rowe *et al.*, 2009).

2.3.2 Solvent (*N*-methyl-2-pyrrolidone)

N-methyl-2-pyrrolidone (NMP) is a chemical compound with 5-membered lactam structure. NMP is a colorless to slightly yellow liquid, with a faint amine odor. It is miscible with water and conventional organic solvents. It has low volatility and low flammability. It is thermally stable, and not corrosive. The mechanism of solubilization of drugs by NMP is uncertainly, and there are various theories, including its action as a cosolvent (Tarantino *et al.*, 1994; Reynolds *et al.*, 2012), complexing agent (Uch *et al.*, 1999), and surfactant (Bachhav *et al.*, 2006). The chemical structure of NMP is shown in Figure 3. It has non-polar carbons, which can weaken the hydrogen-bonded structure of water, thus allowing it to act as a cosolvent (Jouyban *et al.*, 2010; Liu and Venkatraman, 2012). The presence of a large planar nonpolar region can lead to hydrophobic interactions between NMP and drugs (Sanghvi, 2008).

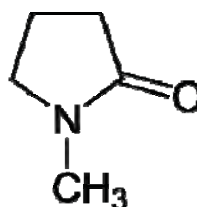


Figure 3 Chemical structure of *N*-Methyl-2-Pyrrolidone (NMP), $\text{C}_5\text{H}_9\text{NO}$

NMP is widely used in the petrochemical industry, and in the manufacturing of various compounds, including pigments, cosmetics, insecticides,

herbicides, and fungicides. NMP is an acceptable pharmaceutical solvent (Jouyban *et al.*, 2010) that is a solubilizing excipient used in parenteral and oral medications (Strickley, 2004). The solubility parameter of NMP is similar to those of ethanol and dimethyl sulfoxide (DMSO) (Hansen and Just, 2001). It has been reported that skin irritation is low for pyrrolidone derivatives (Sasaki *et al.*, 1990). NMP is a penetration enhancer (Godavarthy *et al.*, 2009; Rachakonda *et al.*, 2008) that is used for enhancement of transdermal delivery of hydrophilic and hydrophobic drugs (Lee *et al.*, 2005). NMP increased the transdermal absorption of some drugs such as phenolsulfonphthalein, ibuprofen and flurbiprofen (Akhter and Barry, 1985) and also estradiol (Koizumi *et al.*, 2004). It has been reported that the Atrigel[®] commercial injectable systems comprising PLA or PLGA dissolved in a biocompatible solvent *N*-methyl-2-pyrrolidone (NMP) was used for the periodontal therapy (Polson *et al.*, 1997; Kranz and Bodmeier, 2008)

2.3.3 Drugs

2.3.3.1 Doxycycline hyclate

Doxycycline hyclate is a broad-spectrum antibiotic synthetically derived from oxytetracycline. It is a yellow crystalline powder soluble in water and slightly soluble in alcohol. The chemical structure of doxycycline hyclate is shown in Figure 4. Doxycycline is more active than tetracycline against many bacterial species including *Streptococcus pyrogene*, enterococci, *Nocardia* spp., *Staphylococcus aureus* and various anaerobes. It exhibits greater oral absorption. They have the more prolonged half-lives, and show the enhanced lipid solubility, which is important for antibacterial action (Seymour and Heasman, 1995). Doxycycline is used widely in periodontal treatment. It is bacteriostatic antibiotics and also inhibits tissue collagenase activity (Yu, 1993; Levy, 1984; Seymour and Heasman, 1995). Doxycycline enters through porins in Gram-negative bacteria, and through their lipophilicity in Gram-positive bacteria. It passes through the cytoplasmic membrane via active transport. It bind with RNA on the ribosome by chelating divalent cations like magnesium (Mg^{++}), which are attached to the phosphates on RNA. However, free magnesium ions in the cytosol may chelate with the drug prior to its binding, disabling

their interaction with the ribosome (Takahashi *et al.*, 1986). It has a broad spectrum of activity inhibiting both gram negative and gram positive organisms, including beta-lactamase producing strains which occur in deep periodontal pockets and against which penicillins are ineffective.

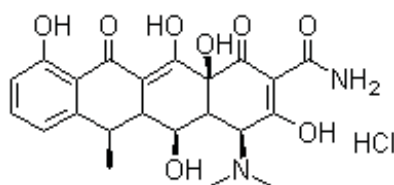


Figure 4 Chemical structure of doxycycline hyclate (C₂₂H₂₄N₂O₈, HCl).

Doxycycline therapy at sub-antimicrobial dose has been shown to reduce periodontal disease activity by reducing matrix metalloproteinases (MMPs) and pro-inflammatory cytokines (Choi *et al.*, 2004; Gapski *et al.*, 2004). The doxycycline hyclate preparation was used for treatment of periodontitis such as poly (lactide-co-glycolide)(PLGA) containing doxycycline hydrochloride were used to formulate the *in situ* implants (Gad *et al.*, 2008). Doxycycline hyclate loaded microspheres composed of SynBiosysTM could be obtained with injectable systems (Gillissen, 2009). The Atrigel[®] is injectable biodegradable delivery system containing 10% doxycycline hyclate. This system is based on poly (DL-lactide) dissolved in a biocompatible solvent *N*-methyl-2-pyrrolidone (NMP) (Schwach *et al.*, 2000). Doxycycline hyclate can also alter the cytoplasmic membrane and causes leakage of nucleotides and other compounds out of cell.

2.3.3.2 Metronidazole

Metronidazole is bactericidal agent against anaerobic bacteria. Its exact mechanism of action has not been entirely determined as yet. It has been proposed that metronidazole is intracellularly activated by reduction and the toxic effect of the reduced intermediates binding to DNA leading to loss of helical structure, strand breakage and impairment of DNA function (Rizzo *et al.*, 2010; Cavalcanti *et al.*, 2004; Oliveira *et al.*, 2009). Metronidazole has been used in the field of periodontal therapy either with a systemic administration, mostly in combination with amoxicillin

or ciprofloxacin, or with local biodegradable sustained-release agents (Rizzo *et al.*, 2010; Haffajee *et al.*, 2003; Noyan *et al.*, 1997). However, the drawbacks observed are the adverse effects of metronidazole involving the gastrointestinal tract with high doses. The reduction of adverse effects was thus desirable by the use of controlled release devices (Ozyazici *et al.*, 2006; Oliveira *et al.*, 2009). The drug controlled release is necessary for a good compliance and the drug released must possess the required bioactivity (Oliveira *et al.*, 2009). The chemical structure of metronidazole is shown in Figure 5. Metronidazole has a high solubility in water (10.61 mg/mL) and particularly at a low pH in HCl 0.1 N (32.30 mg/mL) (Martino *et al.*, 2007; Oliveira *et al.*, 2009).

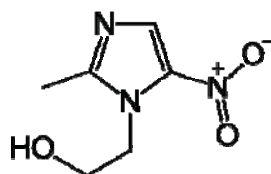


Figure 5 Chemical structure of metronidazole

2.3.3.3 Benzoyl peroxide

Benzoyl peroxide (BP) is an organic compound in the peroxide family. It consists of two benzoyl peroxide groups bridged by a peroxide link. The chemical structure of benzoyl peroxide is shown in Figure 6. It is one of the most widely prescribed drugs in acne therapy. It is an anti-bacterial agent which releases free radical oxygen species capable of oxidizing bacterial proteins. Additionally, it also has a mild keratolytic effect (Matsui *et al.*, 1995; Waller *et al.*, 2005). It is commonly available as cream and gel, either alone or in combination with other drugs. However, the drug is known to cause considerable skin dryness and irritation, sometimes leading to the discontinuation of the treatment. The benzoyl peroxide formulation containing sesame oil (6%w/w) exhibited the maximum drug release, antimicrobial activity and the least skin irritation potential (Thakur *et al.*, 2012). The benzoyl peroxide in Eudragit RS systems containing peppermint oil was studied for periodontitis treatment (Mahadlek *et al.*, 2013).

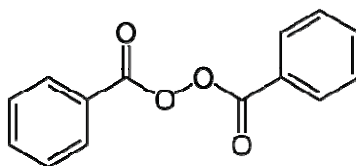


Figure 6 Chemical structure of benzoyl peroxide $(C_6H_5C(O))_2O_2$.

2.3.4 Plasticizers

Plasticizers are additives which cause a reduction in polymer-polymer chain secondary bonding (e.g. hydrogen bonding), forming secondary bonds with the polymer chains instead (Feldstein *et al.*, 2007; Bodmeier *et al.*, 1997). Plasticizers increase the elongation and flexibility, and decrease the tensile strength, Young's modulus and the glass transition temperature (Rahman and Brazel, 2004). Plasticizers are used in several applications including food packaging (Giam and Wong, 1987; Bonini *et al.*, 2008), plastics, agricultures, papers and tapes. Plasticizers are also used to control the drug release in pharmaceutical applications. The plasticizer plays the major role in the all process of the oral dosage form coating and significantly influences the quality of the coatings, particularly the release of incorporated drugs. Plasticizers can be divided into hydrophilic and hydrophobic. Hydrophilic plasticizers can lead to an increase in water diffusion in the polymer and can leach out from the film and increase drug release rate (Bodmeier and Paeratakul, 1992; Turner and Abell, 1987). In contrast, hydrophobic plasticizers can to a decrease in water uptake thus it decrease drug release rate (Turner and Abell, 1987). The type and the amount of plasticizer strongly affect the film formation from polymeric aqueous dispersions (Bodmeier *et al.*, 1997). Plasticizers are also added to the polymeric solutions and dispersion to increase the flexibility of the polymeric material since the plasticizer weakening the polymer intermolecular attractions and increasing the polymer's free volume, thus allowing the polymer molecules to move more freely to cause an increase in their flexibility (Gutierrez-Rocca and McGinity, 1994). The degree of plasticization of a polymer is dependent to a large extent on the amount of plasticizer in the film and the interactions between the plasticizer and the polymer.

The physicochemical properties, particularly the solubility parameters and extent leaching of the plasticizer act the major role in the drug release from a plasticized polymer system. The differences in the drug release patterns are observed in the case of using either hydrophilic or hydrophobic plasticizers. The hydrophobic plasticizers (dibutyl sebacate) are shown to remain within the polymeric system upon exposure to the release media, assuring integral and mechanically resistant coatings during drug release. In contrast, hydrophilic plasticizers leached out of the system, resulting either in decreased mechanical resistance and thus cracking, or in facilitated pore formation. As drug release was controlled by diffusion through the intact membrane and water-filled cracks (with significantly different diffusion coefficients), the mechanical stability of the polymeric system and the onset of crack formation are of major importance for the resulting drug release profiles (Snejdrova and Dittrich, 2012). Plasticized polymers used in drug delivery systems come to contact with liquid after application into the body. Plasticizers tend to diffuse down the concentration gradient to the interface between the polymer surface and the external medium. The hydrophilic plasticizer can be released from polymer and thus conditions for incorporated drug release are changed. The hydrophobic plasticizer remains in the system and ensures standard conditions during the process of drug release. In high concentration of hydrophilic plasticizer can lead to an increase in water diffusion into the polymer (Snejdrova and Dittrich, 2012). Moreover, the affinity of the polymer to the polymer is found to be decisive. The plasticizer redistribution within the polymeric systems during coating, curing and storage affects the drug release rate (Bodmeier and paeratakul, 1997). The extent of the initial release can be controlled by plasticizers. High burst release can be minimized by hydrophobic plasticizers, whereas the opposite effect is achieved by hydrophilic plasticizers, which leach out of polymer in the hydrophilic medium (Snejdrova and Dittrich, 2012). The primary role of all plasticizers as low molecular weight non-volatile additive is to improve the flexibility and processability of polymers by lowering the second order transition temperature (glass transition temperature, T_g) (Snejdrova and Dittrich, 2012). In this study, the hydrophilic (polyethylene glycol 1500) and hydrophobic (peppermint oil) substances were selected to used as plasticizer for *in situ* gels systems.

2.3.4.1 Polyethylene glycol 1500 (PEG1500)

Polyethylene glycols (PEGs) are widely used in a variety of pharmaceutical formulations, including parenteral, topical, ophthalmic, oral, and rectal preparations. Polyethylene glycol has been used verified in biodegradable polymeric matrices used in controlled-release systems (Mohl *et al.*, 2004). It is hydrophilic substance that is essentially nonirritant to the skin. It does not readily penetrate the skin, although the polyethylene glycols are water-soluble. The rate of release of water-soluble drugs decreases with the increasing molecular weight of the polyethylene glycol. Polyethylene glycols are useful as plasticizers in microencapsulated products to avoid rupture of the coating film when the microcapsules are compressed into tablets. Polyethylene glycol has been used in insulin-loaded microparticles for the oral delivery of insulin (Morçöl *et al.*, 2004; Morishita *et al.*, 2004) and self-assembled polymeric nanoparticles as a drug carrier to improve the oral bioavailability of cyclosporine (Jaiswal *et al.*, 2004). Copolymer networks of poly(methacrylic acid) grafted with poly(ethylene glycol) has been used as bioadhesive controlled drug delivery (Peppas, 2004). The chemical structure of PEG1500 is shown in Figure 7.

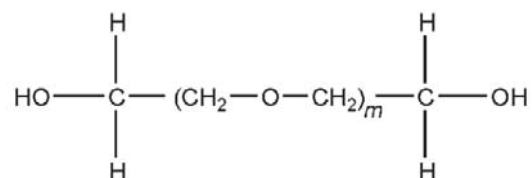


Figure 7 Chemical structure of polyethylene glycol 1500: m represents the number of oxyethylen groups; m = 32.5 (Rowe *et al.*, 2009).

2.3.4.2 Peppermint oil

Peppermint oil is an essential oil derived from leaves of *Mentha piperita*, which is a medicinally important plant. It has been used to extend the shelf life of food, showing inhibition against bacteria, fungi and yeast (Jeyakumar *et al.*, 2011). It is helpful in symptomatic relief of the common cold. It is also used topically as an analgesic and to treat headache (Göbel *et al.*, 1995). Peppermint oil is used for flavoring pharmaceuticals and oral preparations, such as toothpastes, dental

creams, and mouth washes. Peppermint oil is found to be strongly effective against *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Escherichia coli* (Sartoratto *et al.*, 2004). Peppermint oil has also the virucidal activity against herpes simplex virus (Schuhmacher *et al.*, 2003; Mohsenzadeh *et al.*, 2007) and fungicidal activity (Barrera-Necha *et al.*, 2009).

2.4 Antimicrobial combinations

Antimicrobial combinations are used most frequently to provide broad-spectrum empiric coverage in the treatment. Less frequently, combinations of antimicrobials are chosen because an identified pathogen is resistant to inhibition and/or killing by conventional doses of single antimicrobials but against it the combination may exert the desired antimicrobial activity. In both instances, the clinical outcome may depend on the effects of antimicrobial combinations against individual microorganisms. Two types of antimicrobial interactions have been excluded from consideration and are not discussed. The first is enhanced effectiveness of an antimicrobial due to the other drug that interferes with the elimination or metabolism of the first drug but is not itself an antimicrobial. The second is the inactivation of one antimicrobial by another, unrelated to the presence of a microorganism (Black, 2008; Lorian, 1996; Mahon *et al.*, 2007)

2.4.1 Types of drug combination interaction

When two antimicrobial agents act simultaneously on a homogeneous microbial population, the effect may be one of the following:

- **Synergism:** The combined action is significantly greater than the sum of both effects.

- **Antagonism:** The combined action is less than that of the more effective agent when used alone.

- **Indifference:** The combined action is no greater than that of the more effective agent when used alone.

- **Addition:** The combined action is equivalent to the sum of the actions of each drug when used alone.

2.4.2 Rationales for the use of antimicrobial combinations

The rationales of the use of antimicrobial combinations have various reasons including a decreased emergence of resistant strains, a decreased dose-related toxicity as a result of reduced dosage, another important use of antimicrobial combinations is in the treatment of documented or suspected mixed (polymicrobial) infections and antimicrobial synergism.

2.4.3 Methods for assessing interaction of drug combination (Checkerboard)

The checkerboard (or chessboard) method is the technique that has been used most frequently to assess antimicrobial combinations *in vitro*, presumably because (a) its rationale is easy to understand, (b) the mathematics necessary to calculate and interpret the results are simple, (c) it can be readily performed in clinical laboratories using microdilution systems that are obtainable commercially, and (d) it has been the technique most frequently used in studies that have suggested an advantage of synergistic therapy. The term “checkerboard” refers to the pattern (of tubes or microtiter wells) formed by multiple dilutions of the two antimicrobials being tested, in concentrations equal to, above, and below their minimal inhibitory concentrations (MICs) for the organisms being tested.

The checkerboard consists of columns in which each tube (or well) contains the same amount of the drug (drug A) being diluted along the x axis and rows in which each tube (or well) contains the same amount of the drug (drug B) being diluted on the y axis (Figure 8). The result is that each square in the checkerboard contains a unique combination of the two drugs being tested. The results are calculated mathematically and expressed in terms of a fractional inhibitory concentration (FIC) index equal to the sum of the FICs for each drug. The FIC for a drug is defined as the MIC of the drug in combination divided by the MIC of the drug used alone. If the FIC index is < 0.5 , the antimicrobial combination is interpreted as being synergistic; between 1 and 4 as indifferent; and >4 as antagonistic (Black, 2008; Lorian, 1996; Mahon *et al.*, 2007)

A wide variety of combinations of concentrations is tested by dispensing drugs in a two-dimensional checkerboard format, and each drug tested in the combination is also tested by itself. A combination is said to show synergism if its antibacterial activity is significantly greater than that of the single agents—that is, when the MIC for each drug in the combination is less than or equal to one fourth of the single-agent MICs. Conversely, antagonism is defined as the activity of the combination less than (and MICs are greater than) that of the single agents. In indifference the activity of the combination is equal to that of the single agents (Figure 9).

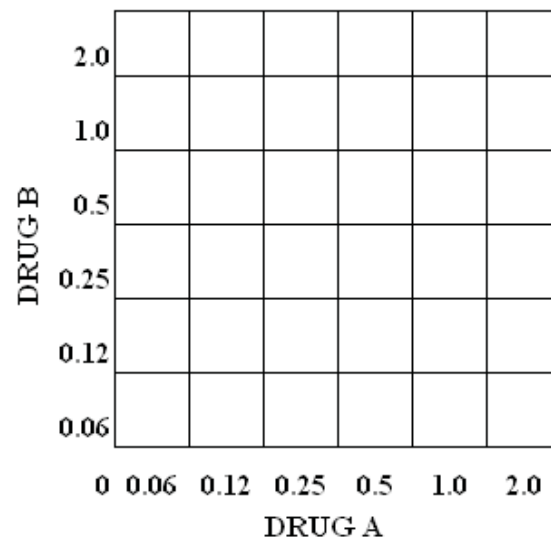


Figure 8 Checkerboard techniques. In the checkerboard, serial dilutions of two drugs are performed using drug concentrations proportional to the MICs of the drugs being tested.

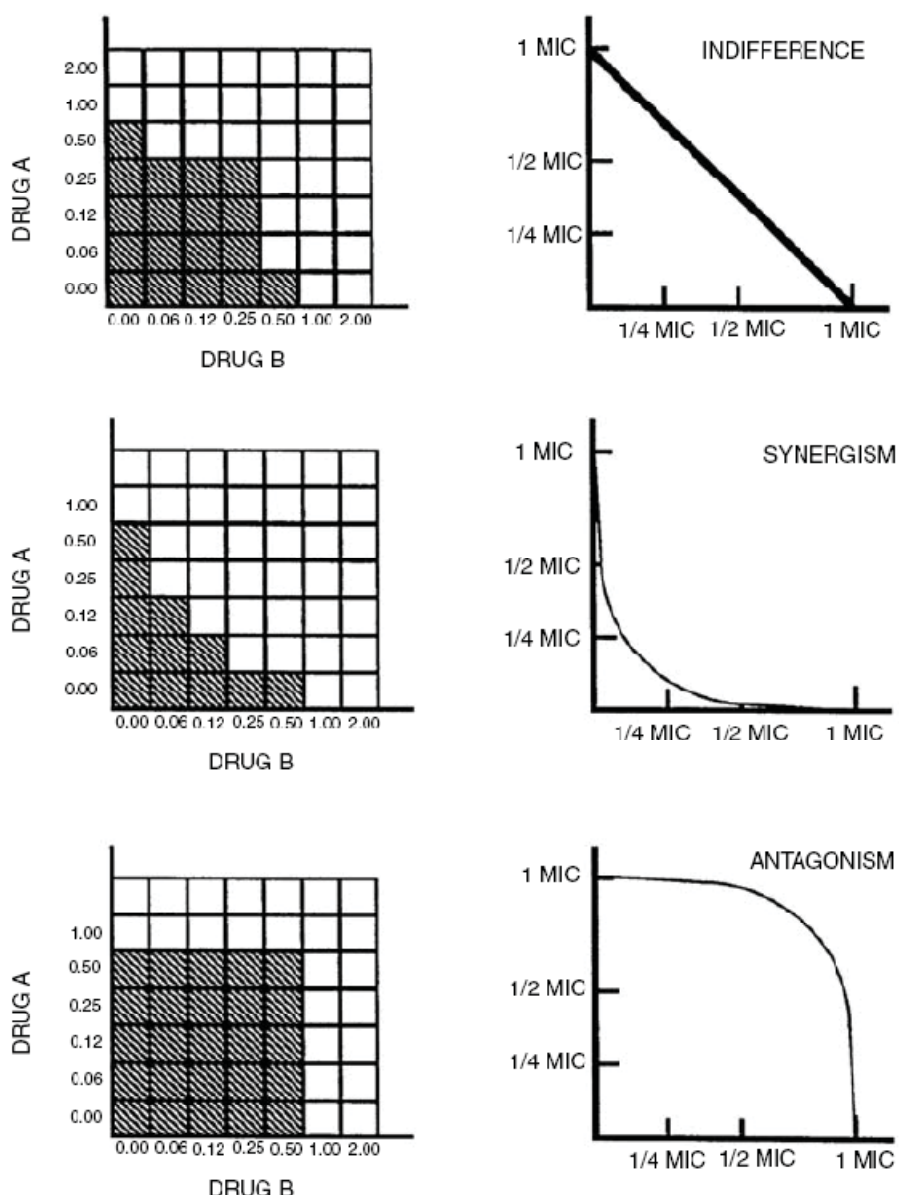


Figure 9 Results of checkerboard represented as isobolograms

2.5 Rheological studies

Rheology is the study of flow and deformation of materials and allows determination of the material viscoelastic behavior. Rheology studies describe for viscosity measurements, characterization of flow behavior and determination of material structure. Shearing of a substance is the key to knowledge of flow behavior and structure. To enable study of the viscosity of a material, the shearing must induce

stationary flow of the materials. The flow occurs through rearrangement and deformation of particles and through breaking of bonds in the structure of material. The measuring result obtained with a viscometer or rheometer is always a flow curve. However, the viscosity function can be calculated based on the measured values.

Newtonian behavior

For Newtonian fluids, the rate of deformation is in proportion to the force applied. Deformation leaves when the applied force is removed. The apparent viscosity is constant with changing shear rates. This behavior is typical of simple liquids such as water and mineral oil.

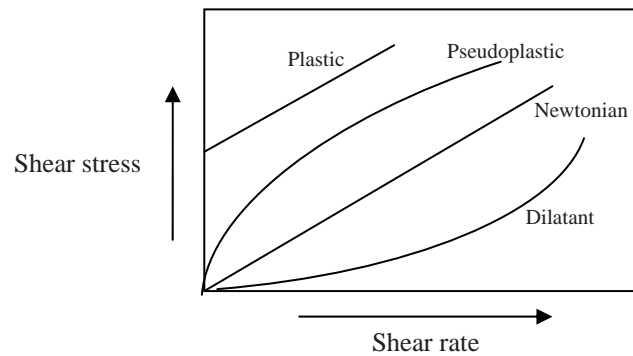


Figure 10 Flow behaviors. The shear stress means the applied force; the shear rate means the rate of deformation.

Non-Newtonian behavior

Non-Newtonian behavior depends on the micro- or nanostructure of the material (breakdown, arrangement, or entanglement).

- Time independent

1. Pseudoplastic

Materials are referred to as pseudoplastic if force acting on the body causes the particle size to change, the particles to be oriented in the direction of flow, or an agglomerate to dissolve. The samples of fluid display a decreasing viscosity with an

increasing shear rate, some examples include paints and emulsions. This type of behavior is called shear-thinning.

2. *Dilatant*

A dilatant material resists deformation more than in proportion to the applied force. For example, the more effort you put into stirring a dilatant material, the more resistant it becomes to stirring. This is usually an indication that the applied force is causing the material to adopt a more ordered structure. A thick slurry of wet beach sand is often dilatant. It is also referred to as shear-thickening liquids.

3. *Plastic (Bingham)*

Plastic materials initially resist deformation, until a yield stress is reached. When that stress is exceeded, the shear rate becomes measurable. Further stress leads finally to linear (Newtonian) behaviour.

- **Time dependent (*Thixotropy and Rheopexy*)**

The thixotropic materials become more fluid with increasing time of the applied force, which called that “the work softening”. This system is reversible. On the other hands, the rheopectic materials become more viscous with increasing time of the applied force, which called that “the work hardening”.

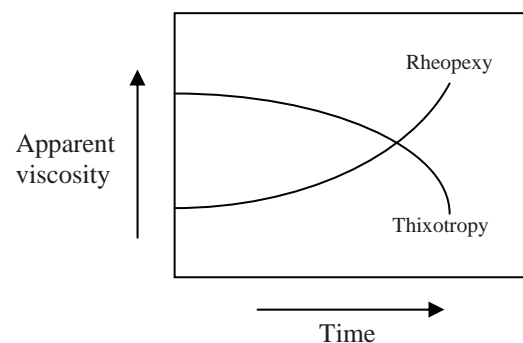


Figure 11 Viscosity and time curves of thixotropy and rheopexy.

2.6 Release kinetics

The aim of the controlled release systems is an ideal kinetic profile of drug release to maintain drug concentration in the blood or in target tissues at a desired value during the long time period. The drug delivery systems referred to as controlled release systems and composed of biodegradable polymeric matrix enclosing therapeutic agent reveal a complex heterogeneous release profiles. The initial stage is a rapid dissolution of part of drug which is not protected effectively by a carrier so called “the burst effect”. The following stage is a slow release of drug fraction enclosed in matrix, which is induced by a polymer hydrolytic degradation. Therefore the drug release kinetics follows a well defined behavior in order to supply the maintenance dose enabling the attainment of the desired drug concentration. The use of mathematical modeling can predict the best release kinetics of the systems for the suitable controlled release systems. The release profiles can be divided into three types including zero order, square root time and first order release models. The release models of zero-order, square-root time and first-order release model is illustrated in Figure 12 (Baker *et al.*, 1987).

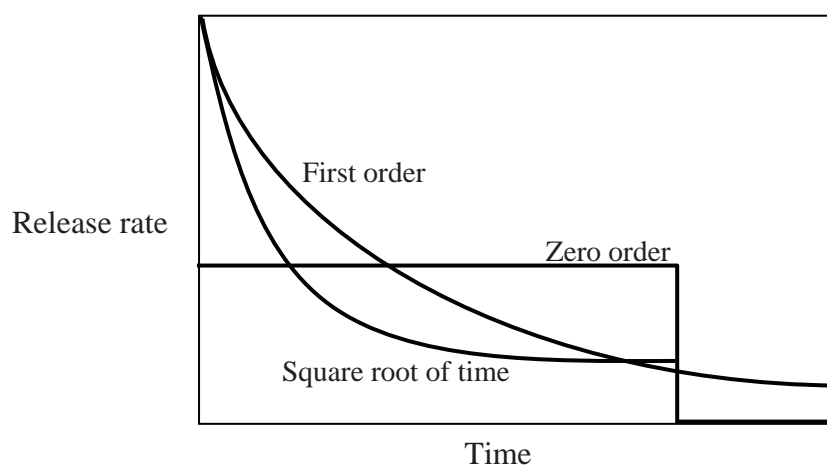


Figure 12 Zero-order, first-order and square-root time release patterns from devices containing the same initial active agent content (Baker *et al.*, 1987).

2.6.1 Zero order release model

Zero order release is an ideal controlled release, which can deliver the drug at constant rate until the device is finished of active agent. The zero order release model is given as:

$$Q_t = Q_0 + k_0 t \quad [1]$$

With Q_t is the total amount of drug released after time t (%), Q_0 is the initial amount of drug (%) and k_0 is the zero order release rate constant (h^{-1}).

2.6.2 Square root time release model (Higuchi's model)

The plot of drug released amount from matrix versus square root of time should be increased linearity when drug release from the matrix is diffusion controlled. This equation is based on release from a single face, thus it may use to describe diffusion-controlled release from all surface matrices. Higuchi's equation is usually desired and used as (Higuchi, 1963):

$$Q_t = k_H \sqrt{t} \quad [2]$$

With Q_t is the total amount of drug released at time t (%) and k_H is the Higuchi release rate constant (h^{-1}).

Higuchi's equation is converted into logarithmic to verify the drug release follows Higuchi's model as:

$$\log Q_t = \log k_H + \frac{1}{2} \log t \quad [3]$$

$$Q = [D\varepsilon/\tau (2A - \varepsilon C_s) C_s t]^{1/2} \quad [4]$$

With Q is weight of drug release (g) per unit surface area, D is diffusion coefficient of drug in the release medium, ε is porosity of the matrix, τ is tortuosity of matrix, C_s is solubility of drug in the release medium and A is concentration of drug in the tablet, expressed as g/mL.

2.6.3 First order release model

The release rate of first order is proportional to the mass of active agent contained in the device. The exposed surface area of matrix decreases exponentially with time, approaching a release rate of zero as the device approaches exhaustion (Wagner, 1969).

$$Q_t = Q_0 e^{-k_1 t} \quad [5]$$

With Q_t is the total amount of drug released at time t (%), Q_0 is the initial amount of drug (%) and k_1 is the first order release rate constant (h^{-1}).

Simplifying and taking the logarithm of first order equation:

$$\log Q_t = \log Q_0 - \frac{k_1 t}{2.303} \quad [6]$$

The relationship between the logarithms of the percentage of drug remaining versus time is linear, the drug release pattern follows first order model.

7. Release mechanism of controlled release systems

The release mechanism of the matrices can predict using the semi-empirical equation of Peppas (1985) given below.

$$M_t = \frac{k t^n}{M_\alpha} \quad [7]$$

Where M_t/M_α is the fractional of release of drug up to time t , t is the release time, k is a constant incorporating the structure and geometric characteristics of the drug/polymer system and n is the release exponent indicative of the drug release mechanism (Peppas, 1985).

The drug release data from thin polymer film interpret the diffusional release mechanism as shown in Table 2. The n value of 1 corresponds to zero order release behavior, $0.5 < n < 1$ means a non-Fickian release model and $n = 0.5$ indicates Fickian diffusion (Peppas, 1985)

Table 2 Interpretation of diffusional release mechanism from drug release data from thin polymer film (Peppas, 1985).

Release exponent (n)	Drug transport mechanism	Rate as a function
0.5	Fickian diffusion	$t^{1/2}$
$0.5 < n < 1.0$	Anomalous (non-Fickian) Transport	t^{n-1}
1.0	Case-II transport	Zero-order (time-independent)
$n > 1.0$	Super case-II transport	t^{n-1}

The empirical equation 7 could be modified for application to non-planar geometric. This equation can be used to analyze drug release from sheets, cylinders, spheres, tablets and polydisperse microspheres under perfect sink conditions. Characteristic diffusional exponents for Fickian diffusional release have been defined in each case, for fitting of the first 60% of release curve. Limiting the analysis of experimental data to the first 15% of the release process could render any value of n obtained statistically insignificant. The relationship between the diffusional exponent (n) and corresponding release mechanism is clearly dependent upon the geometry employed as shown in Table 3 and 4. In case of pure Fickian release the diffusional exponent (n) has the limiting values of 0.50, 0.45 and 0.43 for release from slabs, cylinders and sphere, respectively. For tablets, depending on the ration of diameter to thickness, the Fickian diffusion mechanism is described by $0.43 < n < 0.5$. For drug release from spherical polymer particles of a wide distribution, the diffusional exponent for Fickian diffusion depends on the width of the distribution (Rigger and Peppas, 1987). In swellable controlled release systems, case-II (Fickian diffusion) and case-II solute release behavior are unique in that each can be described in terms of a single parameter. Case-I transport described by diffusion coefficient, while case-II transport described by a characteristic constant. Non-Fickian behavior, by comparison, requires two or more parameters to describe the coupling of diffusion and relaxation phenomena.

Table 3 Diffusional exponent and mechanisms of diffusional release from various non-swellaible controlled release systems (Rittger and Peppas, 1987).

Diffusional exponent (n)			Drug release mechanism
Thin film	Cylindrical sample	Spherical sample	
0.5	0.45	0.43	Fickian diffusion
$0.5 < n < 1.0$	$0.45 < n < 1.0$	$0.43 < n < 1.0$	Anomalous (non-Fickian) transport
1.0	1.0	1.0	Case II transport

In swellaible matrices, the system does not swell more than 25% of its original volume, the values of n of spherical sample are 0.43 and 0.85 for Fickian and case-II transport, respectively. When the value of n is > 0.43 and < 0.85 , the release was said to be non-Fickian (Siepmann and Peppas, 2001). When the value of n was greater than that of the case-II transport, the release is said to be super case-II transport. The diffusional exponent and mechanism of drug from various swellaible controlled release systems are shown in Table 4 (Siepmann and Peppas, 2001).

Table 4 Diffusional exponent and mechanisms of drug from various swellaible controlled release systems (Siepmann and Peppas, 2001).

Diffusion exponent (n)			Drug release mechanism
Thin film	Cylindrical sample	Spherical sample	
0.5	0.45	0.43	Fickian diffusion
$0.5 < n < 1.0$	$0.45 < n < 0.89$	$0.43 < n < 0.85$	Anomalous (non-Fickian) transport
1.0	0.89	0.85	Case II transport

8. MicroMath Scientist[®] for Windows

MicroMath Scientist[®] for Windows is a general mathematical modeling and data analysis application. It is specifically designed to fit model equations to experimental data. Scientist[®] can fit almost any mathematical model from the simplest linear functions to complex systems of differential equations, non-linear algebraic equations or models expressed as Laplace transforms. MicroMath has

prepared several model libraries to aid users working in the fields of pharmacokinetics, chemical kinetics and diffusion. The Scientist Chemical Kinetic Library is a set of chemical kinetics models that can be used to simulate or analyze experimental data. The Scientist Chemical Kinetic Library includes models for zero, first and second order irreversible reactions, first order reversible reactions, and parallel first order irreversible reactions with up to three products.

Least square fitting the experimental dissolution data (cumulative drug release > 10% and up to 80%) to the mathematical equations (power law, first order, Higuchi's and zero order) was carried out using a nonlinear computer programme, Scientist for Windows, version 2.1 (MicroMath Scientific Software, Salt Lake City, UT, USA). The parameters of each model in the software were T, F, K, Tl and N. The T was as time in minute of drug release, F was fractional drug release, K was the constant of each model, Tl was lag time of drug release and N was the n exponent value of power law model. These parameters are shown in Table 5. The coefficient of determination (r^2) was used to indicate the degree of curve fitting. Goodness-of-fit was also evaluated using the Model Selection Criterion (msc) (MicroMath Scientist Handbook, 1995), given below.

$$msc = \ln \left\{ \frac{\sum_{i=1}^n w_i (Y_{obs_i} - \bar{Y}_{obs})^2}{\sum_{i=1}^n w_i (Y_{obs_i} - Y_{cal_i})^2} \right\} - \frac{2p}{n} \quad [8]$$

Where Y_{obs_i} and Y_{cal_i} are observed and calculated values of the i-th point, respectively, and w_i is weight that applies to the i-th point, n is number of points and p is number of parameters.

Table 5 Model files used with Scientist[®]

// MicroMath Scientist Model File (ZERO ORDER) IndVars: T DepVars: F Params: K,Tl $F=K*(T-Tl)$
// MicroMath Scientist Model File (FIRST ORDER) IndVars: T DepVars: F Params: K,Tl $F=1-EXP(-K*(T-Tl))$
// MicroMath Scientist Model File (HIGUCHI's) IndVars: T DepVars: F Params: K,Tl $F=K*((T-Tl)^{(1/2)})$
// MicroMath Scientist Model File (POWER LAW EXPRESSION) IndVars: T DepVars: F Params: K,Tl,N $F=K*((T-Tl)^N)$

CHAPTER 3

MATERIALS AND METHODS

- 3.1 Materials
- 3.2 Microbials
- 3.3 Equipments
- 3.4 Methods
 - 3.4.1 Synergy effect studies of antimicrobial agents
 - 3.4.1.1 Preparation of inoculums
 - 3.4.1.2 Minimal inhibitory concentration (MIC) determination
 - 3.4.1.3 Checkerboard test
 - 3.4.2 Preparation and evaluation of the prepared injectable *in situ* forming gel base systems from various polymers
 - 3.4.2.1 Preparation of the *in situ* forming gel base system
 - 3.4.2.2 Evaluation of gel properties
 - 3.4.2.2.1 Gel appearance and pH measurement
 - 3.4.2.2.2 Viscosity studies
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 - 3.4.2.2.5 *In vitro* gel formation
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 - 3.4.3 Preparation and evaluation of the *in situ* forming gel systems containing antimicrobial agents
 - 3.4.3.1 Preparation of the *in situ* forming gel systems containing antimicrobial agents
 - 3.4.3.2 Evaluation of gel properties
 - 3.4.3.2.1 *In vitro* drug release studies
 - 3.4.3.2.1.1 Dialysis membrane method
 - 3.4.3.2.1.2 Membrane-less diffusion method

- 3.4.3.2.1.3 Calibration curves
- 3.4.3.2.2 Analysis of drug release data
- 3.4.3.2.3 Determination of surface morphology of gels
- 3.4.3.2.4 *In vitro* degradation studies
- 3.4.3.2.5 Antimicrobial activity studies
- 3.4.4 Investigation of an influence of hydrophilic (polyethylene glycol 1500) and hydrophobic (peppermint oil) agents on physicochemical properties of the *in situ* forming gel systems
- 3.4.5 Stability study
- 3.4.6 Statistical analysis

3.1. Materials

Benzoyl peroxide (BP) (T.MAN Pharma Ltd., Part, Bangkok, Thailand)

Bleached shellac (BS) (Ake shellac Co., Ltd., Lumpang, Thailand)

Brain Heart Infusion (BHI) (lot no. 0270845, Bacto™, USA)

Brain Heart Infusion Agar (BHA) (lot no. 0298038, Bacto™, USA)

Ciprofloxacin hydrochloride (T.MAN Pharma Ltd., Part, Bangkok, Thailand)

Doxycycline hyclate (DH) (Batch No. 20071121, Huashu pharmaceutical corporation, Shijiazhuang, China)

Ethocel 10 (EC) (The Dow Chemical Company, USA)

Eudragit RS PO (ERS) (EVONIK Röhm GmbH, Germany)

Metronidazole (MT) (T.MAN Pharma Ltd., Part, Bangkok, Thailand)

Microtiter plate (96 well plates-U bottoms with lid, Corning Incorporated, USA)

Mitis Salivarius Agar (MSA) (lot no. 0118681, Difco™, USA)

N-methyl-2-pyrrolidone (NMP) (lot no. A0251390, Fluka, New Jersey, USA)

Peppermint oil (PO) (SR Lab Co., Bangkok, Thailand)

Polyethylene glycol 1500 (PEG1500) (lot no. 1289946, Fluka, Sigma-Aldrich Chemie GmbH, Switzerland)

Potassium dihydrogen orthophosphate (lot no. E23W60, Ajax Finechem, Australia)

Sabouraud Dextrose Agar (SDA) (lot no. 7312647, Difco™, USA)

Sabouraud Dextrose Broth (SDB) (lot no. 6345690, Difco™, USA)

Sodium hydroxide (lot no. AF 310204, Ajax Finechem, Australia)

Tryptic Soy Agar (TSA) (lot no. 7341698, Difco™, USA)

Tryptic Soy Broth (TSB) (lot no. 8091999, Difco™, USA)

Dialysis tube (Spectra / Por® membrane MWCO: 6,000 - 8,000, lot no. 32644, Spectrum Laboratories, Inc., CAL, USA)

3.2 Microbials

3.2.1 Standard microbes (Aerobic microbes)

Staphylococcus aureus ATCC 6853P

Escherichia coli ATCC 25922

Candida albicans ATCC 17110

3.2.2 Anaerobic microbes

Streptococcus mutans ATCC 27175

Porphyromonas gingivalis ATCC 33277

3.3 Equipments

Analytical balance (Sartorius model BP2100S and Sartorius model CP224S, Germany)

Anaerobic incubator (Forma Anaerobic System, Thermo Scientific, Ohio, USA)

Autoclave (Rexall model LS-2D, Rexall industries co., Ltd, Taiwan)

Brookfield viscometer DV-III ULTRA (Brookfield Engineering Laboratories.Inc., USA)

Freeze dryer (Triad™ Labconco, Missouri, USA)

Fusion Microplate Analyzer (A153601, Packard Bioscience Company, USA)

Hot air oven (Binder, Scientific promotion co.Ltd, Thailand)

pH meter (Ultra Basic UB-10, Denver Instrument, Bohemia, New York)

Scanning electron microscope (Maxim 200 Camscan, Cambridge, England)

Shaking incubator Model SI4 (Shel Lab, Cornelius, USA)

Texture analyzer (TA.XT plus, Charpa Techcenter, Godalming, Stable micro Systems Ltd., UK)

UV-vis spectrophotometer (Perkin-Elmer, Germany)

Water bath (Buchi Heating bath B-490, New Hampshire, USA)

3.4 Methods

3.4.1 Synergy effect studies of antimicrobial agents

The antimicrobial activity of the antimicrobial agents (doxycycline hyclate, ciprofloxacin hydrochloride, metronidazole and benzoyl peroxide) and their combination were investigated. The antimicrobial interactions between their combinations were evaluated using checkerboard method (as described by Hemaiswarya *et al.*, 2008; Lv *et al.*, 2011) against standard microbes (*Staphylococcus aureus* ATCC 6538P, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 17110) and anaerobic microbes (*Streptococcus mutans* ATCC 27175 and *Porphyromonas gingivalis* ATCC 33277).

3.4.1.1 Preparation of inoculums

Microbes were inoculated to media (Tryptic soy broth for *S. aureus* and *E. coli*, Sabouraud dextrose broth for *C. albicans* and Brain heart infusion broth for *S. mutans* and *P. gingivalis*) and incubated in an aerobic incubator (for standard microbes) or anaerobic incubator (for anaerobic microbes) at 37 °C, for 24-48 h. Then the culture was adjusted the turbidity by an optical density at 540 nm. The suspension was further diluted to provide a final inoculum density of 2×10^5 cfu/mL. The standard curves for each microbe were presented in Appendix C.

3.4.1.2 Minimal inhibitory concentration (MIC) determination

MICs of each antimicrobial agents, doxycycline hyclate, ciprofloxacin hydrochloride and metronidazole, against standard microbes and anaerobic microbes were determined by the broth microdilution method. MIC was defined as the lowest concentration of sample which inhibited the microbial growth.

3.4.1.3 Checkerboard test

Checkerboard test was performed in microtiter plate (96 well plates-U bottoms with lid, Corning Incorporated, USA). The microdilution method was the most popular technique used for *in vitro* antimicrobial combination assessment. Eight doubling dilutions of each antimicrobial agent were prepared and distributed into microtiter trays.

Microbial inoculums were prepared and added to all wells to give a final microbial count of approximately 1×10^5 cfu/mL. After incubation at 37 °C for 24 h, the antimicrobial activity of each combination was evaluated. The fractional inhibitory concentration index (FICI) was the sum of the FIC of the individual drugs which was calculated as the following formula:

$$\text{FICI} = \text{FIC}_A + \text{FIC}_B \quad [9]$$

FIC_A and FIC_B were the drug concentration in the well containing the lowest dual drug concentrations divided by the MIC of the respective drug A and B, respectively. The results were interpreted as follows: synergistic (<0.5), additive (0.5–1.0), indifferent ($>1 - \leq 4.0$) or antagonistic (>4.0) (Schelz *et al*, 2006).

3.4.2 Preparation and evaluation of the prepared injectable *in situ* forming gel base systems from various polymers

3.4.2.1 Preparation of the *in situ* forming gel base system

Injectable *in situ* forming gel base was prepared using various polymers such as Ethocel, bleached shellac and Eudragit RS. The different amount of polymer (5, 10, 15, 20, 25, 30, 35 and 40% w/w) was prepared. *N*-methyl pyrrolidone (NMP) was used as the solvent. Each of dispersion was kept for 24 hrs then a clear solution was formed. The formula containing different amounts of polymer in *N*-methyl-2-pyrrolidone are shown in Table 6.

Table 6 Composition formula of the gels containing different types and amounts of polymer in *N*-methyl-2-pyrrolidone.

Formula	Polymers (% w/w)			Solvent (% w/w)
	EC	BS	ERS	NMP
EC-1	5	-	-	95
EC-2	10	-	-	90
EC-3	15	-	-	85
EC-4	20	-	-	80
EC-5	25	-	-	75
EC-6	30	-	-	70
EC-7	35	-	-	65
EC-8	40	-	-	60
BS-1	-	5	-	95
BS-2	-	10	-	90
BS-3	-	15	-	85
BS-4	-	20	-	80
BS-5	-	25	-	75
BS-6	-	30	-	70
BS-7	-	35	-	65
BS-8	-	40	-	60
ERS-1	-	-	5	95
ERS-2	-	-	10	90
ERS-3	-	-	15	85
ERS-4	-	-	20	80
ERS-5	-	-	25	75
ERS-6	-	-	30	70
ERS-7	-	-	35	65
ERS-8	-	-	40	60

3.4.2.2 Evaluation of gel properties

3.4.2.2.1 Gel appearance and pH measurement

The appearances of formulations, color and homogeneity were observed by visual observation. The pH value of each formulation was measured using a pH meter (n=3).

3.4.2.2.2 Viscosity studies

The viscosity of the prepared gels was determined using Brookfield DV-III Ultra programmable rheometer (Brookfield Engineering Laboratories

Inc, Middleboro, MA, USA) with spindles (CP-40 and CP-52). Viscosity parameters were collected at different shear rate with 15 seconds equilibration time at every shear rate. The viscosity measurements were made at 25°C and 37°C which were room temperature and physiological temperature, respectively. It had been reported that the flow property correlated with the viscosity and the viscosity about 1×10^4 mPas was found to be adequate for a proper flow property (Sato *et al.*, 2012).

3.4.2.2.3 Rheological behavior studies

Rheological studies were performed by Brookfield programmable rheometer (Brookfield DV-III Ultra, Brookfield Engineering Laboratories, Middleboro, MA, USA) fitted with CP-40 and CP-52 spindles. The shear stress of the samples was measured at various shear rates at 25°C and 37°C. The temperature was maintained within $\pm 0.1^\circ\text{C}$ by water bath (Buchi Heating bath B-490, New Hampshire, USA) connected to the sample cup of rheometer. The samples were equilibrated on the plate to reach the running temperature prior to each measurement. The flow parameters were characterized using the exponential formula (Martin, 1993):

$$F^N = \eta G \quad [10]$$

$$\text{Log } G = N \text{Log } F - \text{Log } \eta \quad [11]$$

Where F is shear stress, G is shear rate, N is an exponential constant and η is a viscosity coefficient.

3.4.2.2.4 Syringeability test

Syringeability of the gel systems is an important factor to consider for the ease of administration by injection. This is the force required to expel the prepared product via a needle. Syringeability of each sample was evaluated using texture analyzer (TA.XT plus, Stable Micro Systems, UK) in compression mode. The sample was filled into 1 mL syringe with 18-gauge needle that was clamped with stand. The 18-gauge needle was widely used in the dental field (Sato *et al.*, 2012). The upper probe of the texture analyzer moved downwards at constant speed (1.0 mm s^{-1}) until it came in contact with the syringe barrel base. A constant force of 0.1 N was applied to the base and

the distance required to expel the contents for a barrel length of 20 mm was measured at room temperature (n=3). Force displacement profiles were performed, which the force at distance of 10 mm were selected for analysis. The area under the resulting curve was used to determine the work of expulsion (Kelly *et al.*, 2004).

3.4.2.2.5 *In vitro* gel formation

In order to investigate the injectability and gel formation of the polymer solutions, samples (1 mL) were injected into phosphate buffer solution pH 6.8 in test tube (5 mL) with 18-gauge needle. Then the gel formation was observed visually at various times (0, 1, 5 and 30 min) (Le Renard *et al.*, 2010).

3.4.2.2.6 Rate of water diffusion into the gels

The water diffusion into the gels was studied using the observation of the system change in transparent tube immersed in phosphate buffer solution pH 6.8 (15 mL) in test tube. Samples were filled in transparent tube (diameter 6 mm). Then the transparent tube was placed into phosphate buffer solution pH 6.8. When the water diffused into the gels, the apparent system was changed from transparent to opaque. The distance of water front diffusion was observed at various times (0, 4 and 24 hours). The rate of water diffusion into the gels was calculated as the following formula:

Rate of water diffusion into the gels

$$= \frac{\text{distance of water front diffusion (mm.)}}{\text{time (min)}} \quad [12]$$

3.4.3 Preparation and evaluation of the *in situ* forming gel systems containing antimicrobial agents

3.4.3.1 Preparation of the *in situ* forming gel systems containing antimicrobial agents

The antimicrobial agents (doxycycline hyclate, metronidazole and benzoyl peroxide) were incorporated into the prepared gels. Each dispersion was kept for 24 hrs then a clear solution was formed. The formula containing different types and amounts of polymers and drugs in *N*-methyl-2-pyrrolidone are shown in Table 7.

3.4.3.2 Evaluation of gel properties

The prepared gels were evaluated with the methods as described in 3.4.2.2.1 - 3.4.2.2.6.

3.4.3.2.1 *In vitro* drug release studies

In vitro drug release studies were evaluated using dialysis membrane method and membrane-less diffusion method as following.

3.4.3.2.1.1 Dialysis membrane method

A dialysis tube (Spectrapor, MW cutoff: 6,000-8,000) containing 1 g gel formulation was immersed in 100 mL of phosphate buffer pH 6.8 (to simulate the gingival crevicular fluid) (Esposito *et al.*, 1996) at 37 °C and maintained the rotational speed at 50 rpm. Aliquots, each of 10 mL, were withdrawn from the release medium at time intervals of 5, 15, 30, 45, 60, 90, 120, 180, 240, 360, 480, 720, 960, 1200, 1440, 1680, 1920, 2160 and 2880 min and each aliquot was replaced with 10 mL of fresh medium. The amount of samples was determined by UV-vis spectrophotometer. All of the experiments were triplicately done, and the mean cumulative drug release \pm S.D. were calculated.

Table 7 Composition formula of various gel systems containing different types of drugs (5% w/w).

Formula	Polymers (% w/w)			Solvent (% w/w)	Drugs (% w/w)		
	EC	BS	ERS	NMP	DH	MT	BP
EC-9	5	-	-	90	5	-	-
EC-10	10	-	-	85	5	-	-
EC-11	15	-	-	80	5	-	-
EC-12	20	-	-	75	5	-	-
EC-13	5	-	-	90	-	5	-
EC-14	10	-	-	85	-	5	-
EC-15	15	-	-	80	-	5	-
EC-16	20	-	-	75	-	5	-
EC-17	5	-	-	90	-	-	5
EC-18	10	-	-	85	-	-	5
EC-19	15	-	-	80	-	-	5
EC-20	20	-	-	75	-	-	5
BS-9	-	15	-	85	5	-	-
BS-10	-	20	-	75	5	-	-
BS-11	-	25	-	70	5	-	-
BS-12	-	30	-	65	5	-	-
BS-13	-	15	-	85	-	5	-
BS-14	-	20	-	75	-	5	-
BS-15	-	25	-	70	-	5	-
BS-16	-	30	-	65	-	5	-
BS-17	-	15	-	85	-	-	5
BS-18	-	20	-	75	-	-	5
BS-19	-	25	-	70	-	-	5
BS-20	-	30	-	65	-	-	5
ERS-9	-	-	15	70	5	-	-
ERS-10	-	-	25	65	5	-	-
ERS-11	-	-	30	60	5	-	-
ERS-12	-	-	35	55	5	-	-
ERS-13	-	-	15	70	-	5	-
ERS-14	-	-	25	65	-	5	-
ERS-15	-	-	30	60	-	5	-
ERS-16	-	-	35	55	-	5	-
ERS-17	-	-	15	70	-	-	5
ERS-18	-	-	25	65	-	-	5
ERS-19	-	-	30	60	-	-	5
ERS-20	-	-	35	55	-	-	5

3.4.3.2.1.2 Membrane-less diffusion method

A membrane-less diffusion system was used for studying drug release from *in situ* gel systems. Sample (about 0.4 g) was added into the cup (10 mm x 12 mm) and then placed in 100 mL of phosphate buffer solution pH 6.8 at 37°C and maintained the speed of rotation at 50 rpm. Aliquots, each of 10 mL, were withdrawn from the release medium at time intervals of 5, 15, 30, 45, 60, 90, 120, 180, 240, 360, 480, 720, 960, 1200, 1440, 1680, 1920, 2160 and 2880 min and each aliquot was replaced by 10 mL of fresh medium. The amount of samples was determined by UV-vis spectrophotometer. All of the experiments were triplicately done, and the mean cumulative drug release \pm S.D. were calculated

3.4.3.2.1.3 Calibration curve

Calibration curve of doxycycline hyclate in phosphate buffer pH 6.8

Doxycycline hyclate of 7.5 mg was accurately weighed, dissolved in 25 mL distilled water and adjusted the volume to 50 mL with distilled water. This solution was used as a standard stock solution A. Stock solution A (5 mL) was pipetted and adjusted with phosphate buffer pH 6.8 to volume 50 mL as stock solution B. The 0.80, 1.7, 2.5, 4.2 and 5.8 mL stock solution B was pipetted and adjusted with phosphate buffer pH 6.8 to volume in 25 mL volumetric flask to make approximately 0.005 - 0.035 mg/mL concentration of doxycycline hyclate. The relationship between concentration and absorbance was determined using UV-vis spectrophotometer at 349 nm. The calibration curve of doxycycline hyclate in phosphate buffer pH 6.8 was exhibited in Appendix A (Figure 93).

Calibration curve of metronidazole in phosphate buffer pH 6.8

Metronidazole of 7.5 mg was accurately weighed, dissolved in 25 mL absolute ethanol and adjusted the volume to 50 mL with absolute ethanol. This solution was used as a standard stock solution A. Stock solution A (5 mL) was pipetted and adjusted with phosphate buffer pH 6.8 to volume 50 mL as stock solution B. The 0.05, 0.10, 0.15, 0.25 and 0.3 mL stock solution B was pipetted and adjusted with phosphate buffer pH 6.8 to volume in 25 mL volumetric flask to make approximately 0.0003 - 0.0018 mg/mL

concentration of metronidazole. The relationship between concentration and absorbance was determined using UV-vis spectrophotometer at 320 nm. The calibration curve of metronidazole in phosphate buffer pH 6.8 was exhibited in Appendix A (Figure 94).

Calibration curve of benzoyl peroxide in phosphate buffer pH 6.8

Benzoyl peroxide of 7.5 mg was accurately weighed, dissolved in 25 mL absolute ethanol and adjusted the volume to 50 mL with absolute ethanol. This solution was used as a standard stock solution A. Stock solution A (5 mL) was pipetted and adjusted with phosphate buffer pH 6.8: ethanol (5:2) to volume 50 mL as stock solution B. The 0.05, 0.10, 0.15, 0.25 and 0.3 mL stock solution B was pipetted and adjusted with phosphate buffer pH 6.8: ethanol (5:2) to volume in 25 mL volumetric flask to make approximately 0.0003 - 0.0018 mg/mL concentration of benzoyl peroxide. The relationship between concentration and absorbance was determined using UV-vis spectrophotometer at 275 nm. The calibration curve of benzoyl peroxide in phosphate buffer pH 6.8: ethanol (5:2) was exhibited in Appendix A (Figure 95).

3.4.3.2.2 Analysis of drug release data

The data obtained from the *in vitro* release experiments were analyzed by a nonlinear computer programme, Scientist[®] for Windows, version 2.1 (MicroMath Scientific Software, SaltLake City, UT, USA). The cumulative percentage of drug release profiles were fitted with different mathematical release equations. Least square fitting the experimental dissolution data (cumulative drug release > 10% and up to 80%) to the mathematical equations (power law, zero order, first order and Higuchi's) was carried out. The coefficient of determination (r^2) was used to indicate the degree of curve fitting. Model files used in this study are shown in Table 5.

3.4.3.2.3 Determination of surface morphology of gels

Samples were determined after the release studies under conditions identical to those described above in 3.3.2.1.1 and then they were dried using the freeze dryer for 48 hours in order to avoid the collapse of porous structures. Samples were coated with gold prior to examine by scanning electron microscope (SEM). The surface and cross-sectional morphology of the dried samples were determined.

Micrographs were taken with a scanning electron microscope at an accelerating voltage of 15 kV. The morphology of samples was observed as the porosity of structures, surface structure and drug crystalline.

3.4.3.2.4 *In vitro* degradation studies

Degradation studies of the prepared gels were studied by incubating in phosphate buffer solution pH 6.8. The sample was injected into phosphate buffer pH 6.8 (10 mL). Each sample was incubated in shaking bath at 37 °C with 50 rpm. Fresh phosphate buffer solution was replaced every week for 1 month. Then the sample was dried in hot air oven at 65 °C. The percentage of weight loss was carried out following:

$$\% \text{ Weight loss} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100 \quad [13]$$

3.4.3.2.5 Antimicrobial activity studies

Antimicrobial activities of the samples were evaluated. Both the standard microbes (*Staphylococcus aureus* ATCC 6538P, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 17110) and anaerobic microbes (*Streptococcus mutans* ATCC 27175 and *Porphyromonas gingivalis* ATCC 33277) were used in this study. Antimicrobial activities of prepared systems were determined using agar-cup diffusion method (Ji *et al.*, 2010). The actively growing broth culture of microbes were prepared, the turbidity was approximately 10^8 cells/mL. Then, the swab spread onto the agar plate and dried. The sterilized cylinder cups were carefully placed on the surface of the swabbed agar. The prepared gels were filled into the cylinder cup (8 mm diameter and 10 mm height) and incubated at 37°C for 24-48 h. The antimicrobial activities were measured as the diameter (mm) of inhibition zone. The tests were carried in triplicate and the mean of inhibition zone \pm S.D. were calculated.

3.4.4 Investigation of an influence of hydrophilic (polyethylene glycol 1500) and hydrophobic (peppermint oil) agents on physicochemical properties of the *in situ* forming gel systems

The hydrophilic (polyethylene glycol 1500) and hydrophobic (peppermint oil) agents were added into the selected systems by varying their amount in the ranges of 0-10 %w/w concentration. The prepared gels were evaluated as the method mentioned previously.

Table 8 Composition formula of Eudragit RS systems containing different amounts of plasticizers (polyethylene glycol 1500 and peppermint oil) without and with doxycycline hyclate (5% w/w).

Formula	Polymers (%w/w)		Solvent (%w/w)		Drug (%w/w)		Plasticizers (%w/w)	
	ERS	NMP	DH	PEG1500	PO			
ERS-21	35	62.5	-	2.5	-			
ERS-22	35	60	-	5	-			
ERS-23	35	57.5	-	7.5	-			
ERS-24	35	55	-	10	-			
ERS-25	35	62.5	-	-	2.5			
ERS-26	35	60	-	-	5			
ERS-27	35	57.5	-	-	7.5			
ERS-28	35	55	-	-	10			
ERS-29	35	57.5	5	2.5	-			
ERS-30	35	55	5	5	-			
ERS-31	35	52.5	5	7.5	-			
ERS-32	35	50	5	10	-			
ERS-33	35	57.5	5	-	2.5			
ERS-34	35	55	5	-	5			
ERS-35	35	52.5	5	-	7.5			
ERS-36	35	50	5	-	10			

3.4.5 Stability study

Selected formulation was stored at 4 ± 1 °C, room temperature (25 ± 1 °C) and 45 ± 1 °C for period of 3 months (Wu *et al.*, 2011; Mathews, 1999). The formulations were evaluated including the physicochemical properties, drug release and antimicrobial activities.

3.4.6 Statistical analysis

All experimental measurements were collected in triplicate. Values were expressed as mean \pm standard deviation (S.D.). Statistical significance of the drug release studies was examined using one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) post hoc test or Duncan. The significance level was set at $p < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Synergistic effect of antimicrobial agents

4.1.1 Minimal inhibitory concentration (MIC) determination and Checkerboard test

The efficacy of doxycycline hyclate, ciprofloxacin hydrochloride, metronidazole and their combinations against aerobic microbes (*S. aureus* ATCC 6538P, *E. coli* ATCC 25922 and *C. albicans* ATCC 17110) and anaerobic microbes (*S. mutans* ATCC 27175 and *P. gingivalis* ATCC 33277) were evaluated by MICs and FIC index. The MIC results of each sample against *S. aureus* ATCC 6538P, *E. coli* ATCC 25922, *C. albicans* ATCC 17110, *S. mutans* ATCC 27175 and *P. gingivalis* ATCC 33277 obtained with the broth microdilution method are summarized in Table 9. The MIC values of the samples were determined as the reference point for defining the interactions. It has been reported that MICs range of doxycycline, ciprofloxacin and metronidazole against *P. gingivalis* were ≤ 0.125 -1 mg/L, 0.25-1 mg/L and ≤ 0.25 -0.5 mg/L, respectively (Eick *et al.*, 1999). The antimicrobial agents (doxycycline hyclate, ciprofloxacin HCl and metronidazole) and their combinations against aerobic and anaerobic microbes were evaluated by the checkerboard method. The results of antimicrobial combinations are defined. Synergism is observed when the effect of the combined drugs is greater than the sum of the individual effects. Additivity is observed that the result with two drugs is equal to the sum of the results of each drugs used separately. Whereas, the indifference shows the result of two drugs do not significantly differ from that of the most effective drug alone. Antagonism is recorded when the effect of two drugs is significantly less than the additive response (Pillai *et al.*, 2005). In this investigation the synergistic results were shown in Table 10. Doxycycline hyclate-ciprofloxacin HCl combination was indifferent against all aerobic microbes (*S. aureus* ATCC 6538P, *E. coli* ATCC 25922 and *C. albicans* ATCC 17110) and only anaerobic microbe (*S. mutans* ATCC 27175) (FIC index >1 , ≤ 4.0) whereas, only doxycycline hyclate-ciprofloxacin HCl combination had additive effects

against *P. gingivalis* ATCC 33277 (FIC index 0.5-1.0) as shown in Table 10. For the additive effect (FIC index 0.5-1.0), the combination between their antimicrobial agents decrease the amount of antimicrobial agents and minimized the side effect regarding the used of high dose antimicrobial agents.

Doxycycline hyclate-metronidazole and ciprofloxacin HCl-metronidazole combinations were additive against *S. aureus* and *S. mutans* and their combinations were indifferent against *E. coli* and *C. albicans*. Doxycycline hyclate-metronidazole combination was synergistic against *P. gingivalis* (FIC index < 0.5) whereas, ciprofloxacin HCl-metronidazole combination was indifferent against *P. gingivalis*. Doxycycline inhibits 30S ribosome (Edwards, 1993) that occurs in the early stage of the protein synthesis pathway and ciprofloxacin inhibits DNA gyrase (Ng *et al.*, 1996) that function in the later stage of the pathway. Therefore, if doxycycline was completely effective in the early stage, the later stage of ciprofloxacin could not promote the function. Doxycycline is used widely in periodontal treatment. It is bacteriostatic antibiotics and also inhibits tissue collagenase activity (Yu *et al.*, 1993; Levy, 1984; Seymour *et al.*, 1995). The doxycycline activity related to the drug's ability to bind with chelating divalent cations like magnesium (Mg^{++}), which are attached to the phosphates on RNA (Seymour *et al.*, 1995). It can reduce tissue destruction and bone resorption (Sapadin *et al.*, 2006; Preshaw *et al.*, 2004). Metronidazole is nitroimidazole antimicrobials that inhibits DNA replication of anaerobic bacteria by the nitro group of metronidazole and reduced an electron transport protein in anaerobic bacteria. The reduced metronidazole causes strand breaks in the DNA (Edwards, 1993). However, the combination of metronidazole and ciprofloxacin (500 mg each BID for 5-7 days) was a drug of choice in the systemic antibiotics in periodontal therapy (Serio and Hawley, 2002).

Doxycycline hyclate and metronidazole showed the synergistic activity against *P. gingivalis* ATCC 33277. The results indicated that the antimicrobial activity of doxycycline hyclate-metronidazole combination against *P. gingivalis* ATCC 33277 was more active than that of metronidazole or doxycycline hyclate alone. Therefore the combination of doxycycline hyclate and metronidazole was the most interesting alternative for periodontal treatment. However, the antimicrobial combinations showed the additive activity against microbes could reduce the amount of each drug

that reduced side effect or toxicity of drug. The antimicrobial combination could reduce the drug resistant of microbes (Eliopoulos and Moellering, 1996).

Table 9 Minimal inhibitory concentrations (MICs) of antimicrobial agents against *S. aureus* ATCC 6538P, *E. coli* ATCC 25922, *C. albicans* ATCC 17110, *S. mutans* ATCC 27175 and *P. gingivalis* ATCC 33277 determined by broth microdilution method

Drugs	MIC				
	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>	<i>S. mutans</i>	<i>P. gingivalis</i>
Doxycycline hyclate	0.08 µg/mL	0.20 µg/mL	2.50 µg/mL	0.21 µg/mL	1.88 µg/mL
Ciprofloxacin HCl	0.25 µg/mL	0.27 µg/mL	1.06 µg/mL	0.97 µg/mL	0.31 µg/mL
Metronidazole	1.60 mg/mL	5.69 mg/mL	11.38 mg/mL	3.20 mg/mL	0.80 mg/mL

Table 10 Fractional inhibitory concentration (FIC) index values of the combinations

Combined drugs	FIC in the combination		FICI	Interpretation
	FIC _A	FIC _B		
<i>S. aureus</i>				
D + C	0.67	0.40	1.07	ID
D + M	0.40	0.40	0.80	AD
C + M	0.50	0.33	0.83	AD
<i>E. coli</i>				
D + C	0.40	0.75	1.15	ID
D + M	0.50	0.83	1.33	ID
C + M	1.00	0.25	1.25	ID
<i>C. albicans</i>				
D + C	1.00	0.25	1.25	ID
D + M	1.00	0.20	1.20	ID
C + M	0.83	0.20	1.03	ID
<i>S. mutans</i>				
D + C	0.50	0.63	1.13	ID
D + M	0.30	0.20	0.50	AD
C + M	0.10	0.75	0.85	AD
<i>P. gingivalis</i>				
D + C	0.10	0.40	0.50	AD
D + M	0.10	0.30	0.40	S
C + M	0.30	0.75	1.05	ID

D: Doxycycline hyclate; C: Ciprofloxacin; M: Metronidazole; S: Synergy; AD: Additive; ID: Indifference; AD: Antagonism.

4.2 Evaluation of gel base properties

4.2.1 Gel appearance and pH measurement

The preparation of *in situ* forming system is not sophisticated while is, reliable and reproducible. Different *in situ* forming formula were prepared with various polymers including Ethocel, bleached shellac and Eudragit RS (Table 6). The appearances of Ethocel, bleached shellac and Eudragit RS gel were light yellow, dark yellow and colorless, respectively. All formula were clear. The pH of formulations containing different amounts of Ethocel, bleached shellac and Eudragit RS are shown in Table 11. The pH of Ethocel (5-20%w/w), bleached shellac (15-30%w/w) and Eudragit RS (15-35%w/w) formula were in the range of 9.57 ± 0.06 to 10.58 ± 0.04 , 6.65 ± 0.02 to 7.22 ± 0.01 and 8.69 ± 0.09 to 9.07 ± 0.02 , respectively. The pH of each formula slightly decreased when the amount of polymer increased. *N*-methyl-2-pyrrolidone (NMP) is a strong solubilizing agent and biodegradable, which was used as solvent and pH of NMP is 8.0 to 9.5 (Jouyban *et al.*, 2010). Therefore it suggested that the pH value of formula was influenced by an addition of polymer. The resting pH of the oral cavity is between 5 and 9 (Galgut, 2001). The pH range within gingival pockets is 7.0-8.5, whereas the pH in the periodontal pocket was about 6.92 ± 0.03 (Eggert, 1991). However the pH in the periodontal pocket depends on the depth of periodontal pockets and the host inflammatory response is induced (Gibson *et al.*, 2006).

Table 11 Gel appearance and pH value of various formula (Ethocel, bleached shellac and Eudragit RS)

Formula (%w/w)	Appearance	Clarity	pH \pm S.D. (n=3)
EC			
5%	Light yellow	Clear	10.58 ± 0.04
10%	Light yellow	Clear	9.24 ± 0.02
15%	Light yellow	Clear	9.19 ± 0.02
20%	Light yellow	Clear	9.57 ± 0.06
BS			
15%	Dark yellow	Clear	7.22 ± 0.01
20%	Dark yellow	Clear	7.02 ± 0.03
25%	Dark yellow	Clear	6.81 ± 0.02
30%	Dark yellow	Clear	6.65 ± 0.02
ERS			
15%	Colorless	Clear	9.07 ± 0.02
25%	Colorless	Clear	8.87 ± 0.03
30%	Colorless	Clear	8.76 ± 0.02
35%	Colorless	Clear	8.69 ± 0.09

4.2.2 Viscosity studies

The effects of type and amount of polymers loading and temperature on the viscosity were investigated. The relationships between shear rate and apparent viscosity of the Ethocel, bleached shellac and Eudragit RS formula are shown in Figures 13-15. The apparent viscosity of formulation increased as the polymer concentration was increased. The apparent viscosities of the Ethocel (5-15%w/w), bleached shellac (15-25%w/w) and Eudragit RS (15-35%w/w) formulations were constant when the shear rate was increased indicating Newtonian behavior, which the viscosity unchanged as the shear rate was increased (Zaki *et al.*, 2007). However, the apparent viscosity at a low shear rate was higher than that at the higher shear rate. The higher amount of Ethocel (20%w/w) and bleached shellac (30%w/w) were used indicating pseudoplastic behavior, which the N values from Equation 11 could be used to indicate these flow behaviors. The apparent viscosities of all formula at 37°C were lower than that of formula at 25°C. All formula exhibited a decrease in viscosity with increasing temperature. A decrease in the viscosity with increasing temperature was found to be reflected by a decrease in the number of interactions. It was also found that the higher amount of Ethocel ($\geq 25\%$ w/w), bleached shellac ($\geq 35\%$ w/w) and Eudragit RS ($\geq 40\%$ w/w) in the formulations, the *in situ* gel formulations could not flow, since these formulations exhibited a higher viscosity. The viscosity of gel commercial product for periodontal pocket drug delivery was approximately 1×10^4 mPas (Sato *et al.*, 2012). The viscosity of *in situ* gel systems was important factor for periodontal poket drug delivery systems (Gupta *et al.*, 2008). Thus, the systems with flowable properties were selected for the further studies.

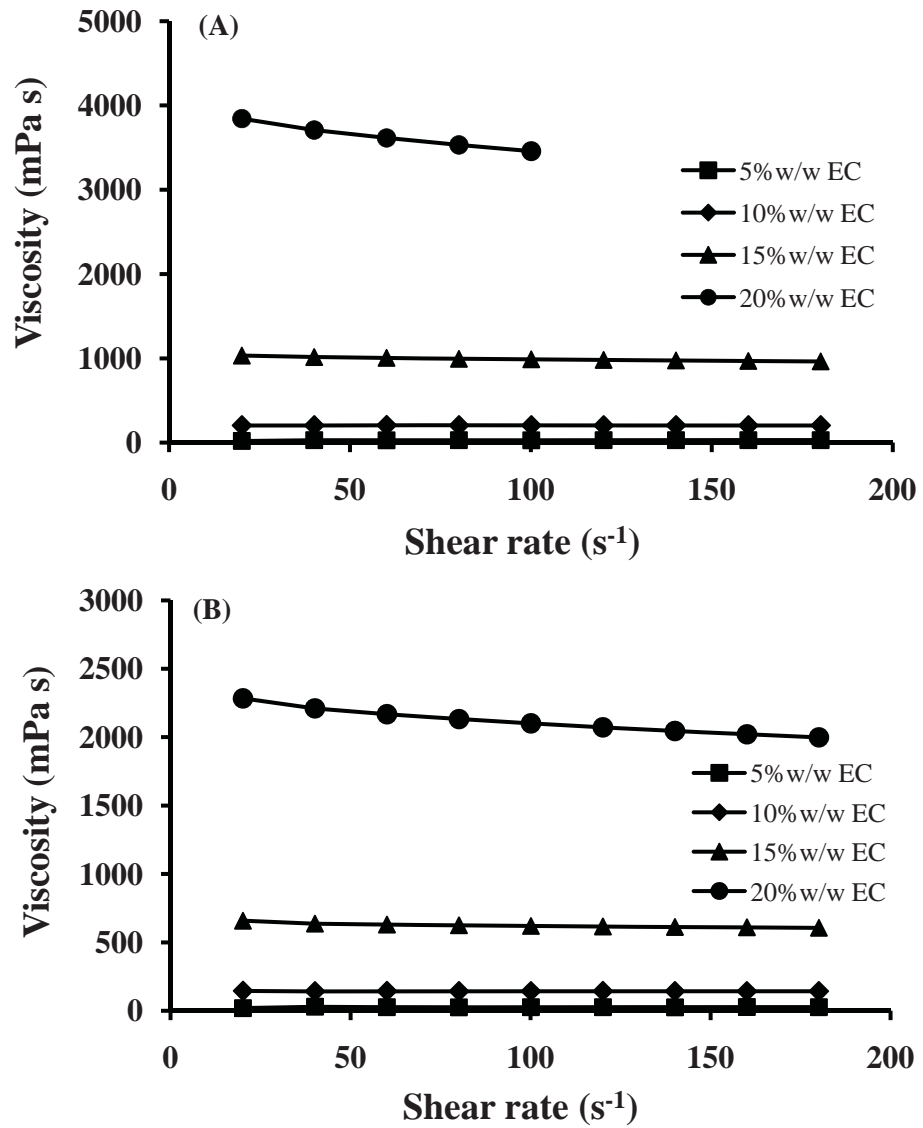


Figure 13 Viscosity curves of Ethocel formula at (A) 25°C and (B) 37°C

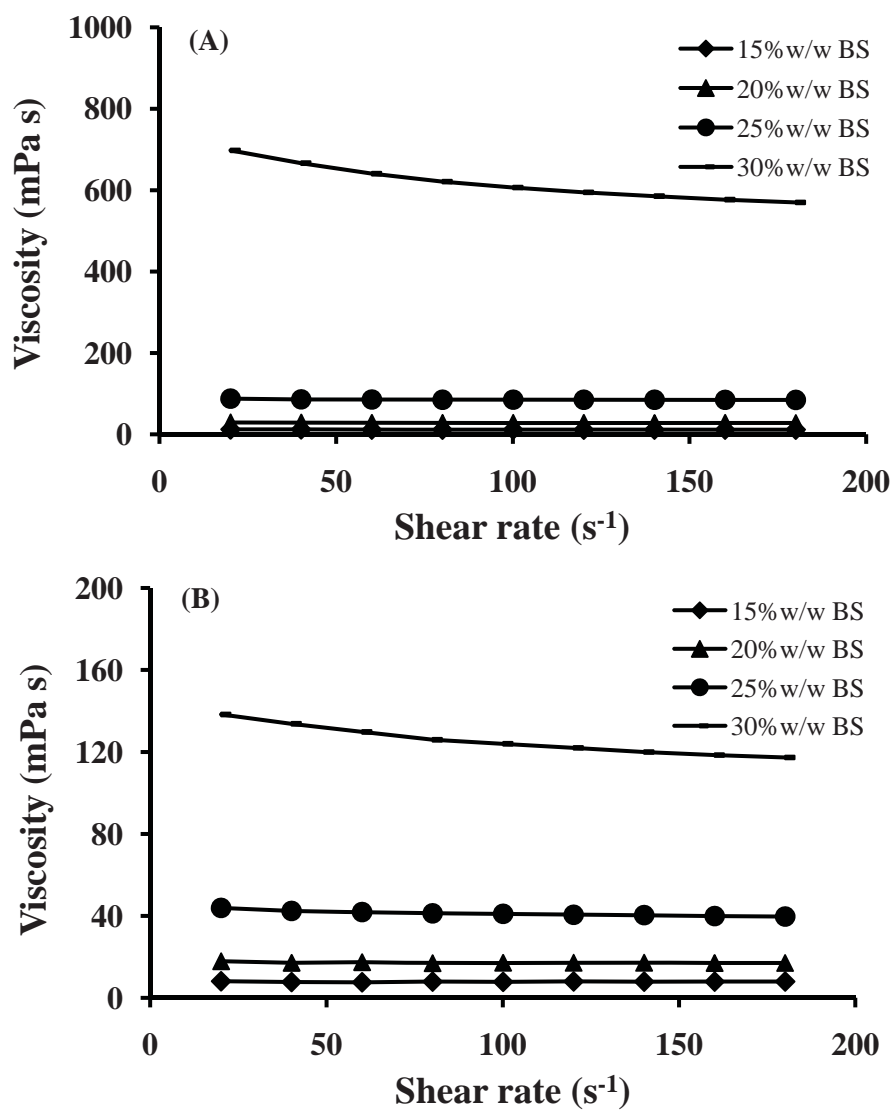


Figure 14 Viscosity curves of bleached shellac formula at (A) 25°C and (B) 37°C

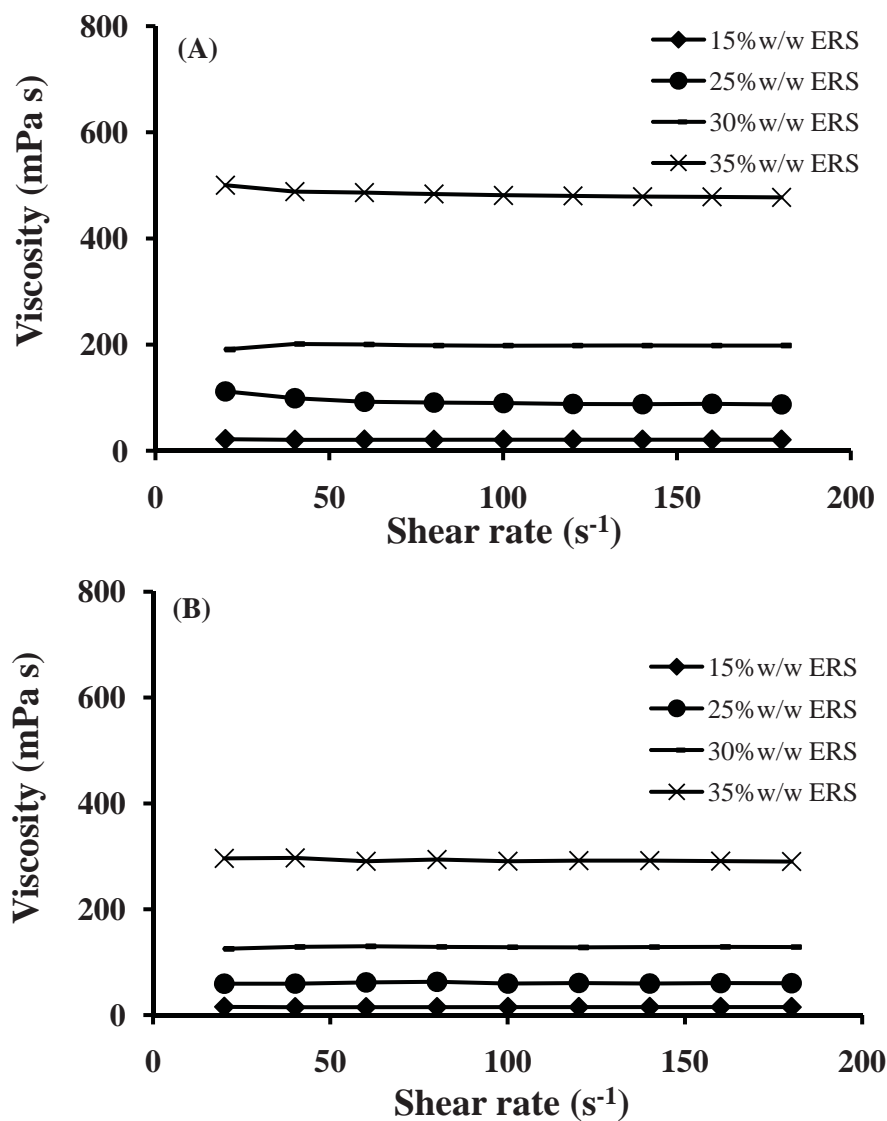


Figure 15 Viscosity curves of Eudragit RS formula at (A) 25°C and (B) 37°C

4.2.3 Rheological behavior studies

The rheological behaviors of various polymer solutions were investigated as a function of amount and temperature. The shear stress versus shear rate flow curves of Ethocel, bleached shellac and Eudragit RS at different temperatures are shown in Figures 16, 17 and 18, respectively. The shear stress of all formula increased as the amount of Ethocel, bleached shellac and Eudragit RS increased. All formula showed Newtonian behavior, indicating the up curve did coincide with the down curve. The curves moved to a higher shear stress value indicating compact structure of the gels (Priyanka and Meenakshi, 2011). However, the shear stress of all formula decreased with an increasing temperature. The curves moved to a lower shear stress value indicating loose structure.

The flow parameters of Ethocel, bleached shellac and Eudragit RS formula are shown in Tables 12. N value of all formulations was close to 1, indicating that the flow type was Newtonian. Temperature did not change the flow behavior of the systems. In the case of the viscosity coefficient (or consistency index, η), the polymer concentration of formula was higher, the viscosity coefficient was also significantly greater ($p < 0.05$). In contrast, the viscosity coefficient significantly decreased when the temperature was increased ($p < 0.05$). These results indicated Newtonian behavior of all formula, which were confirmed by the N values from Equation 11. The previous results showed that Ethocel (20%w/w) and bleached shellac (30%w/w) exhibited pseudoplastic behavior but N values were Newtonian characteristics. It is known that the structure of the gel is destroyed when the shear speed is increased (Ueda *et al.*, 2004). Rheological studies suggested that possession of a gel structure could be an important determinant of retention where shear displacing forces are present *in vivo*. e.g. the oral mucosa. Furthermore, these studies indicated that formulations which could demonstrate resistance to changes in rheological properties on hydration would also favour retention *in situ* (Needleman *et al.*, 1998). Typically, the optimal rheological behavior of the *in situ* forming gel should be a Newtonian or pseudoplastic flow due to the ease of injection. Thus the rheological results were used to understand the performance of *in situ* gels.

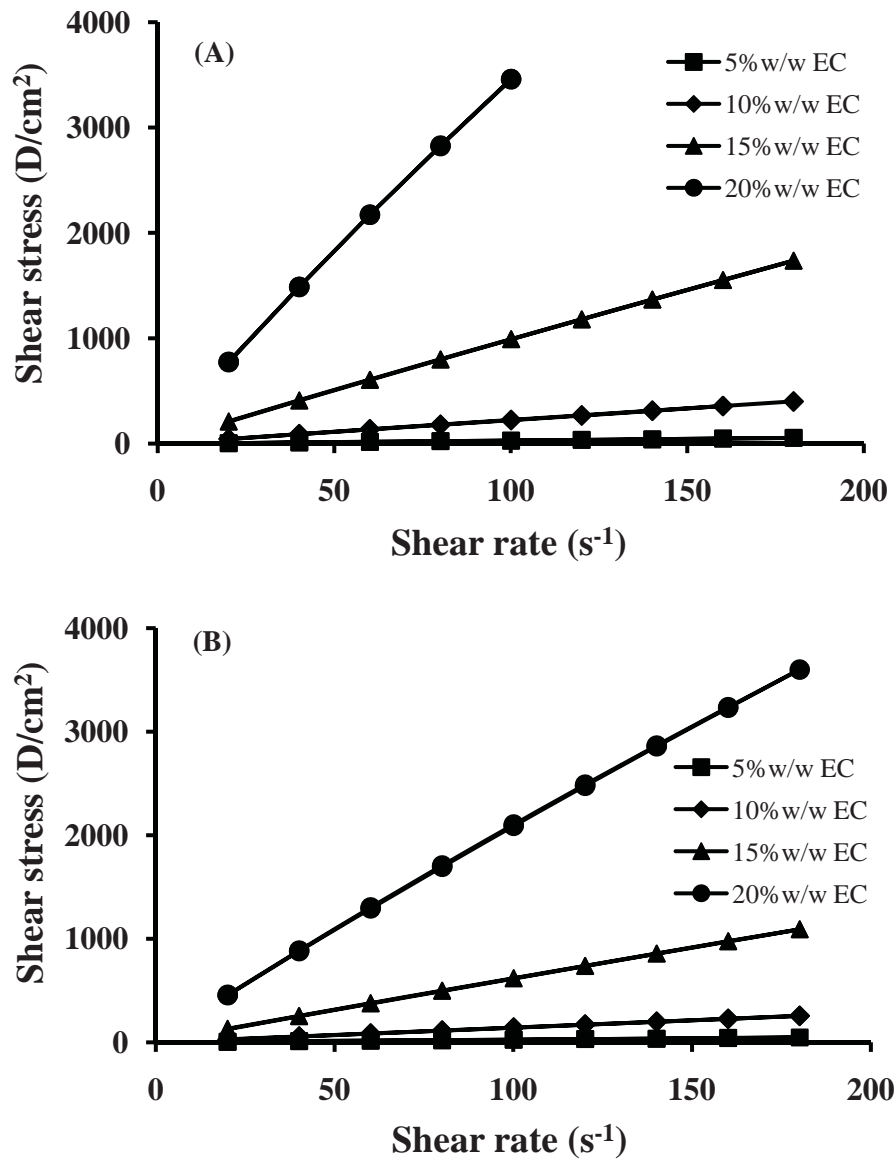


Figure 16 Flow curve of Ethocel formula containing different amount of Ethocel at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

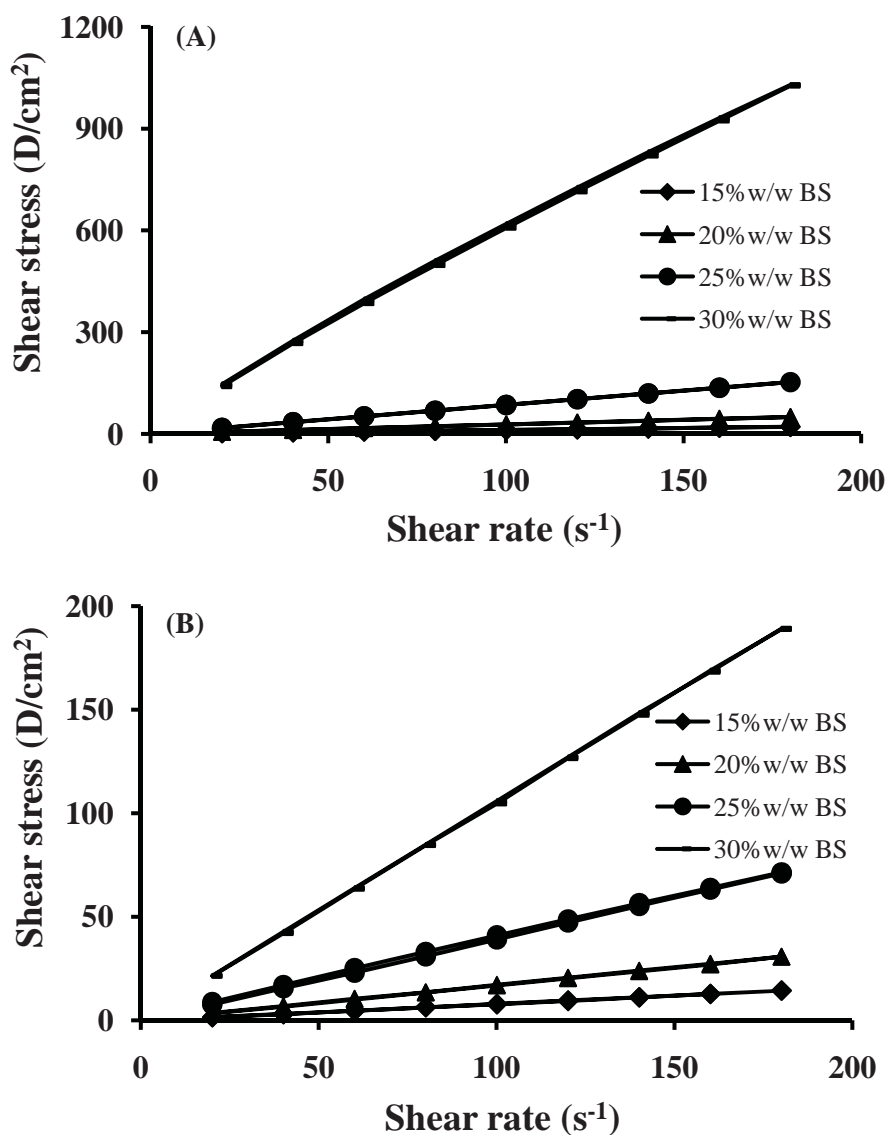


Figure 17 Flow curve of bleached shellac formula containing different amount of bleached shellac at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

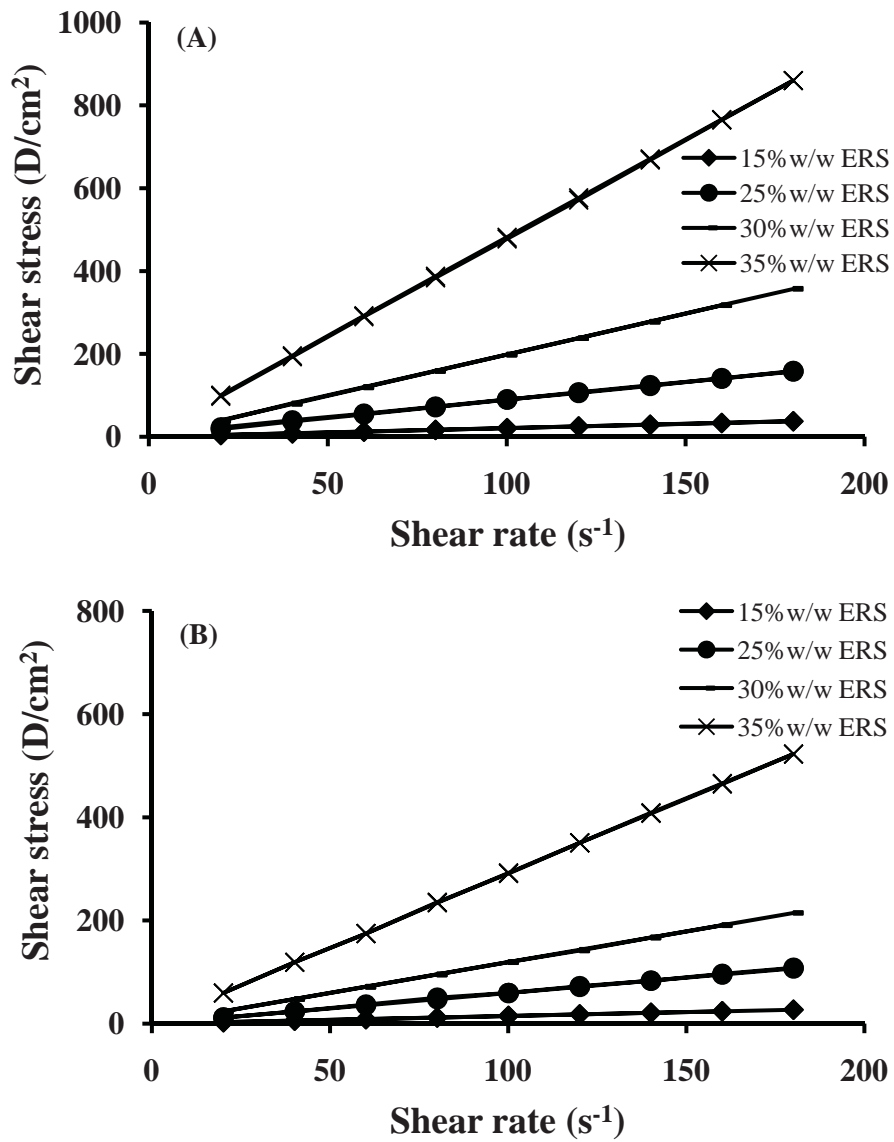


Figure 18 Flow curve of Eudragit RS formula containing different amount of Eudragit RS at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

Table 12 Effect of polymers concentrations and temperature on flow parameters (n=3)

Concentration of polymer (% w/w)	25°C		37°C	
	Flow index (N) (mean ± S.D.)	Consistency index (η) (mean ± S.D., [D/cm ²] ^N s)	Flow index (N) (mean ± S.D.)	Consistency index (η) (mean ± S.D., [D/cm ²] ^N s)
EC				
5%	1.02 ± 0.02	68.13 ± 5.79	0.99 ± 0.02	30.50 ± 3.03
10%	1.00 ± 0.01	221.77 ± 8.61	1.01 ± 0.03	133.07 ± 15.97
15%	0.96 ± 0.01	1164.00 ± 13.61	0.97 ± 0.00	700.57 ± 9.27
20%	0.95 ± 0.03	4464.67 ± 503.89	0.94 ± 0.04	2732.33 ± 69.72
BS				
15%	0.95 ± 0.02	14.63 ± 1.24	1.03 ± 0.03	7.07 ± 1.05
20%	0.98 ± 0.00	31.00 ± 0.44	0.99 ± 0.01	17.50 ± 1.25
25%	0.98 ± 0.01	75.30 ± 20.12	1.01 ± 0.02	37.80 ± 1.11
30%	0.95 ± 0.01	845.80 ± 337.68	0.99 ± 0.00	122.30 ± 21.24
ERS				
15%	0.98 ± 0.00	23.03 ± 0.31	1.00 ± 0.01	14.83 ± 1.04
25%	0.96 ± 0.03	167.10 ± 38.82	1.00 ± 0.01	59.00 ± 0.26
30%	1.01 ± 0.01	193.00 ± 10.02	1.00 ± 0.03	128.93 ± 15.74
35%	0.98 ± 0.01	514.20 ± 15.32	0.99 ± 0.00	305.60 ± 1.93

4.2.4 Syringeability

The syringeability of Ethocel, bleached shellac and Eudragit RS formulations are shown in Tables 13. The work of syringeability is important factor for the product delivery from a syringe through a needle. This study showed the ease for application of products. The work of syringeability of all formulations significantly increased with the increasing concentration of each polymer (Ethocel, bleached shellac and Eudragit RS) ($p < 0.05$). At the same concentration (15% w/w), the work of syringeability of EC was more than that of BS and ERS, respectively. The results indicated that the syringeability of systems was decreased with increasing the polymer concentration due to the increasing of viscosity. The result agreed with the trends of the viscosity and rheological studies. Generally the injection force of in situ gel was approximately 50 N or below (via a needle 20-21 guage) (Rungseevijitprapa and Bodmeier, 2009).

Table 13 Effect of the concentration of polymers (Ethocel, bleached shellac and Eudragit RS) on the work of syringeability (n=3).

Formula (%w/w)	Work of syringeability (N.mm)
EC	
5%	21.73 ± 0.99
10%	23.26 ± 2.11
15%	32.64 ± 0.82
20%	41.98 ± 2.47
BS	
15%	11.73 ± 0.88
20%	12.22 ± 0.83
25%	15.72 ± 2.05
30%	27.69 ± 1.58
ERS	
15%	8.04 ± 0.79
25%	13.19 ± 0.62
30%	16.70 ± 1.37
35%	23.99 ± 2.48

4.2.5 *In vitro* gel formation

For ease of administration and handling, the preparation should typically remain as liquid formulation but on injection undergoes gelation due to the solvent exchange of NMP and gingival fluid (Malik *et al.*, 2010). When the solvent exchange was occurred, the gel would be changed as an opaque formed. The *in vitro* gel formation of Ethocel, bleached shellac and Eudragit RS formulations in phosphate buffer pH 6.8 are shown in Figures 19, 20 and 21, respectively. The effect of amount of polymers on the *in vitro* gel formation was demonstrated. When the formulations contacted with phosphate buffer pH 6.8, the formulations were changed as an instantaneous gelation. The *in vitro* gel formation of Ethocel (5%w/w), bleached shellac (15%w/w) and Eudragit RS (15%w/w) formulations were started to be gel. The formula were formed to be a greater stiff gel with the amount of polymers and longer time. Ethocel formula were formed as a stiff and fragile. The bleached shellac formulations were formed as an elastic gels after injecting into PBS pH 6.8 whereas the Eudragit RS formula were formed to be a soft gel more apparently than that of Ethocel. The polymer at lower concentration did not change into the gel form. Therefore, the suitable polymer concentrations were selected for further studies. However, another manner has been

studied to confirm the gel formation by subcutaneously injecting the polymer solution (about 200 μ L) into rat or mice and the rat or mice was then sacrificed to remove the *in situ* gel formed (Dayananda *et al.*, 2008; Moon *et al.*, 2011; Abashzadeh *et al.*, 2011).

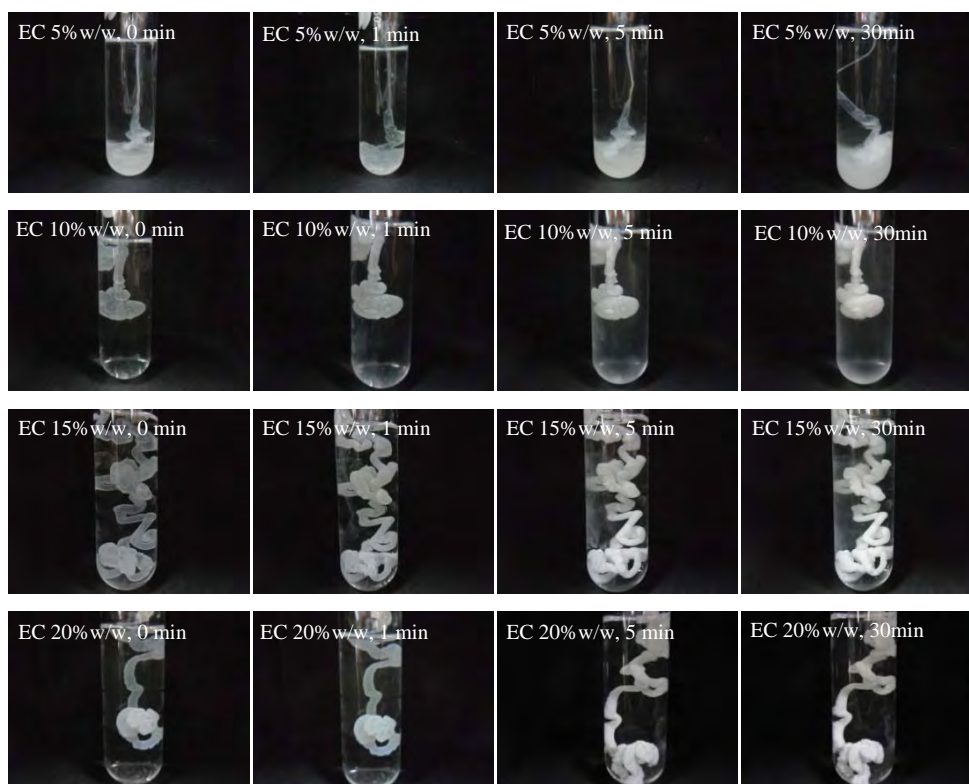


Figure 19 *In vitro* gel formation of Ethocel formula (5-20% w/w) at various times (0, 1, 5 and 30 min)

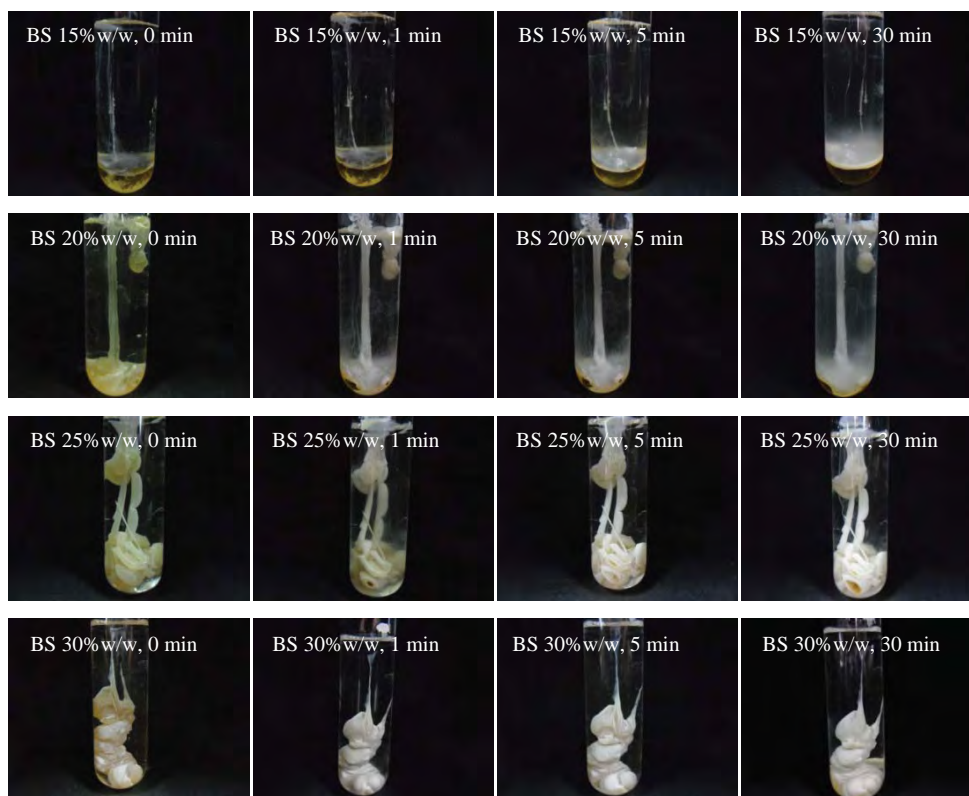


Figure 20 *In vitro* gel formation of bleached shellac formula (15-30% w/w) at various times (0, 1, 5 and 30 min)

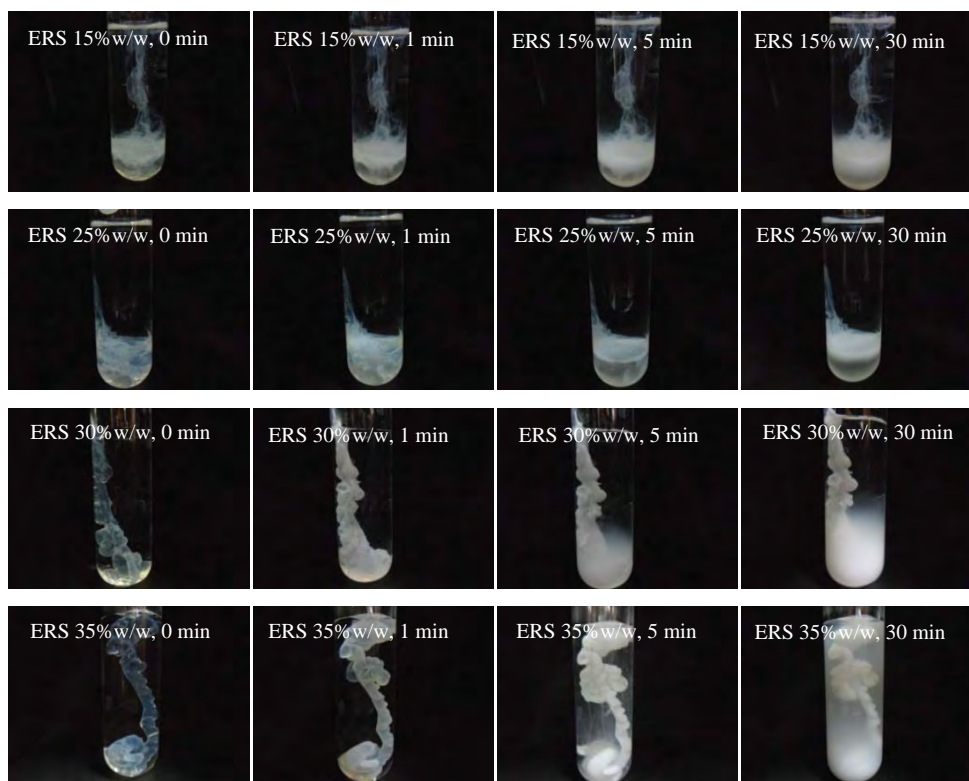


Figure 21 *In vitro* gel formation of Eudragit RS formula (15-35% w/w) at various times (0, 1, 5 and 30 min)

4.2.6 Rate of water diffusion into the gels

The water diffusion has been studied by NMR technique that described self-diffusion coefficient of water in gel (Ray *et al.*, 1998). For this study, the water diffusion into the gels was studied using the observation of the system change in transparent tube which immersed in phosphate buffer solution pH 6.8. When the water diffused into the gels, the apparent system was changed from transparent to opaque. The distance of water front diffusion was then observed at 4 and 24 hours. The rate of water diffusion into gels could be calculated as Equation 12. The rate of water diffusion into the gels of Ethocel, bleached shellac and Eudragit RS formula are shown in Table 14. The rate of water diffusion into the gels at 4 hours of the systems significantly decreased with the increasing concentration of Ethocel and Eudragit RS ($p < 0.05$), whereas the rate of water diffusion into the gels at 24 hours of each polymer (Ethocel, bleached shellac and Eudragit RS) did not significantly differ ($p > 0.05$). However, the rate of water diffusion into the gels of the Ethocel and Eudragit RS

systems at 24 hours was significantly slower than that of the systems at 4 hours ($p < 0.05$). The rate of water diffusion into the gel of bleached did not change since the greater hydrophobic properties. The results suggested that the water was then diffused into the gels, the front of surface systems was altered opaque and harder, resulting in the decreasing rate of water diffusion. The advantages of this manner was easy and convenient that could describe rate of drug release (Wang *et al.*, 2012).

Table 14 Effect of polymer amounts in the systems on rate of water diffusion into gels.

Concentration of polymer (% w/w)	Rate of water diffusion into gels (mm/min) (mean \pm S.D.)	
	at 4 hours	at 24 hours
EC		
5%	0.0131 \pm 0.0006	0.0034 \pm 0.0001
10%	0.0107 \pm 0.0009	0.0030 \pm 0.0001
15%	0.0064 \pm 0.0010	0.0029 \pm 0.0001
20%	0.0039 \pm 0.0002	0.0022 \pm 0.0002
BS		
15%	0.0035 \pm 0.0012	0.0038 \pm 0.0003
20%	0.0035 \pm 0.0012	0.0032 \pm 0.0004
25%	0.0035 \pm 0.0012	0.0032 \pm 0.0004
30%	0.0035 \pm 0.0012	0.0028 \pm 0.0003
ERS		
15%	0.0097 \pm 0.0012	0.0057 \pm 0.0002
25%	0.0069 \pm 0.0012	0.0045 \pm 0.0003
30%	0.0063 \pm 0.0021	0.0039 \pm 0.0004
35%	0.0056 \pm 0.0024	0.0038 \pm 0.0003

4.3 Evaluation of the *in situ* forming gel systems containing antimicrobial agents

4.3.1 Gel appearance and pH measurement

The *in situ* forming gel systems were incorporated with antimicrobial agents such as doxycycline hyclate, metronidazole and benzoyl peroxide. The 5% w/w of each drug was selected in this study. The pH values of Ethocel, bleached shellac and Eudragit RS formula containing 5% w/w of drugs (doxycycline hyclate (DH), metronidazole (MT) and benzoyl peroxide (BP)) are shown in Tables 15. The

appearances of Ethocel formula containing DH, MT and BP (5%w/w) were yellow, light yellow and light yellow, respectively, whereas the appearances of bleached shellac formula containing DH, MT and BP (5%w/w) were similar to the gel based (dark yellow). The appearances of Eudragit RS formula containing DH, MT and BP (5%w/w) were yellow, light yellow and colorless, respectively. All formula were clear. The pH of Ethocel (5-20%w/w) formula containing DH, MT and BP (5%w/w) were in the range of 3.95 ± 0.05 to 4.42 ± 0.02 , 9.53 ± 0.02 to 10.22 ± 0.02 and 8.15 ± 0.05 to 8.63 ± 0.03 . The pH of bleached shellac (15-30%w/w) formula containing DH, MT and BP (5%w/w) were in the range of 3.93 ± 0.04 to 4.16 ± 0.05 , 6.84 ± 0.01 to 7.69 ± 0.04 and 7.05 ± 0.04 to 7.83 ± 0.01 . The pH of Eudragit RS (15-35%w/w) formula containing DH, MT and BP (5%w/w) were in the range of 3.77 ± 0.05 to 3.93 ± 0.01 , 8.76 ± 0.02 to 9.14 ± 0.02 and 7.50 ± 0.07 to 7.86 ± 0.02 . The results indicated that the incorporation of drug affect the pH values of the *in situ* gel systems. The pH value of doxycycline hyclate was between 2.0 and 3.0 (Kogawa and Salgado, 2012), whereas the pH of a saturated aqueous metronidazole solution was 5.8 (Alexander *et al.*, 1997).

Table 15 pH values of Ethocel, bleached shellac and Eudragit RS systems containing various drugs (5%w/w).

Formula (%w/w)	pH \pm S.D. (n=3)			
	Without drug	With drug (5%w/w)		
		DH	MT	BP
EC				
5%	10.58 ± 0.04	3.95 ± 0.05	10.22 ± 0.02	8.15 ± 0.05
10%	9.24 ± 0.02	4.05 ± 0.01	9.86 ± 0.02	8.49 ± 0.04
15%	9.19 ± 0.02	4.36 ± 0.01	9.63 ± 0.05	8.56 ± 0.02
20%	9.57 ± 0.06	4.42 ± 0.02	9.53 ± 0.02	8.63 ± 0.03
BS				
15%	7.22 ± 0.01	4.16 ± 0.05	7.69 ± 0.04	7.83 ± 0.01
20%	7.02 ± 0.03	4.09 ± 0.01	7.34 ± 0.02	7.70 ± 0.03
25%	6.81 ± 0.02	4.13 ± 0.01	7.06 ± 0.02	7.46 ± 0.01
30%	6.65 ± 0.02	3.93 ± 0.04	6.84 ± 0.01	7.05 ± 0.04
ERS				
15%	9.07 ± 0.02	3.86 ± 0.04	9.14 ± 0.02	7.50 ± 0.07
25%	8.87 ± 0.03	3.92 ± 0.01	9.27 ± 0.04	7.78 ± 0.04
30%	8.76 ± 0.02	3.93 ± 0.01	9.04 ± 0.01	7.82 ± 0.03
35%	8.69 ± 0.09	3.77 ± 0.05	8.76 ± 0.02	7.86 ± 0.02

4.3.2 Viscosity studies

The effects of polymers (Ethocel, bleached shellac and Eudragit RS) loading and comprising DH, MT and BP (5%w/w) and temperature on the viscosity were investigated. The viscosities of the Ethocel (5-20%w/w) formulations comprising DH, MT and BP at different temperatures, 25°C and 37°C, are shown in Figures 22, 23 and 24, respectively. The apparent viscosities of the Ethocel (5-15%w/w) formula comprising DH, MT and BP were constant when the shear rate was increased indicating Newtonian behavior, which the viscosity did not change as the shear rate was increased. While the apparent viscosities of the Ethocel (20%w/w) formula comprising DH, MT and BP at a low shear rate were higher than that at a high shear rate, indicating pseudoplastic behavior. The viscosities of the bleached shellac (15-30%w/w) formula comprising DH, MT and BP at different temperatures, 25°C and 37°C, are shown in Figures 25, 26 and 27, respectively. The viscosities of all beached shellac (15-30%w/w) formulations did not change as the shear rate was increased, indicating Newtonian behavior. The viscosities of the Eudragit RS (15-35%w/w) formula comprising DH, MT and BP at different temperatures, 25°C and 37°C, are shown in Figures 28, 29 and 30, respectively. The apparent viscosities of all Eudragit RS (15-35%w/w) formula comprising DH, MT and BP were constant when the shear rate was increased indicating Newtonian behavior, which the viscosity did not change as the shear rate was increased. While the effects of temperature on the viscosity of the systems containing drugs were described that all formula comprising drugs (DH, MT and BP) exhibited a decrease in viscosity with increasing temperature. The viscosity of systems was increased when incorporated drugs since drug-polymer interaction. These trends were similar to gel bases in previous viscosity study. However some research indicated that the incorporation of drug increased viscosity of the gel system (Mayol *et al.*, 2008).

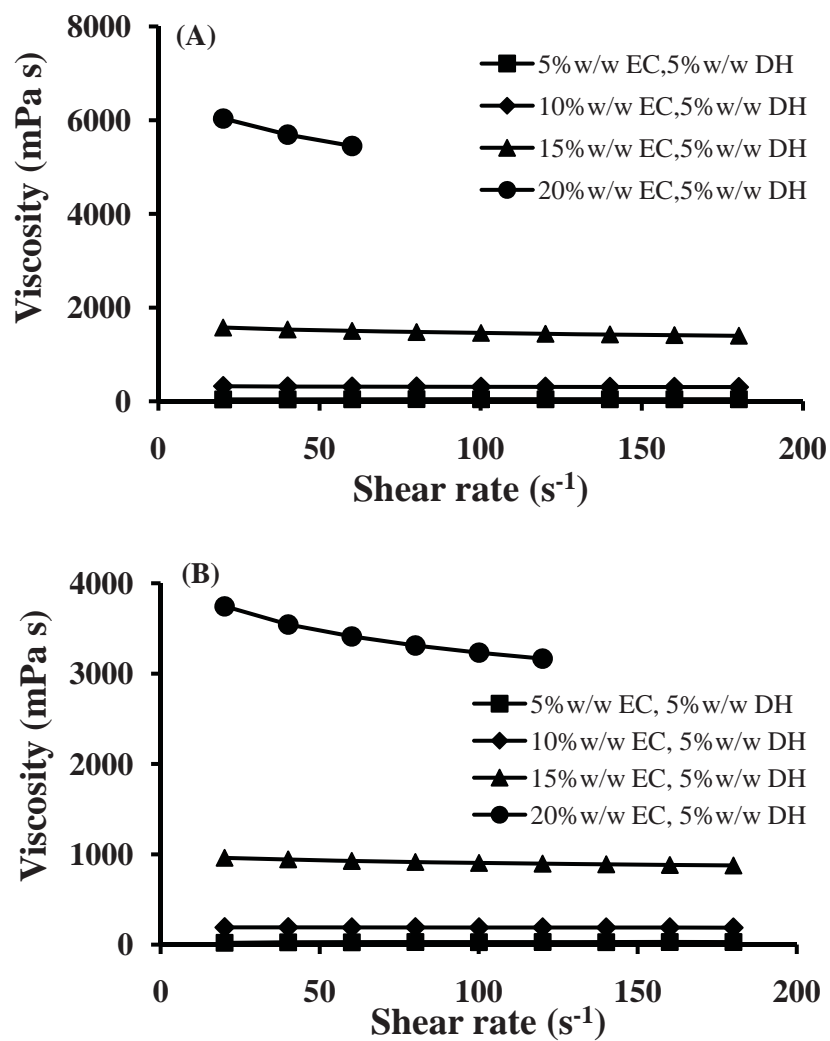


Figure 22 Viscosity curves of Ethocel-5%w/w doxycycline hyclate formula at (A) 25°C and (B) 37°C

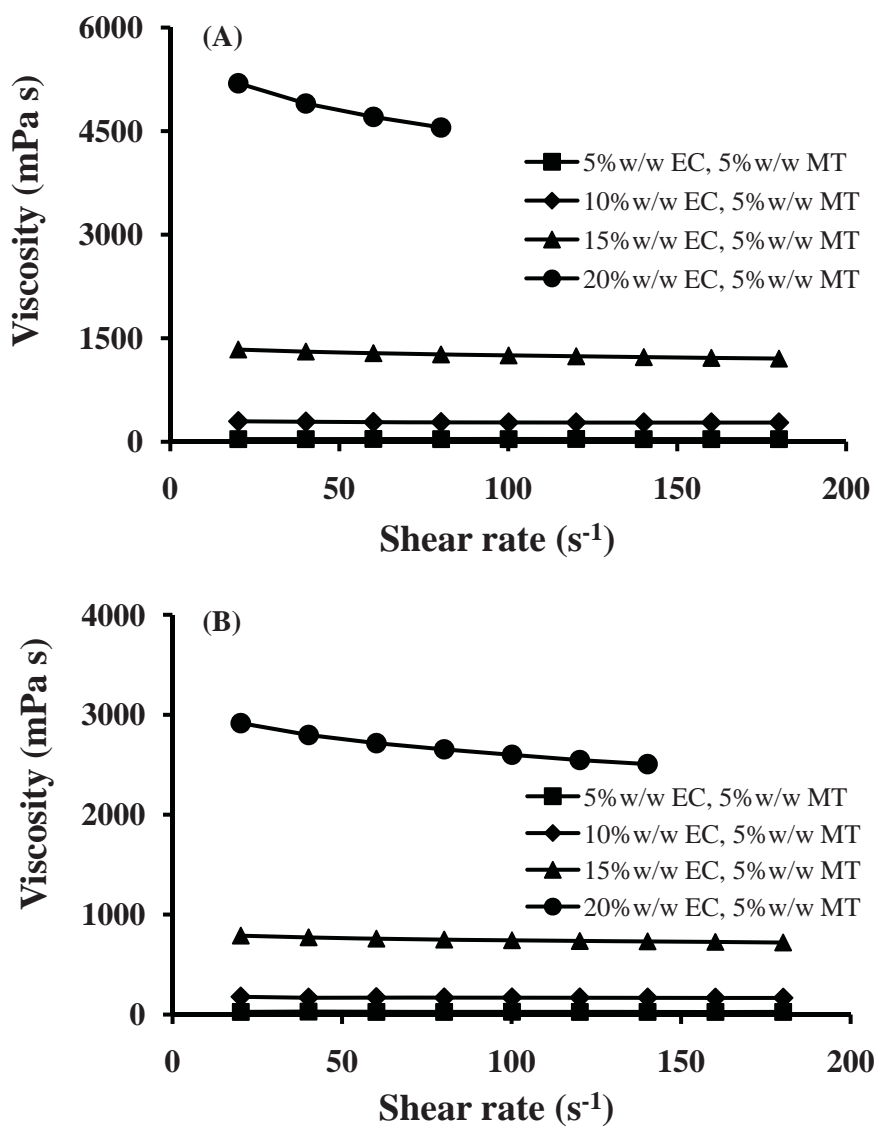


Figure 23 Viscosity curves of Ethocel-5%w/w metronidazole formula at (A) 25°C and (B) 37°C

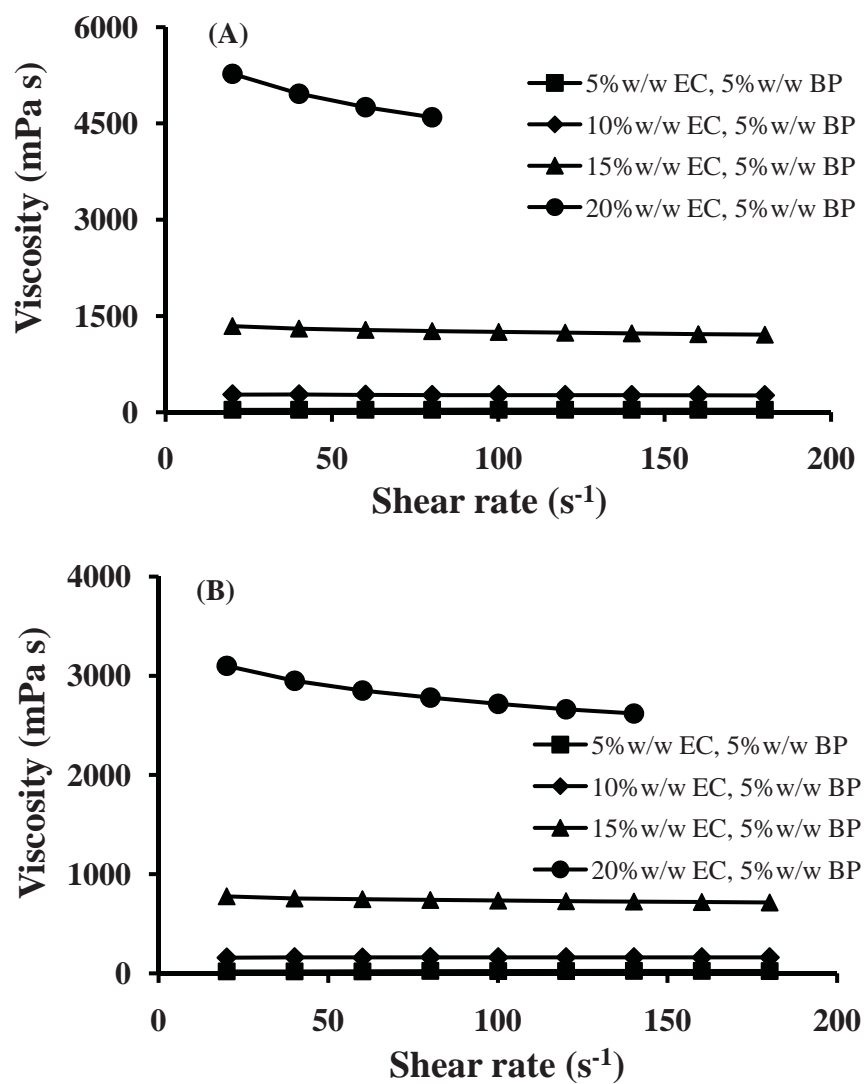


Figure 24 Viscosity curves of Ethocel-5%w/w benzoyl peroxide formula at (A) 25°C and (B) 37°C

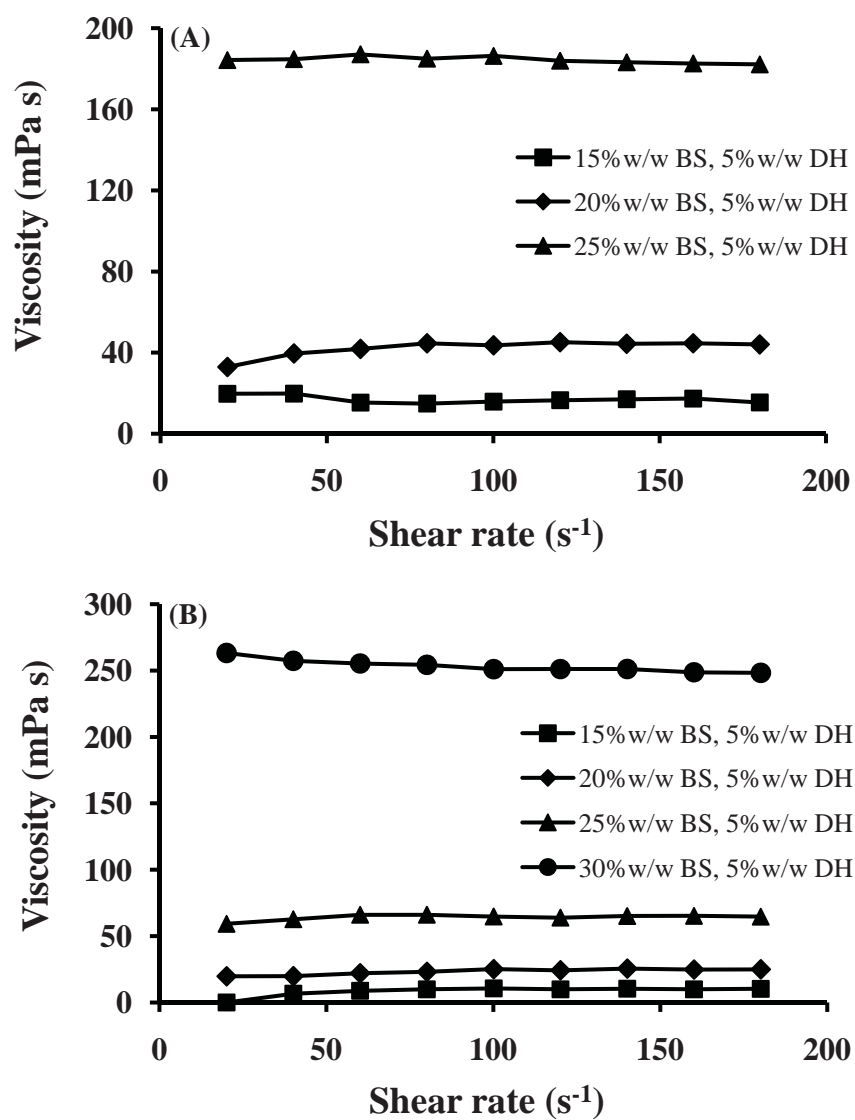


Figure 25 Viscosity curves of bleached shellac-5%w/w doxycycline hyclate formula at (A) 25°C and (B) 37°C

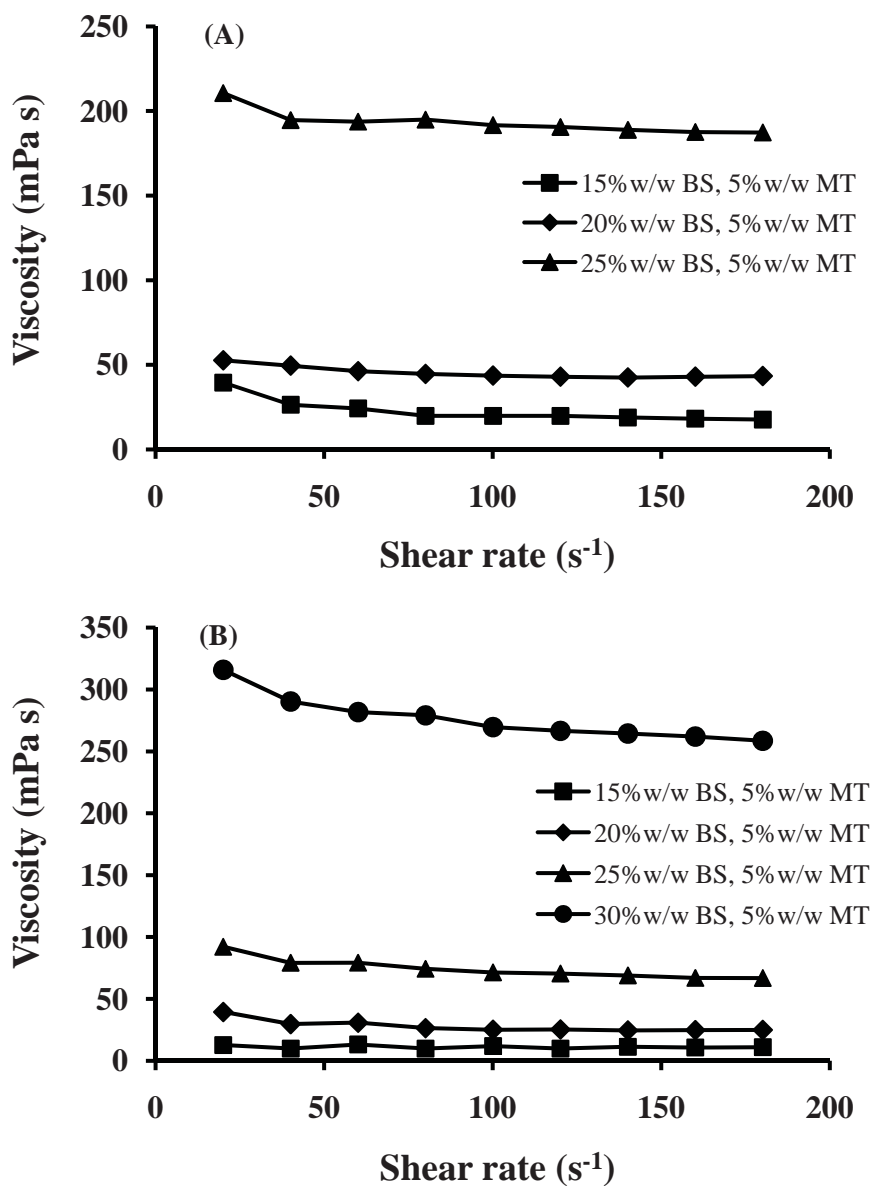


Figure 26 Viscosity curves of bleached shellac-5% w/w metronidazole formula at (A) 25°C and (B) 37°C

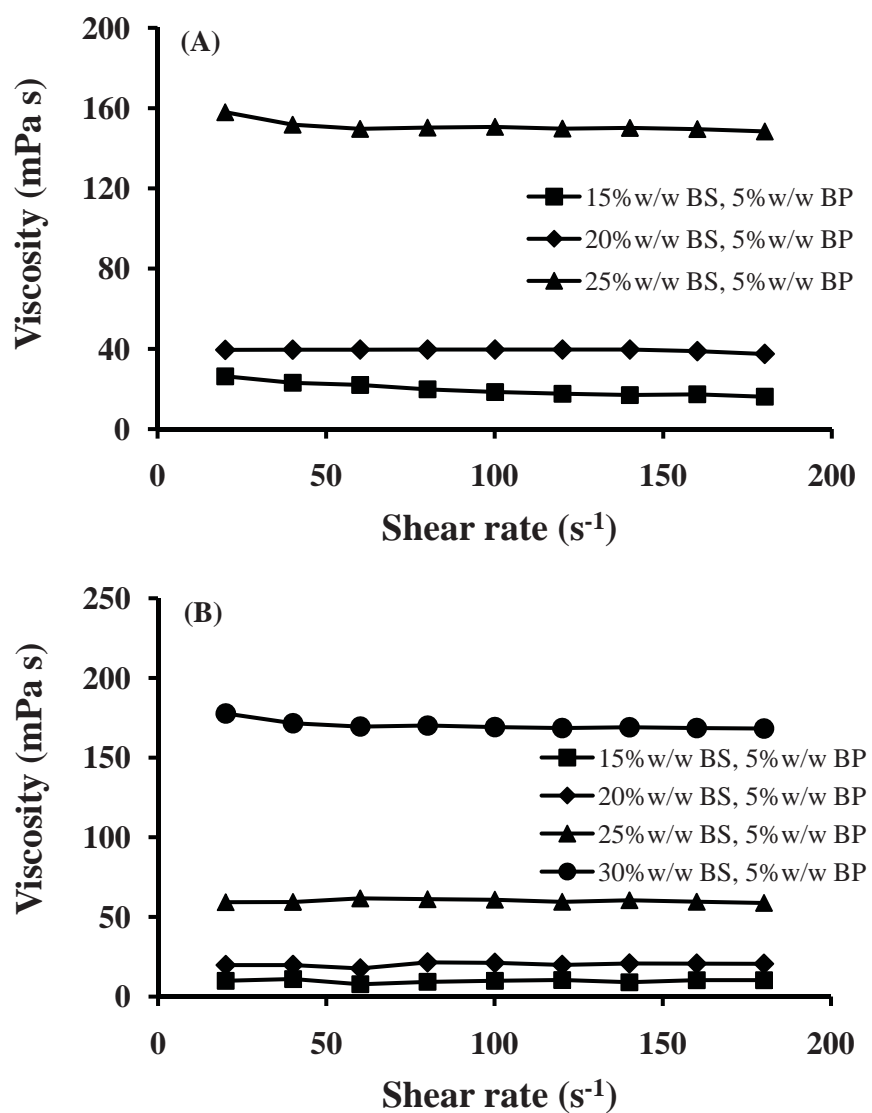


Figure 27 Viscosity curves of bleached shellac-5% w/w benzoyl peroxide formula at (A) 25°C and (B) 37°C

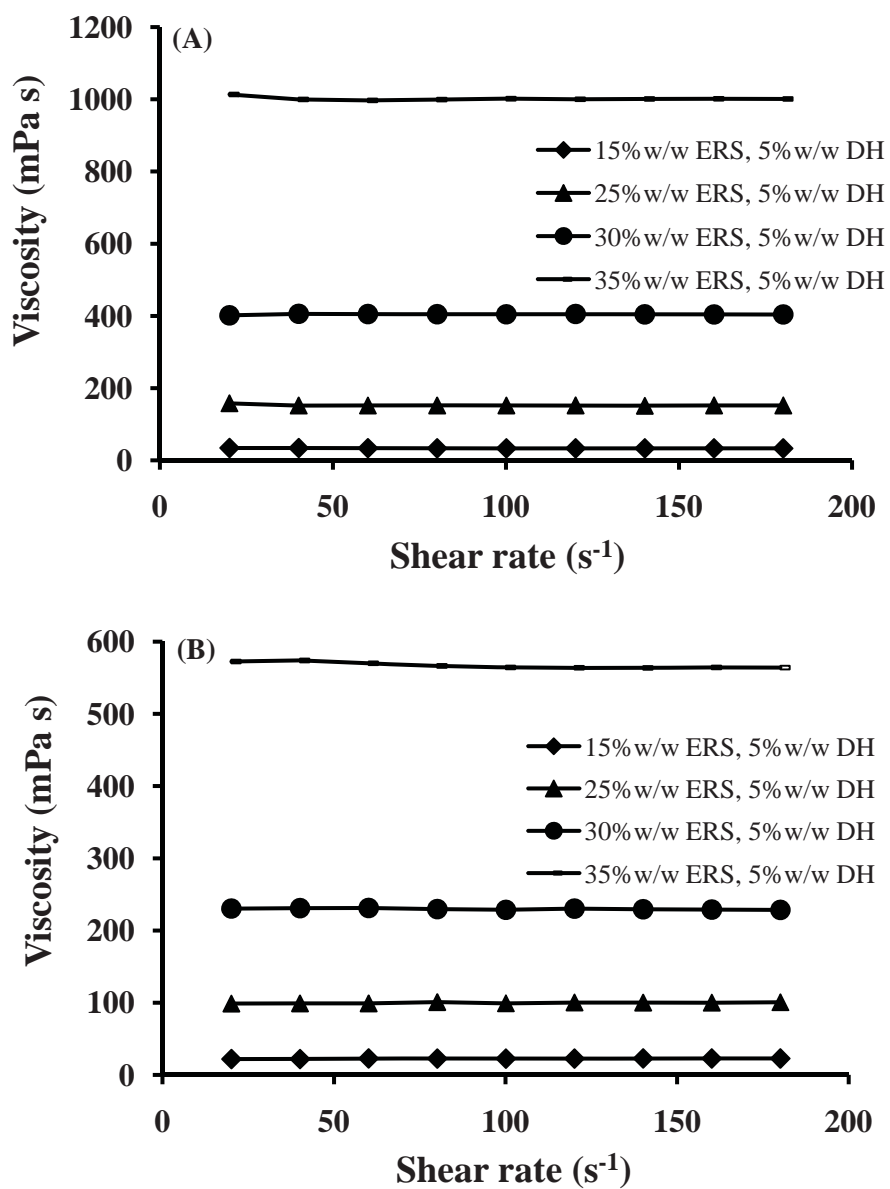


Figure 28 Viscosity curves of Eudragit RS-5%w/w doxycycline hyclate formula at (A) 25°C and (B) 37°C

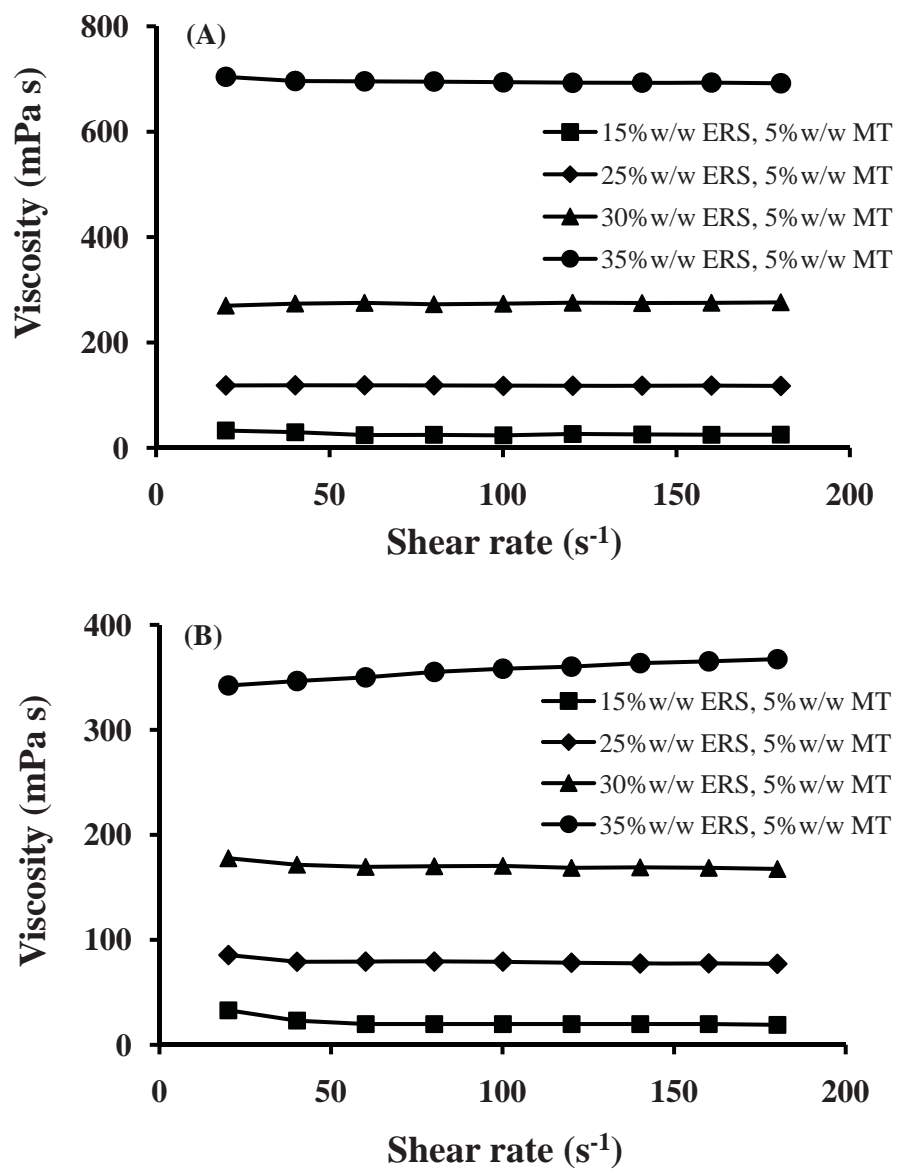


Figure 29 Viscosity curves of Eudragit RS-5% w/w metronidazole formula at (A) 25°C and (B) 37°C

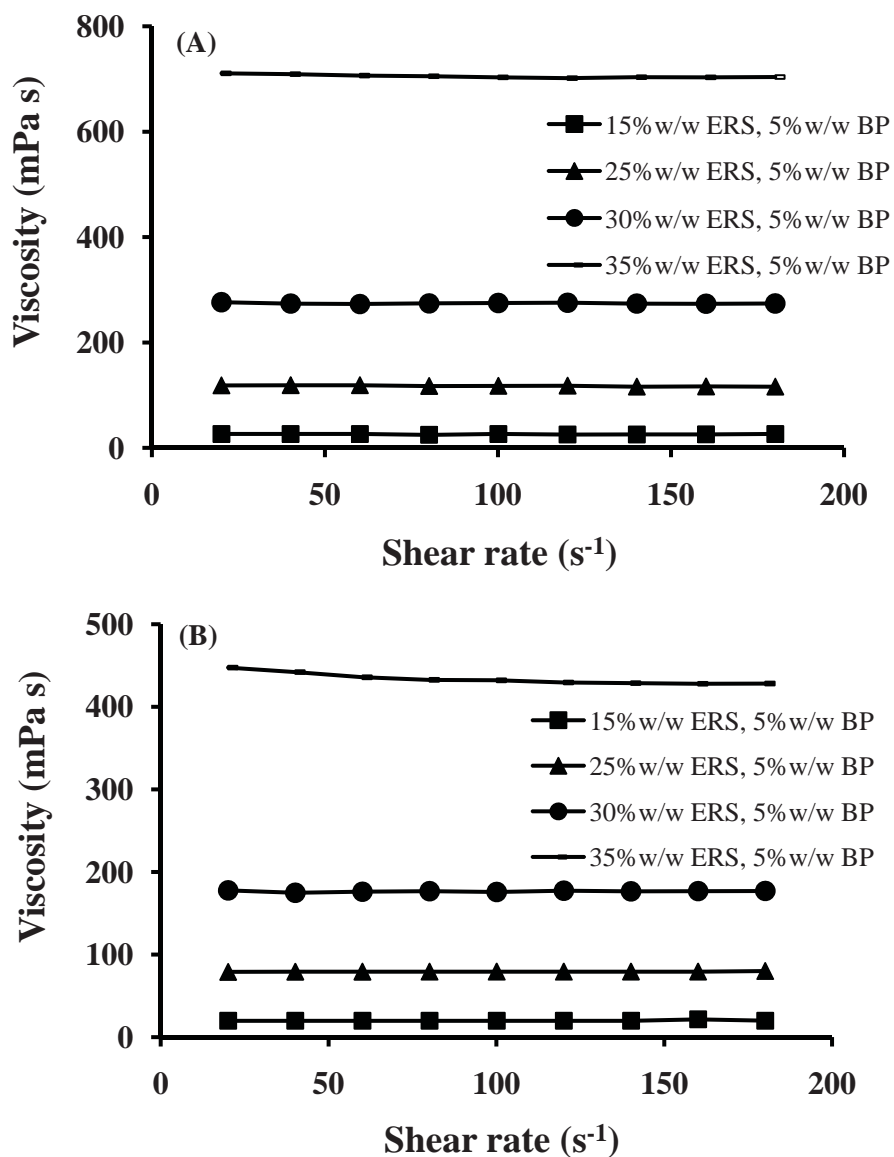


Figure 30 Viscosity curves of Eudragit RS-5%w/w benzoyl peroxide formula at (A) 25°C and (B) 37°C

4.3.3 Rheological behavior studies

The rheological behaviors of various polymer solutions comprising drugs (DH, MT and BP) were investigated as a function of amount and temperature. The shear stress versus shear rate flow curves of Ethocel (5-20% w/w), bleached shellac (15-30% w/w) and Eudragit RS (15-35% w/w) formula comprising DH, MT and BP (5% w/w) at 25°C and 37°C are shown in Figures 31-33, 34-36 and 37-39, respectively. The shear stress of all formula with drugs was higher than that of gel base. The shear stress of all formula comprising 5% w/w drugs (DH, MT and BP) was increased as the shear rate and polymer amounts were increased. All formula were Newtonian flow, indicating the up curve did coincide with the down curve. The curves moved to a higher shear stress value indicating compact structure of the gels. On the other hand, the effect of temperature on rheological study of the formula comprising drugs (DH, MT and BP) was investigated. The shear stress of all formulations comprising 5% w/w drugs (DH, MT and BP) decreased with an increasing temperature. The curves of formulations moved to a lower shear stress value indicating loose structure.

The rheological behaviors of various polymer solutions (Ethocel, bleached shellac and Eudragit RS) comprising 5% w/w drugs (DH, MT and BP) were confirmed by the N value and viscosity coefficient (η). The flow parameters of Ethocel (5-20% w/w), bleached shellac (15-30% w/w) and Eudragit RS (15-35% w/w) formula comprising DH, MT and BP (5% w/w) are shown in Tables 16, 17 and 18, respectively. N value of all formula comprising DH, MT and BP was close to 1, indicating that the flow type was Newtonian similar to gel base. Temperature did not influence the flow types of all systems comprising 5% w/w drugs (DH, MT and BP). In the case of the viscosity coefficient (η), the amount of each formula comprising DH, MT and BP was higher, the viscosity coefficient was also significantly greater ($p < 0.05$). In contrast, the viscosity coefficient was significantly decreased when the temperature was increased ($p < 0.05$). The results indicated that the amount of each polymer affected the viscosity coefficient of each system comprising drugs (DH, MT and BP). However, all formula were Newtonian flow behaviors, the N values were closed to 1. While the temperature did not influence the rheological behavior of systems, the viscosity coefficient of each system was significantly decreased ($p < 0.05$) as the temperature was

increased. These results supported the previous studies. It has been studied that the structure could be altered the deformation by changing in the shape of polymer molecules and in the number of molecular entanglements, possibly by shear rate (Fresno *et al.*, 2002).

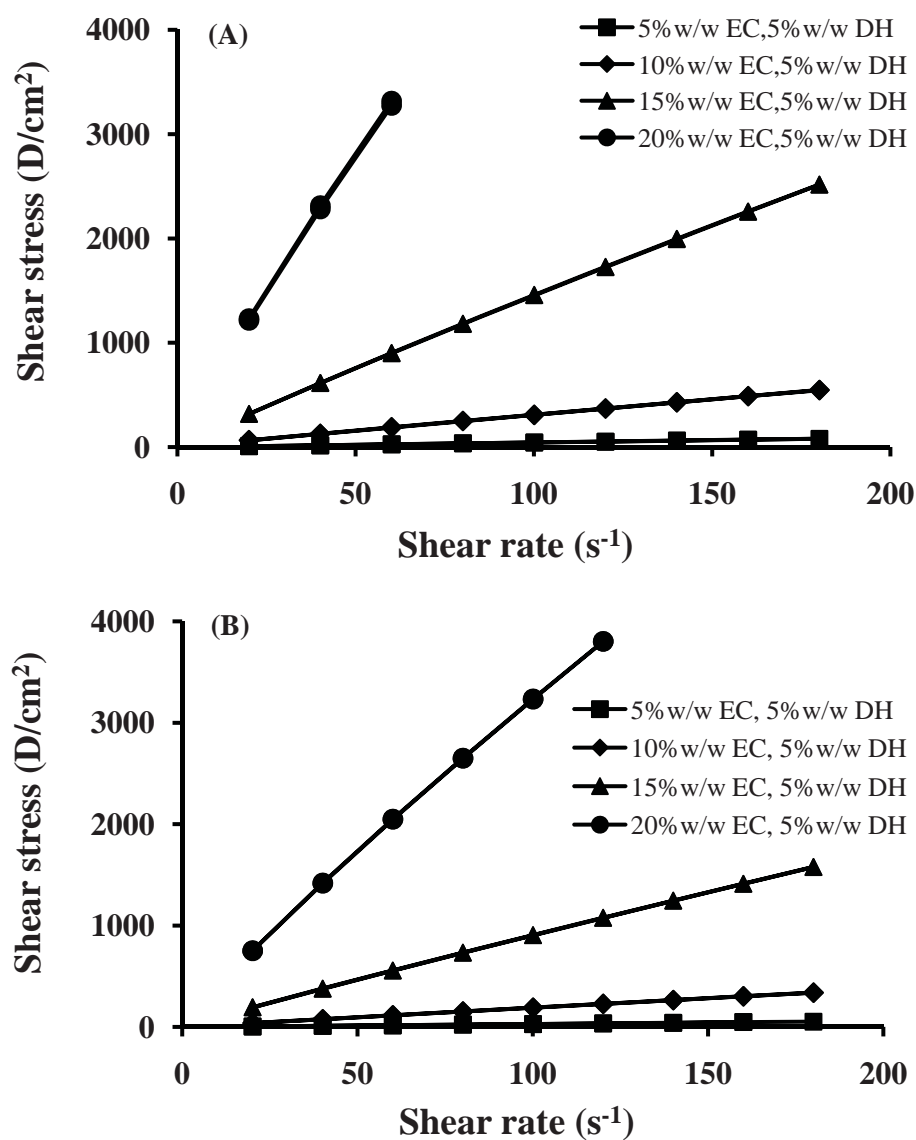


Figure 31 Flow curve of Ethocel formula containing 5% w/w doxycycline hyclate at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

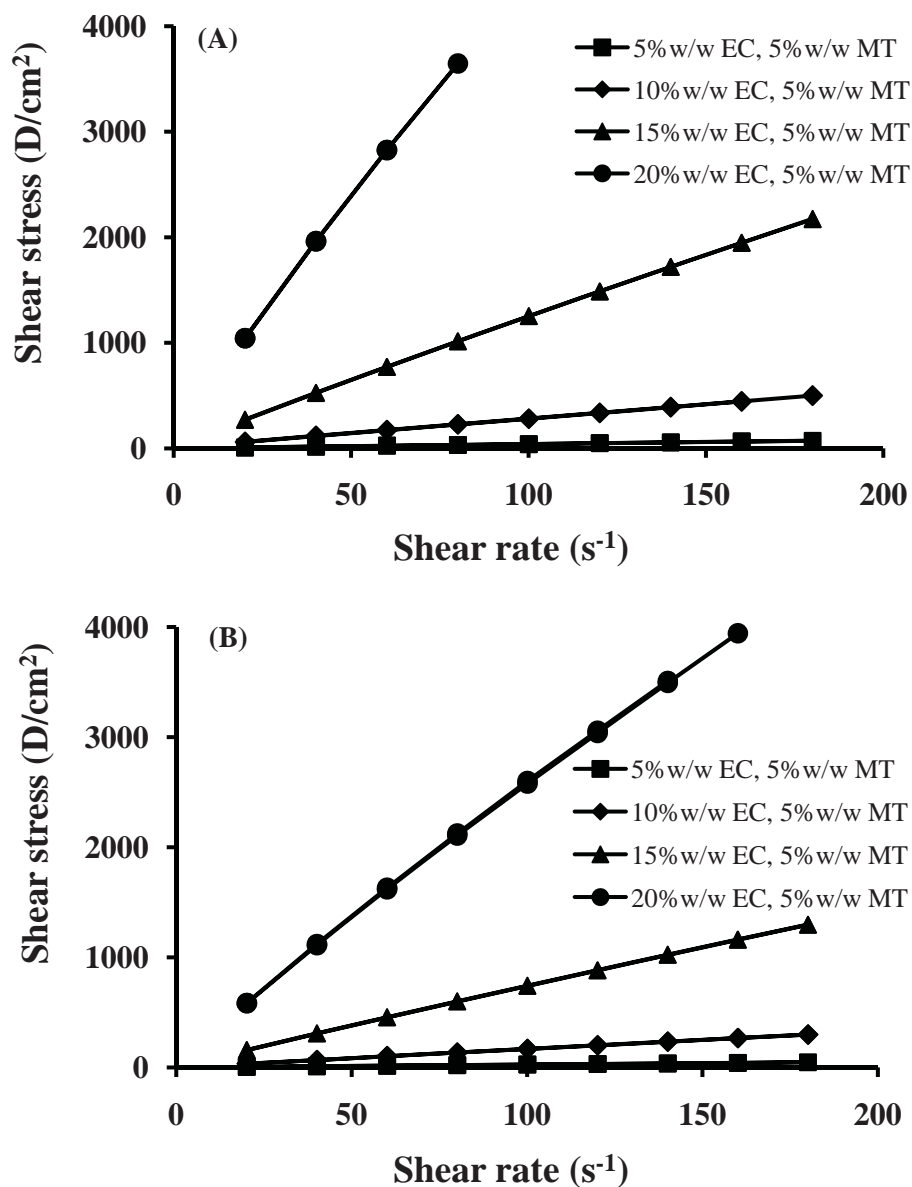


Figure 32 Flow curve of Ethocel formula containing 5% w/w metronidazole at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

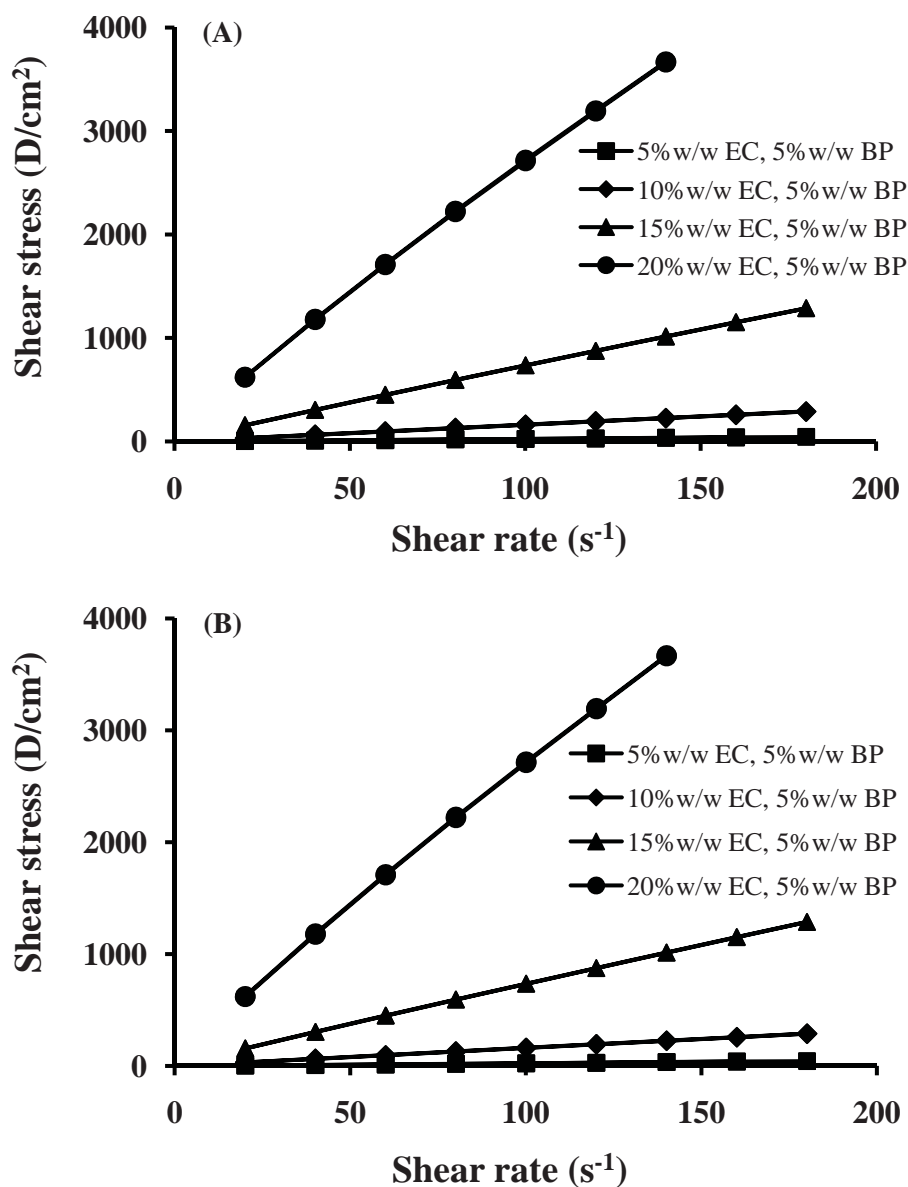


Figure 33 Flow curve of Ethocel formula containing 5% w/w benzoyl peroxide at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

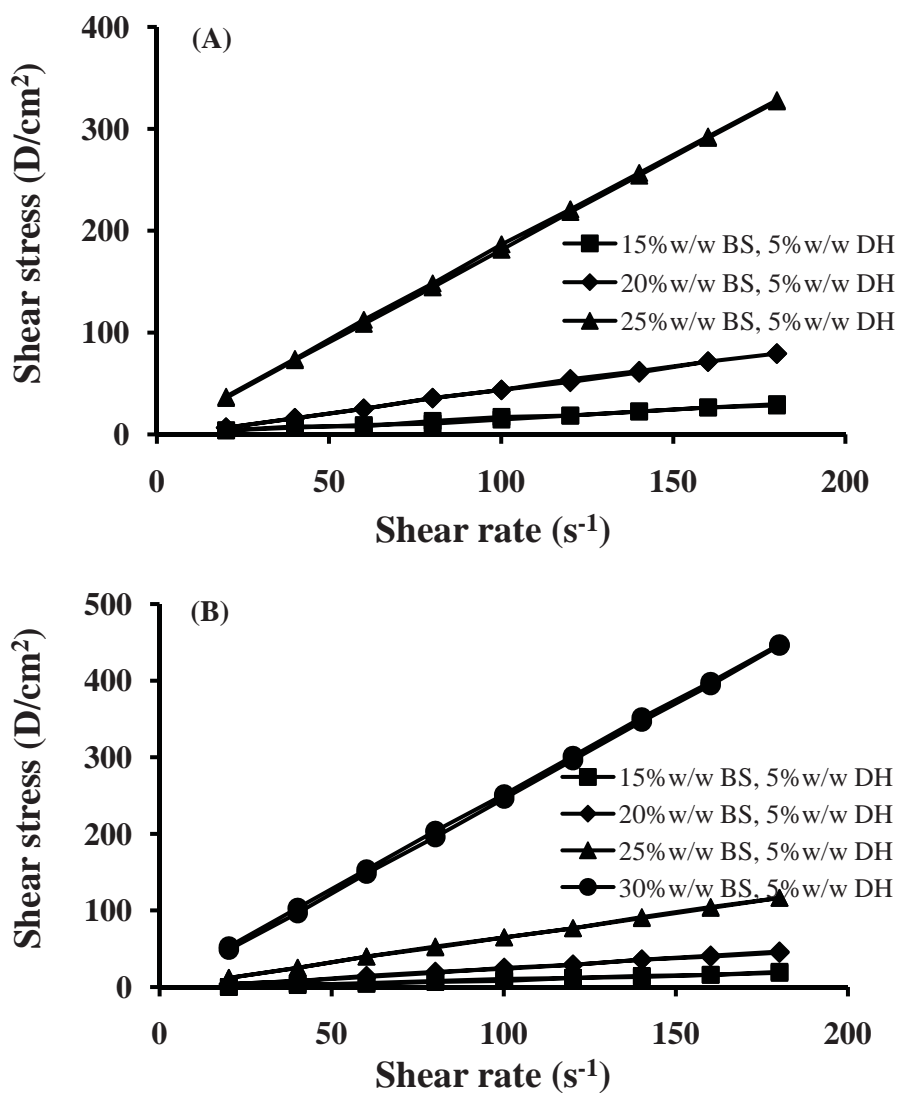


Figure 34 Flow curve of bleached shellac formula containing 5% w/w doxycycline hyclate at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

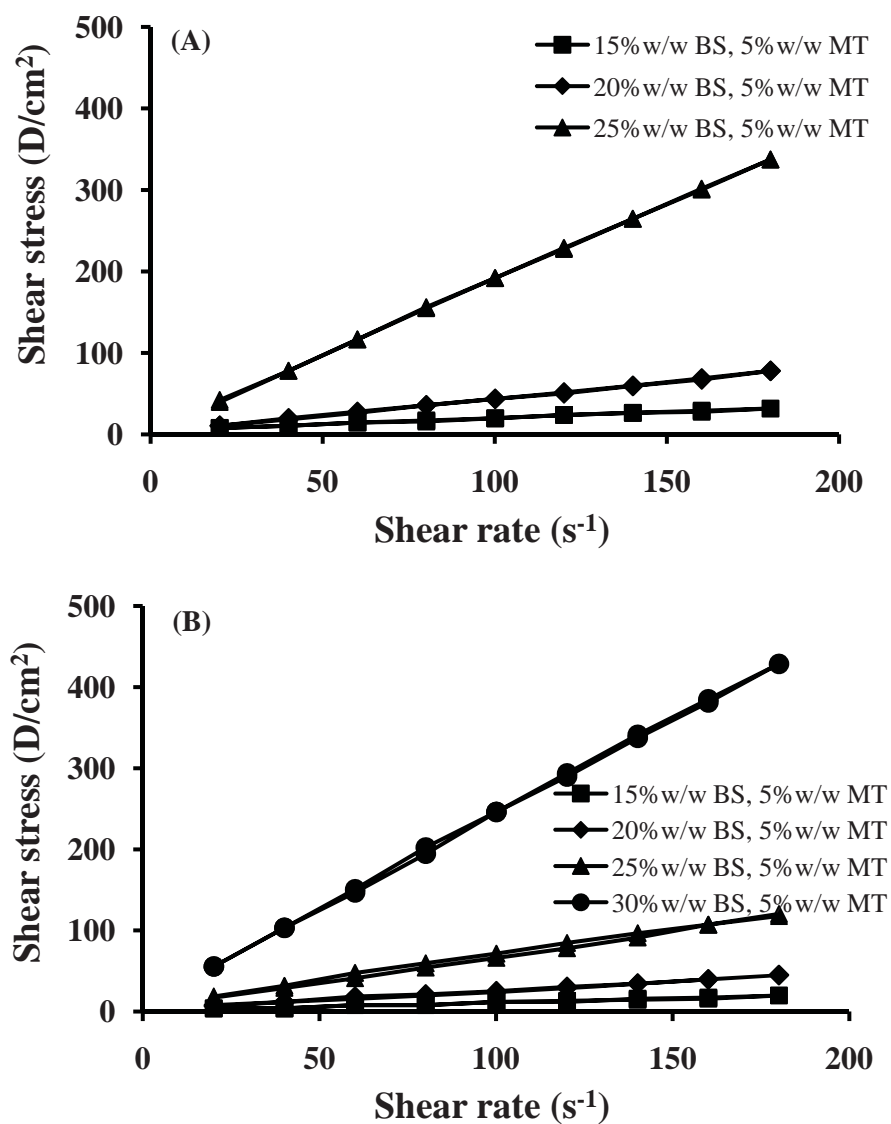


Figure 35 Flow curve of bleached shellac formula containing 5% w/w metronidazole at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

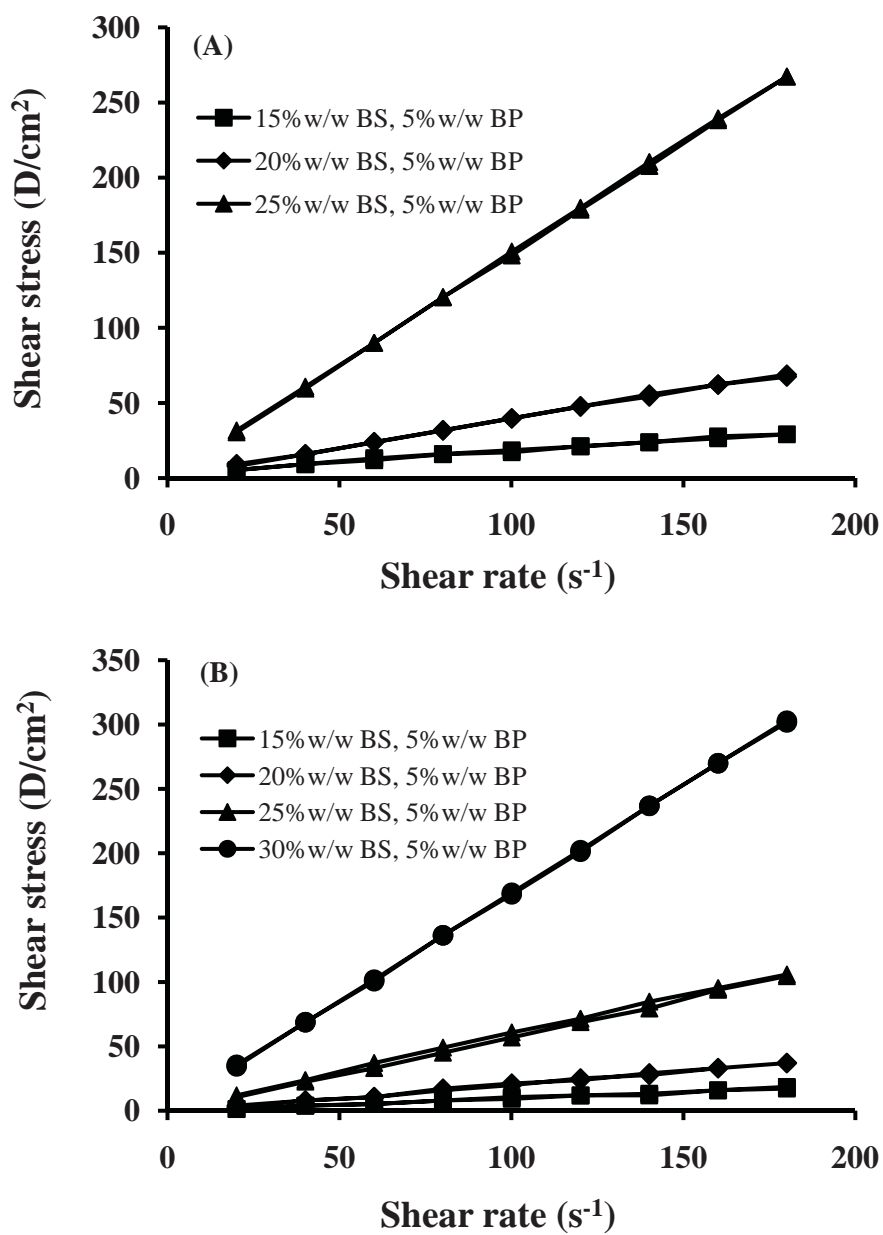


Figure 36 Flow curve of bleached shellac formula containing 5% w/w benzoyl peroxide at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

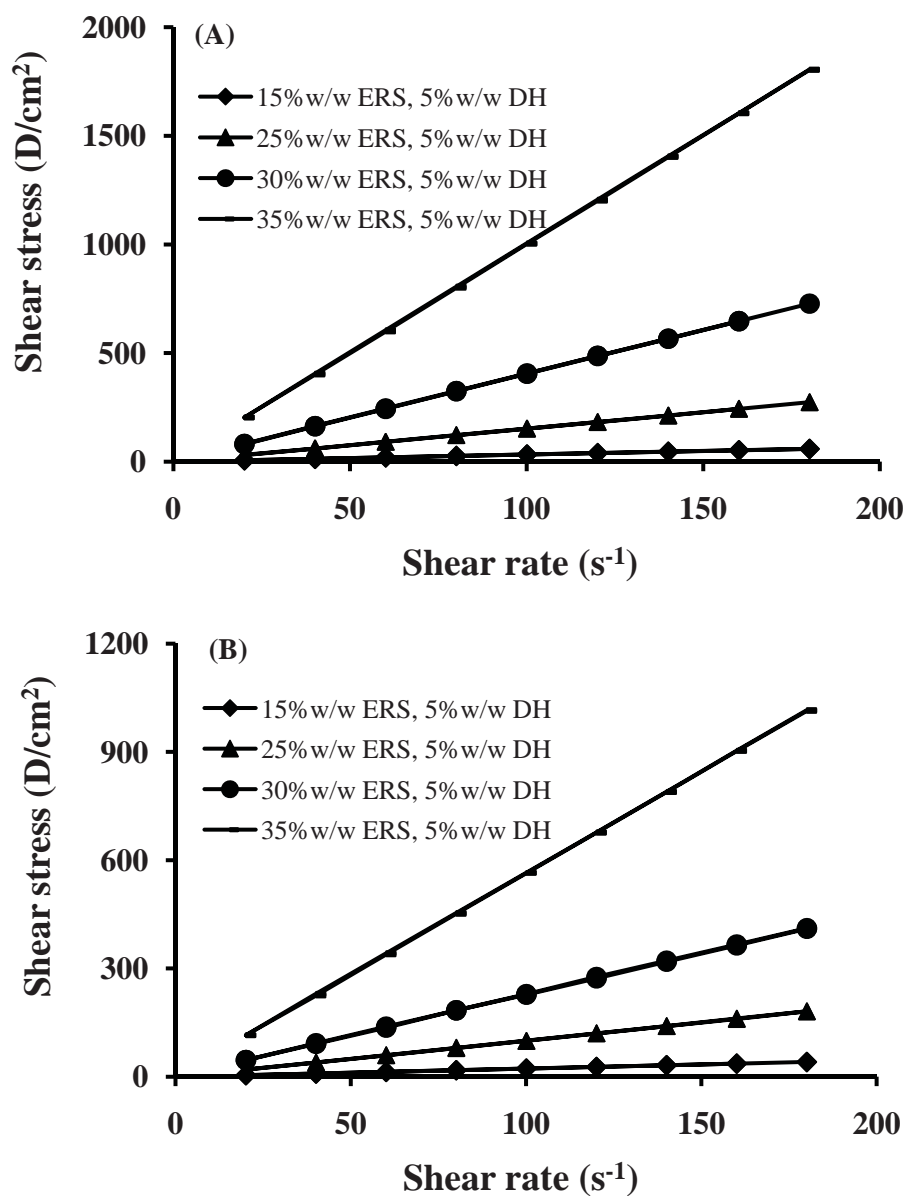


Figure 37 Flow curve of Eudragit RS formula containing 5% w/w doxycycline hyclate at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

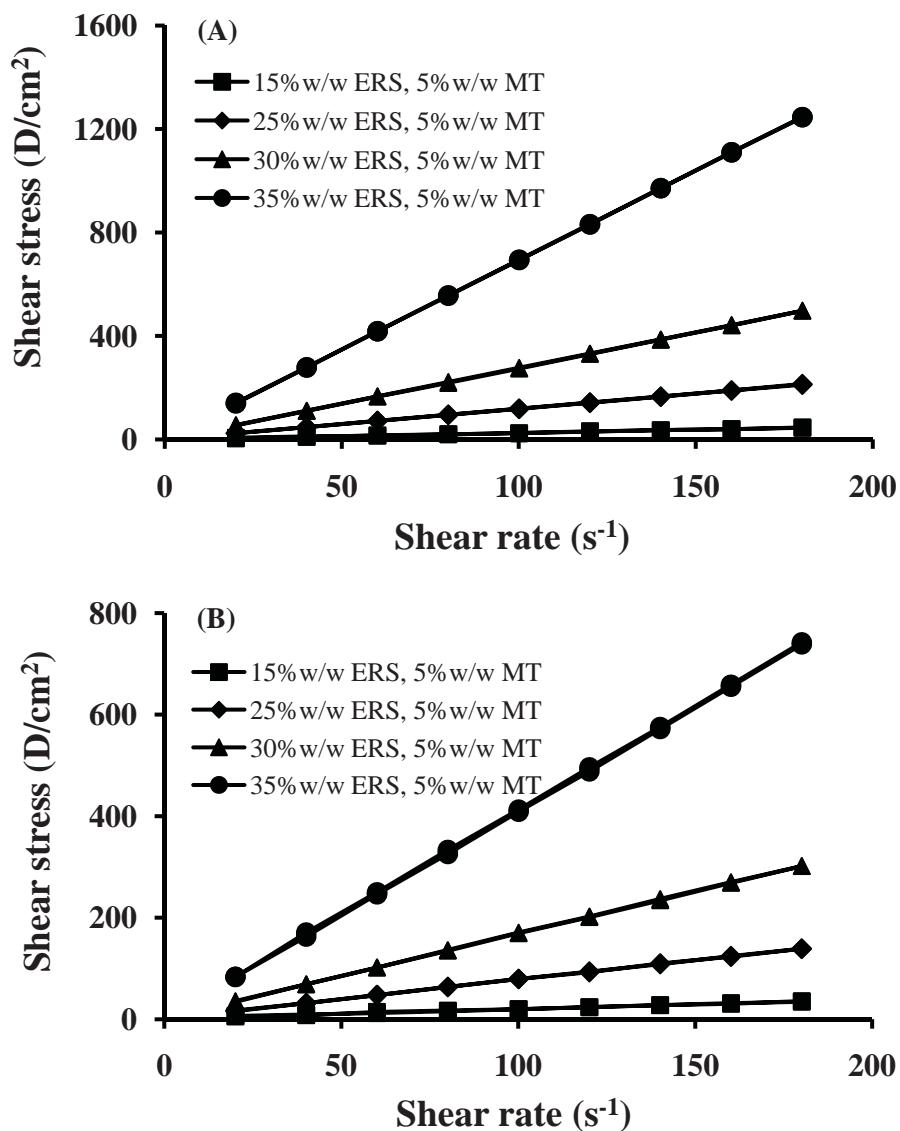


Figure 38 Flow curve of Eudragit RS formula containing 5%w/w metronidazole at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

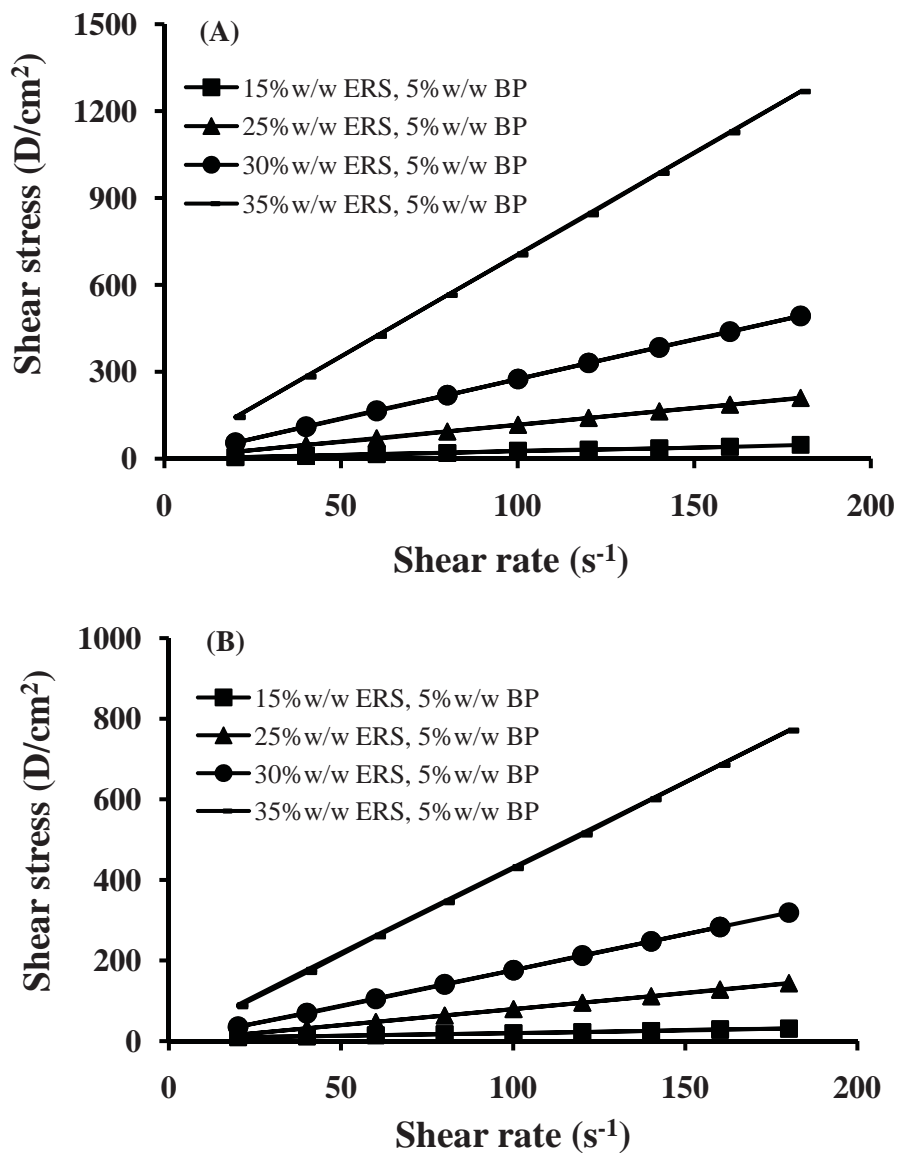


Figure 39 Flow curve of Eudragit RS formula containing 5% w/w benzoyl peroxide at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

Table 16 Flow parameters of Ethocel (5-20% w/w) formula containing different types of drugs (doxycycline hyclate, metronidazole and benzoyl peroxide) at 25°C and 37°C (n=3)

Concentration of EC (% w/w)	25°C		37°C	
	Flow index (N) (mean ± S.D.)	Consistency index (η) (mean ± S.D., [D/cm ²] ^N s)	Flow index (N) (mean ± S.D.)	Consistency index (η) (mean ± S.D., [D/cm ²] ^N s)
With 5% w/w DH				
5%	1.06 ± 0.01	33.50 ± 1.27	1.07 ± 0.01	13.60 ± 1.39
10%	0.99 ± 0.01	348.63 ± 13.98	1.00 ± 0.02	193.63 ± 14.11
15%	0.97 ± 0.01	1856.00 ± 34.77	0.97 ± 0.01	1098.00 ± 14.80
20%	0.95 ± 0.01	8058.67 ± 358.69	0.96 ± 0.00	4963.33 ± 23.86
With 5% w/w MT				
5%	1.00 ± 0.02	40.77 ± 3.69	1.02 ± 0.02	21.83 ± 5.60
10%	0.98 ± 0.01	335.50 ± 8.96	0.99 ± 0.01	182.73 ± 9.27
15%	0.97 ± 0.01	1578.33 ± 12.22	0.98 ± 0.00	891.50 ± 21.62
20%	0.96 ± 0.01	6879.00 ± 112.95	0.96 ± 0.01	3726.00 ± 68.83
With 5% w/w BP				
5%	1.01 ± 0.01	39.33 ± 0.06	1.08 ± 0.01	13.57 ± 0.49
10%	0.99 ± 0.01	297.50 ± 0.52	1.01 ± 0.01	154.50 ± 3.98
15%	0.97 ± 0.01	1564.00 ± 9.85	0.98 ± 0.01	870.13 ± 20.47
20%	0.96 ± 0.00	7085.00 ± 86.60	0.96 ± 0.01	4009.00 ± 102.00

Table 17 Flow parameters of bleached shellac (15-30% w/w) formula containing different types of drugs (doxycycline hyclate, metronidazole and benzoyl peroxide) at 25°C and 37°C (n=3)

Concentration of BS (% w/w)	25°C		37°C	
	Flow index (N) (mean ± S.D.)	Consistency index (η) (mean ± S.D., [D/cm ²] ^N s)	Flow index (N) (mean ± S.D.)	Consistency index (η) (mean ± S.D., [D/cm ²] ^N s)
With 5% w/w DH				
15%	0.99 ± 0.01	17.80 ± 5.91	1.03 ± 0.02	4.98 ± 0.74
20%	1.02 ± 0.01	31.77 ± 2.19	1.02 ± 0.01	13.03 ± 0.15
25%	1.00 ± 0.02	181.80 ± 13.99	1.04 ± 0.01	55.13 ± 2.14
30%	-	-	0.99 ± 0.01	258.30 ± 24.42
With 5% w/w MT				
15%	0.98 ± 0.01	33.53 ± 5.15	0.99 ± 0.02	11.50 ± 1.01
20%	0.97 ± 0.01	57.33 ± 7.32	0.97 ± 0.04	29.00 ± 3.96
25%	0.96 ± 0.01	224.57 ± 6.04	0.98 ± 0.01	97.17 ± 16.74
30%	-	-	0.98 ± 0.05	354.47 ± 26.86
With 5% w/w BP				
15%	0.99 ± 0.01	29.57 ± 0.98	1.01 ± 0.04	9.38 ± 2.89
20%	0.98 ± 0.02	58.00 ± 15.00	1.03 ± 0.03	17.17 ± 1.54
25%	1.01 ± 0.03	160.03 ± 10.79	1.01 ± 0.03	52.97 ± 9.73
30%	-	-	0.99 ± 0.02	181.67 ± 19.99

Table 18 Flow parameters of Eudragit RS (15-35% w/w) formula containing different types of drugs (doxycycline hyclate, metronidazole and benzoyl peroxide) at 25°C and 37°C (n=3)

Concentration of ERS (% w/w)	25°C		37°C	
	Flow index (N) (mean ± S.D.)	Consistency index (η) (mean ± S.D., [D/cm ²] ^N s)	Flow index (N) (mean ± S.D.)	Consistency index (η) (mean ± S.D., [D/cm ²] ^N s)
With 5% w/w DH				
15%	0.99 ± 0.01	7.63 ± 0.40	1.01 ± 0.01	20.73 ± 0.29
25%	0.99 ± 0.02	156.30 ± 11.98	1.00 ± 0.01	95.97 ± 2.08
30%	1.00 ± 0.01	409.00 ± 18.04	1.00 ± 0.01	233.30 ± 18.91
35%	1.00 ± 0.01	1025.00 ± 5.20	1.00 ± 0.02	583.57 ± 7.18
With 5% w/w MT				
15%	0.99 ± 0.01	28.47 ± 1.72	0.98 ± 0.01	21.47 ± 0.58
25%	1.00 ± 0.01	119.60 ± 2.29	0.99 ± 0.01	83.77 ± 2.14
30%	1.00 ± 0.01	271.30 ± 8.06	0.98 ± 0.01	182.73 ± 5.47
35%	1.00 ± 0.01	604.30 ± 167.56	1.01 ± 0.02	363.63 ± 82.93
With 5% w/w BP				
15%	1.00 ± 0.02	25.97 ± 2.31	1.01 ± 0.01	18.87 ± 0.81
25%	0.99 ± 0.01	121.73 ± 2.62	1.00 ± 0.01	78.10 ± 0.87
30%	1.00 ± 0.01	279.57 ± 1.20	1.00 ± 0.00	179.63 ± 5.98
35%	0.99 ± 0.01	725.20 ± 19.69	0.99 ± 0.00	451.47 ± 7.37

4.3.4 Syringeability

The syringeability of various formula comprising drugs (DH, MT and BP) was evaluated to determine the effect of the incorporated drug in gel systems on the force required to expel the product. Syringeability of Ethocel (5-20% w/w), bleached shellac (15-30% w/w) and Eudragit RS (15-35% w/w) formula comprising 5% w/w drugs (DH, MT and BP) are shown in Tables 19. The work of syringeability of all formula comprising drugs (DH, MT and BP) significantly increased ($p < 0.05$) as the amount of polymers was increased. The increasing polymer content increased the work required for expulsion, indicating lower syringeability as previously reported (Chang *et al.*, 2002).

Table 19 Syringeability of Ethocel, bleached shellac and Eudragit RS formula containing different drugs (doxycycline hyclate, metronidazole and benzoyl peroxide)

Formula (% w/w)	Work (N.mm) (n=3)			
	Without drug	With drug (5% w/w)		
		DH	MT	BP
EC				
5%	21.73 ± 0.99	18.58 ± 1.32	17.68 ± 1.80	14.75 ± 2.67
10%	23.26 ± 2.11	24.78 ± 1.32	24.34 ± 2.20	22.36 ± 2.29
15%	32.64 ± 0.82	26.73 ± 1.47	25.96 ± 1.68	23.15 ± 3.07
20%	41.98 ± 2.47	46.33 ± 1.71	53.48 ± 4.56	49.66 ± 2.83
BS				
15%	11.73 ± 0.88	13.32 ± 2.12	5.07 ± 0.28	7.45 ± 1.14
20%	12.22 ± 0.83	25.89 ± 2.89	6.10 ± 0.26	8.13 ± 0.94
25%	15.72 ± 2.05	37.29 ± 4.76	10.04 ± 1.05	9.73 ± 1.00
30%	27.69 ± 1.58	115.23 ± 3.05	18.73 ± 1.48	52.97 ± 4.07
ERS				
15%	8.04 ± 0.79	9.09 ± 0.82	14.19 ± 1.73	6.05 ± 0.76
25%	13.19 ± 0.62	14.02 ± 0.74	16.85 ± 2.62	8.70 ± 2.65
30%	16.70 ± 1.37	17.04 ± 0.86	23.49 ± 0.79	12.30 ± 1.96
35%	23.99 ± 2.48	24.72 ± 1.35	32.18 ± 1.24	16.18 ± 0.98

4.3.5 *In vitro* gel formation

The *in vitro* gel formation of Ethocel (5-20%w/w), bleached shellac (15-30%w/w) and Eudragit RS (15-35%w/w) formula containing DH, MT and BP (5%w/w) in phosphate buffer pH 6.8 are shown in Figures 40-42, 43-45 and 46-48, respectively. The 5%w/w of Ethocel formula containing DH, MT and BP were flaccid gel after injected into phosphate buffer pH 6.8, whereas the 10%w/w of Ethocel formula containing DH, MT and BP were stronger than that of 5%w/w. The increased Ethocel amount in formula containing DH, MT and BP, the formed gel was much more solid and opaque. The 15%w/w of bleached shellac formula containing DH, MT and BP did not form the complete gelation, while the 20%w/w of bleached shellac formula containing DH, MT and BP were formed the elastic gel after injecting into PBS pH 6.8. The increased bleached shellac amount enhanced the elasticity and opacity of the prepared gel. This was due to the hydrophobic property of bleached shellac. The 15%w/w of Eudragit RS formula containing DH was formed gel, while that of formula containing MT and BP were not formed complete gelation. The Eudragit RS formula containing DH, MT and BP formed a greater stiff gel with the increasing amount of

polymers. The *in vitro* injection experiments suggested that the polymer solution could be easily injected into the periodontal pocket and formed *in situ* gel immediately. The higher concentrations of polymer would quickly precipitate. Hence the lower amount of polymer did not result in the gelation of the system, while increasing the polymer amount resulted in the rapid gelation as previously mentioned (Abashzadeh *et al.*, 2011).

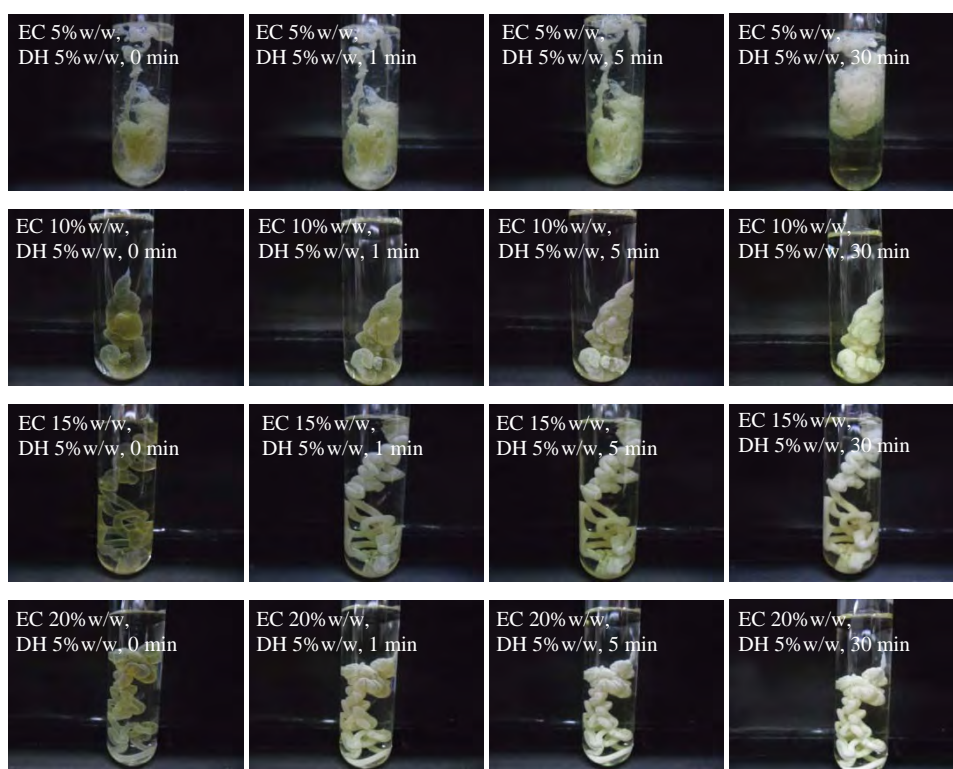


Figure 40 *In vitro* gel formation of Ethocel (5-20% w/w) formula containing 5% w/w doxycycline hyclate at various times (0, 1, 5 and 30 min)

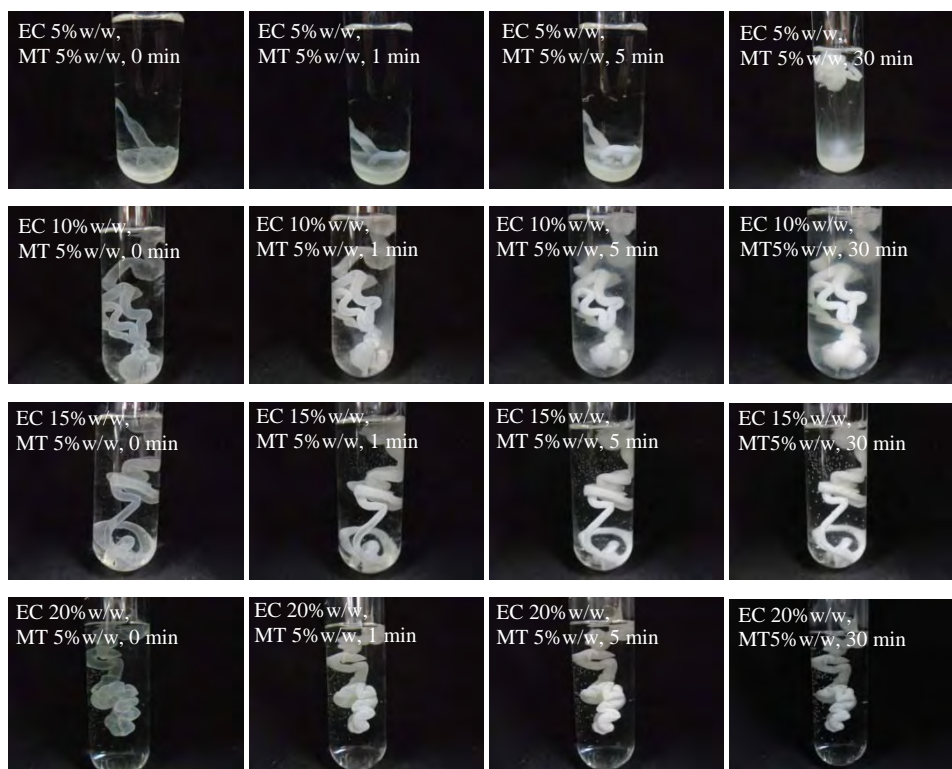


Figure 41 *In vitro* gel formation of Ethocel (5-20% w/w) formula containing 5% w/w metronidazole at various times (0, 1, 5 and 30 min)

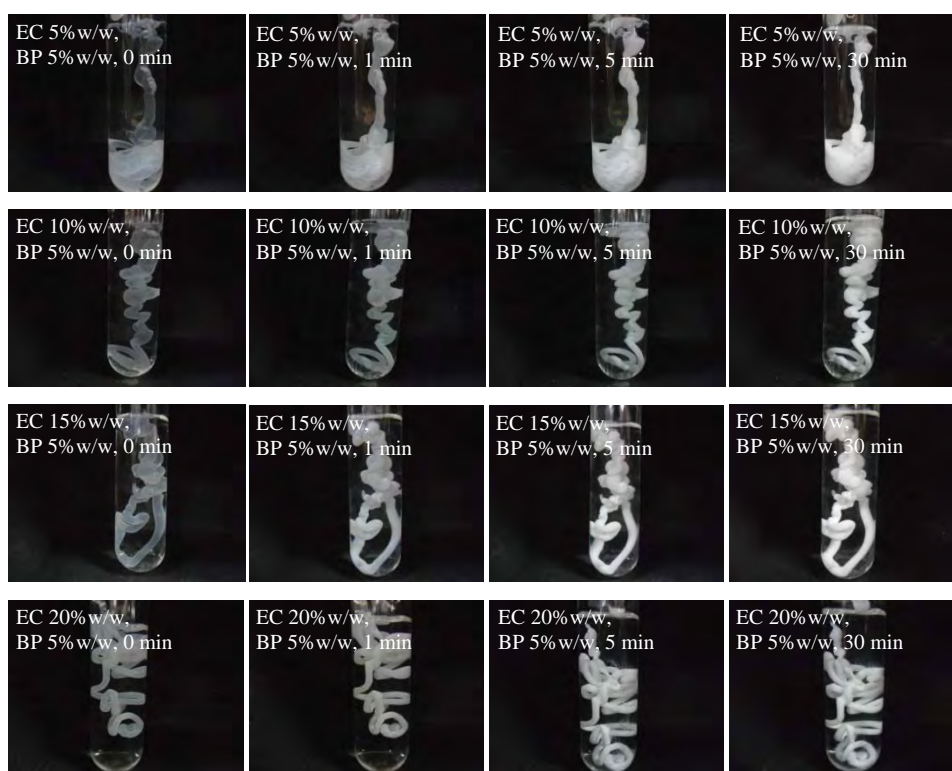


Figure 42 *In vitro* gel formation of Ethocel (5-20% w/w) formula containing 5% w/w benzoyl peroxide at various times (0, 1, 5 and 30 min)

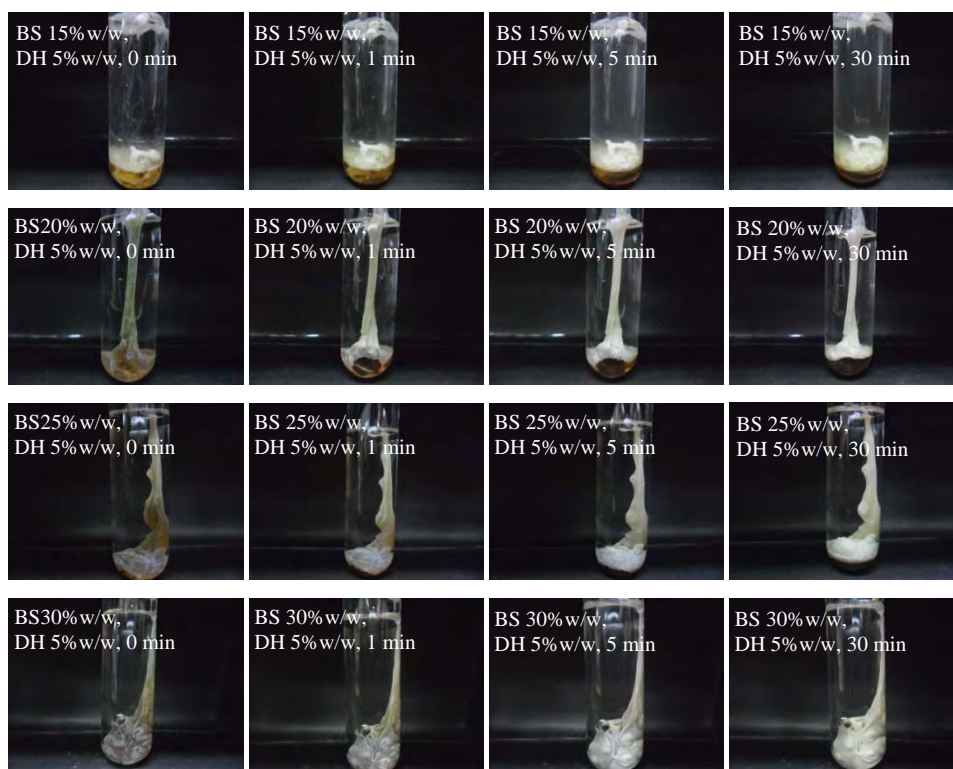


Figure 43 *In vitro* gel formation of bleached shellac (15-30% w/w) formula containing 5% w/w doxycycline hyclate at various times (0, 1, 5 and 30 min)

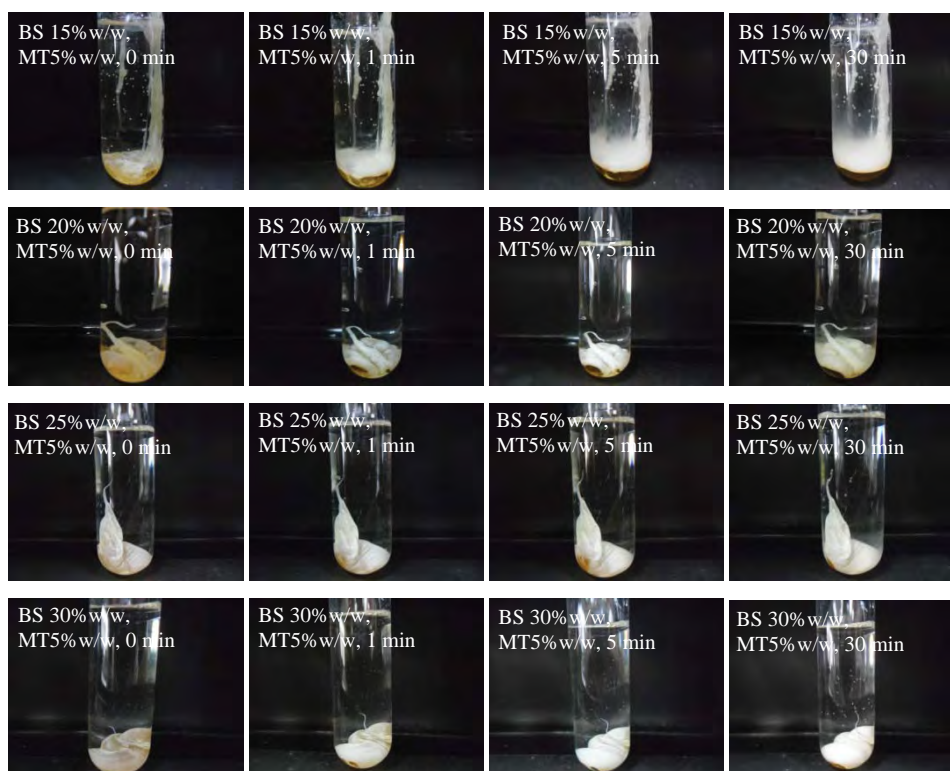


Figure 44 *In vitro* gel formation of bleached shellac (15-30% w/w) formula containing 5% w/w metronidazole at various times (0, 1, 5 and 30 min)

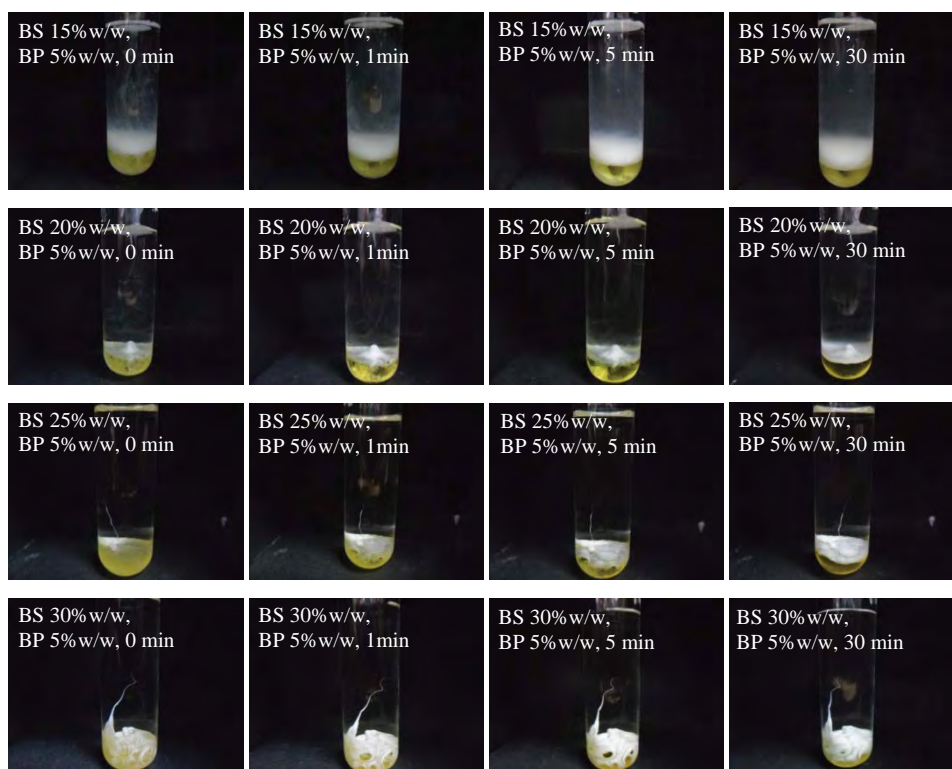


Figure 45 *In vitro* gel formation of bleached shellac (15-30% w/w) formula containing 5% w/w benzoyl peroxide at various times (0, 1, 5 and 30 min)

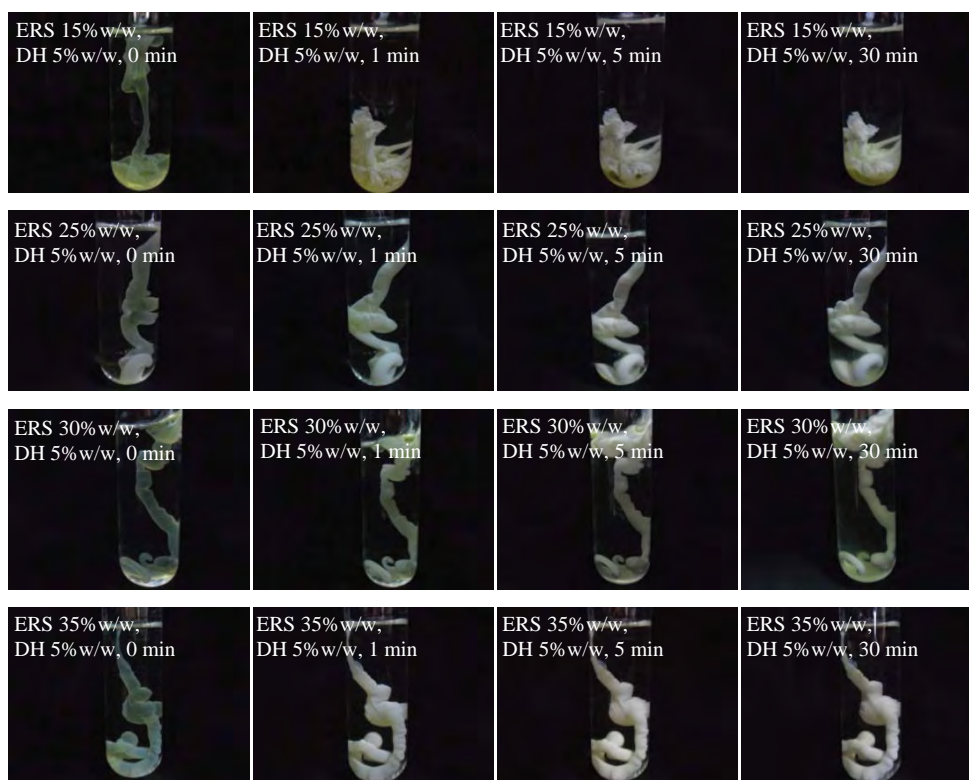


Figure 46 *In vitro* gel formation of Eudragit RS (15-35% w/w) formula containing 5% w/w doxycycline hyclate at various times (0, 1, 5 and 30 min)

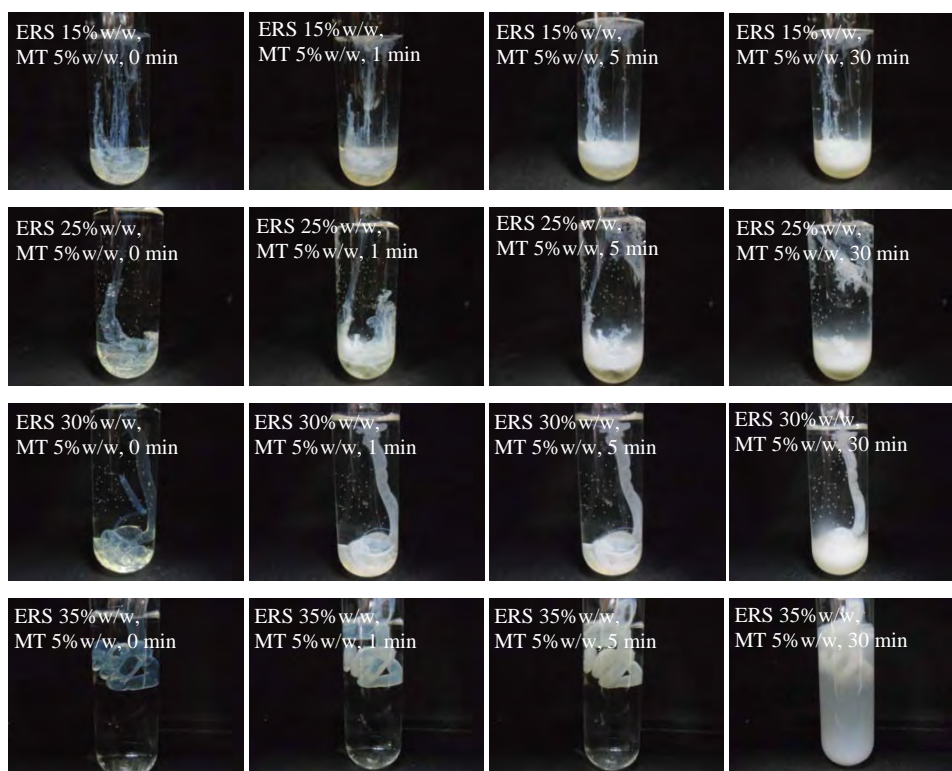


Figure 47 *In vitro* gel formation of (15-35%w/w) Eudragit RS formula containing 5%w/w metronidazole at various times (0, 1, 5 and 30 min)

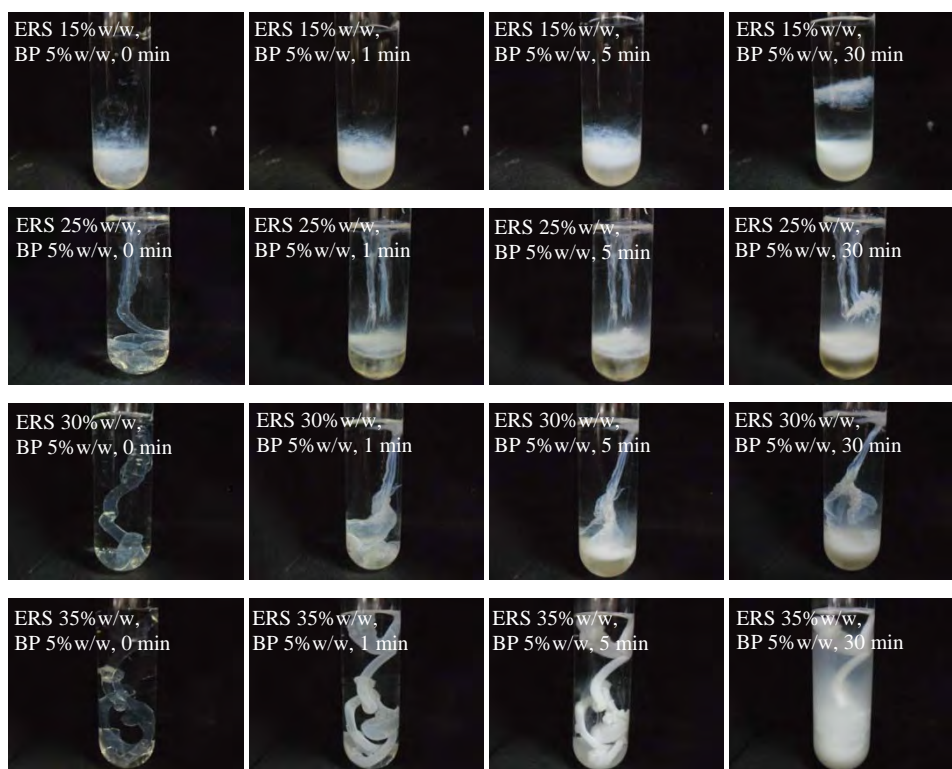


Figure 48 *In vitro* gel formation of Eudragit RS (15-35% w/w) formula containing 5% w/w benzoyl peroxide at various times (0, 1, 5 and 30 min)

4.3.6 Rate of water diffusion into the gels

The rate of water diffusion into the gels of Ethocel (5-20%w/w), bleached shellac (15-30% w/w) and Eudragit RS (15-35% w/w) formula comprising drugs (DH, MT and BP) are shown in Tables 20. The rate of water diffusion into the gels of the Ethocel systems containing drugs (DH, MT and BP) at 4 hours decreased with the increasing of Ethocel amounts ($p < 0.05$), whereas the rate of water diffusion into the gels of both of bleached shellac and Eudragit RS systems containing DH, MT and BP at 4 and 24 hours have the trend to decrease when increasing of polymer amount ($p > 0.05$). The results indicated that increasing polymer amount increased viscosity of *in situ* gel system and decreased rate of water diffusion into gels. It has been studied that the fast solvents extraction often followed by a fast drug release. On the other hand, for the drugs of poor solubility, a fast diffusion of DMSO would lead to a fast solidification of the implant, resulting in a high drug retention rate, which was reflected by the drug release (Wang *et al.*, 2012).

Table 20 Effect of polymer amounts in the formula containing different drugs (doxycycline hyclate, metronidazole and benzoyl peroxide) on rate of water diffusion into gels.

Concentration of polymers (% w/w)	Rate of water diffusion into gels (mm/min) (mean \pm S.D.)					
	with DH (5% w/w)		with MT (5% w/w)		with BP (5% w/w)	
	at 4 hours	at 24 hours	at 4 hours	at 24 hours	at 4 hours	at 24 hours
EC						
5%	0.0104 \pm 0.0013	0.0033 \pm 0.0002	0.0097 \pm 0.0012	0.0072 \pm 0.0004	0.0092 \pm 0.0004	0.0019 \pm 0.0001
10%	0.0101 \pm 0.0005	0.0028 \pm 0.0000	0.0090 \pm 0.0012	0.0047 \pm 0.0005	0.0069 \pm 0.0012	0.0019 \pm 0.0001
15%	0.0088 \pm 0.0004	0.0028 \pm 0.0001	0.0063 \pm 0.0000	0.0041 \pm 0.0005	0.0049 \pm 0.0012	0.0018 \pm 0.0002
20%	0.0078 \pm 0.0005	0.0022 \pm 0.0001	0.0049 \pm 0.0012	0.0020 \pm 0.0002	0.0042 \pm 0.0000	0.0017 \pm 0.0000
BS						
15%	0.0049 \pm 0.0012	0.0029 \pm 0.0005	0.0042 \pm 0.0021	0.0032 \pm 0.0005	0.0049 \pm 0.0012	0.0028 \pm 0.0003
20%	0.0056 \pm 0.0012	0.0032 \pm 0.0002	0.0035 \pm 0.0012	0.0032 \pm 0.0002	0.0028 \pm 0.0012	0.0025 \pm 0.0002
25%	0.0049 \pm 0.0012	0.0025 \pm 0.0002	0.0042 \pm 0.0021	0.0030 \pm 0.0004	0.0042 \pm 0.0000	0.0025 \pm 0.0002
30%	0.0049 \pm 0.0012	0.0025 \pm 0.0004	0.0028 \pm 0.0012	0.0030 \pm 0.0004	0.0028 \pm 0.0012	0.0025 \pm 0.0002
ERS						
15%	0.0049 \pm 0.0012	0.0030 \pm 0.0004	0.0069 \pm 0.0012	0.0049 \pm 0.0007	0.0056 \pm 0.0012	0.0031 \pm 0.0006
25%	0.0035 \pm 0.0012	0.0024 \pm 0.0000	0.0049 \pm 0.0012	0.0037 \pm 0.0005	0.0056 \pm 0.0012	0.0025 \pm 0.0002
30%	0.0028 \pm 0.0012	0.0023 \pm 0.0002	0.0035 \pm 0.0012	0.0035 \pm 0.0007	0.0035 \pm 0.0012	0.0024 \pm 0.0003
35%	0.0035 \pm 0.0012	0.0017 \pm 0.0003	0.0035 \pm 0.0012	0.0027 \pm 0.0005	0.0028 \pm 0.0012	0.0023 \pm 0.0002

4.3.7 *In vitro* drug release studies

4.3.7.1 Dialysis membrane method

The doxycycline hyclate release profiles of Ethocel, bleached shellac and Eudragit RS formula were evaluated using the dialysis membrane method (Figure 49-51). The drug release was tested in phosphate buffer pH 6.8 to simulate the environment of periodontitis. Drug release from all polymer systems was slower than that from a DH solution. The drug release profile of system containing DH (5% w/w) without polymer showed the fastest release rate with about 90% drug release at 3 hours (data not shown), whereas that of 5%, 10%, 15% and 20% w/w Ethocel systems containing DH (5% w/w) were about 90%, 85%, 84% and 80% drug release at 6 hours (Figure 49). The drug release profile of 15%, 20%, 25% and 30% w/w bleached shellac system containing DH (5% w/w) was about 75%, 57%, 49% and 43% drug release at 12 hours, respectively (Figure 50). The drug release profile of 15%, 25%, 30% and 35% w/w Eudragit RS systems containing DH (5% w/w) was about 90%, 86%, 82% and 80% drug release at 7 hours, respectively (Figure 51). The result indicated that the drug release from the *in situ* gel systems decreased with increasing of the polymer amount. It may be noted that all profiles were slower than the formula without polymer. It may occur through physical entanglements between polymers, producing a chemical crosslinked material is able to control the drug diffusion. It has been reported that the initial burst release significantly affected by polymer phase inversion dynamics and the increase in polymer concentrations could reduce the burst release (Liu and Venkatraman, 2012). The solvent type and polymer concentration were the most critical factors determining the drug release (Brodbeck *et al.*, 1999). The release of doxycycline hyclate from three different polymers implants has been studied. The more hydrophobic poly (dl-lactide co-caprolactone) (PLC) showed the slowest release of drug, whereas the hydrophilic poly (dl-lactide-co-glycolide) (PLG) lead to low initial release of drug followed by a more rapid release once the polymer becomes hydrated (Malik *et al.*, 2010). NMP exhibited a rapid phase inversion associated with a high drug burst due to the formation of a porous rubbery gel structure. Whereas other solvents such as triacetin and ethyl benzoate, both weak solvents for PLGA showed the slow gelation with the reducing of drug burst of proteins significantly. Therefore, solvent type and polymer concentration were the important

factors determining the drug release (Malik *et al.*, 2010). It has been studied that the concentration of polymer has few effect on the diffusional exponent, but it has great effect on the kinetic constant in Peppas' equation. Increasing HPMC concentration will decrease kinetic constant, so decrease release rate of a drug from HPMC matrices (Fu *et al.*, 2004). It has been reported that protein release kinetics was also influenced by solvent strength and water miscibility.

The cumulative releases of metronidazole and benzoyl peroxide from Eudragit RS formula using dialysis membrane method are shown in Figure 52. The metronidazole release of all Eudragit RS (15-35%w/w) systems containing metronidazole (5%w/w) were about 10% release in 6 hours and constant for 16 hours whereas, the 35%w/w Eudragit RS systems containing 5%w/w benzoyl peroxide was about 30% drug release in 20 hrs. These results suggested that the amount of Eudragit RS did not affect the metronidazole release. It has been reported that the hydroxyl group of metronidazole formed a conjugate covalently bonding with polymers, which could delay release by the slow hydrolysis of the bond polymer-drug formed (Mocanu *et al.*, 1993). It has been reported that cumulative release and release rate was affected by the drug solubility (Kiortsis *et al.*, 2005). Typically, the release of soluble drugs was faster than that of insoluble drug from all the matrix systems under the investigation (Reza *et al.*, 2003).

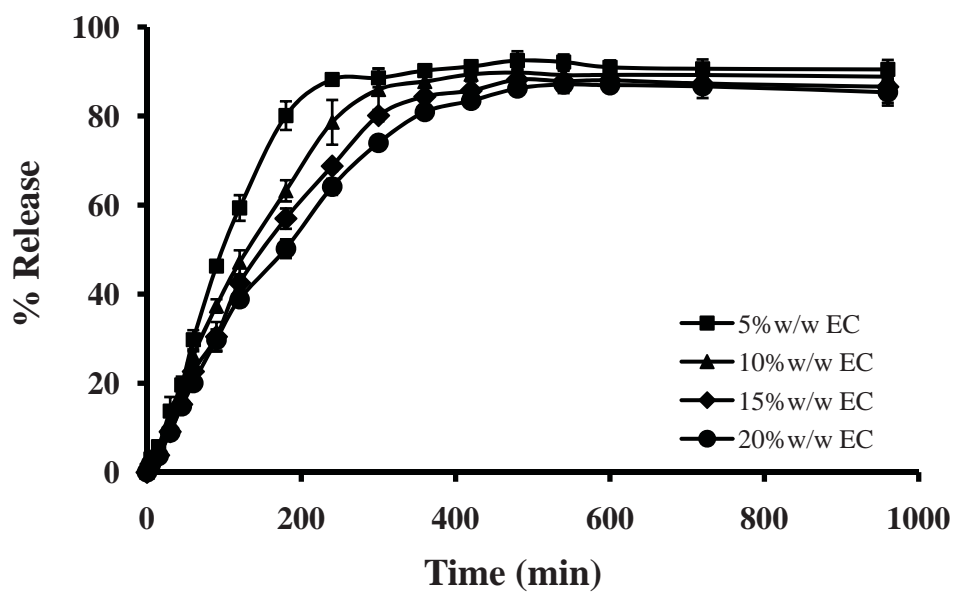


Figure 49 Effect of Ethocel amount on release of doxycycline hyclate using dialysis method.

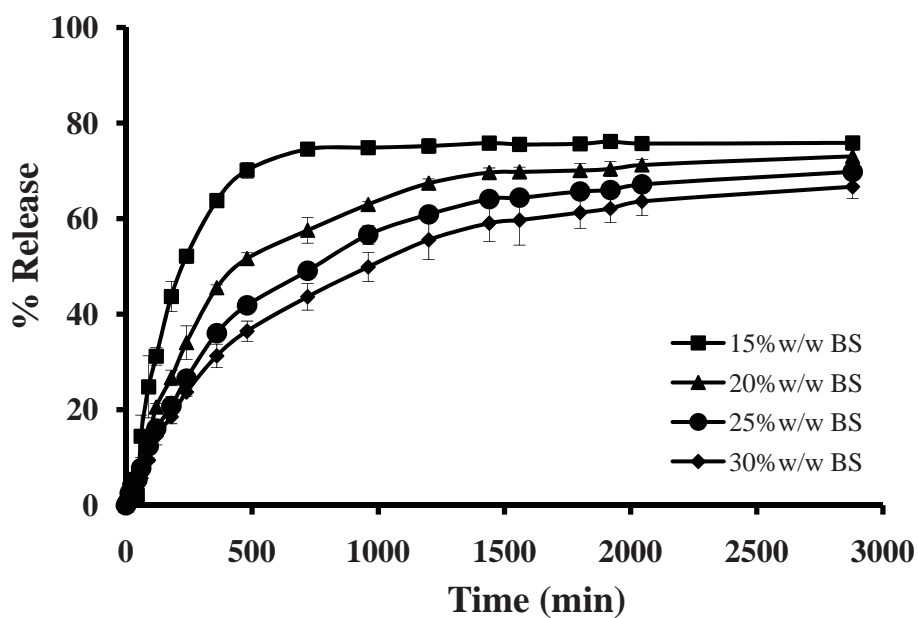


Figure 50 Effect of bleached shellac amount on release of doxycycline hyclate using dialysis method.

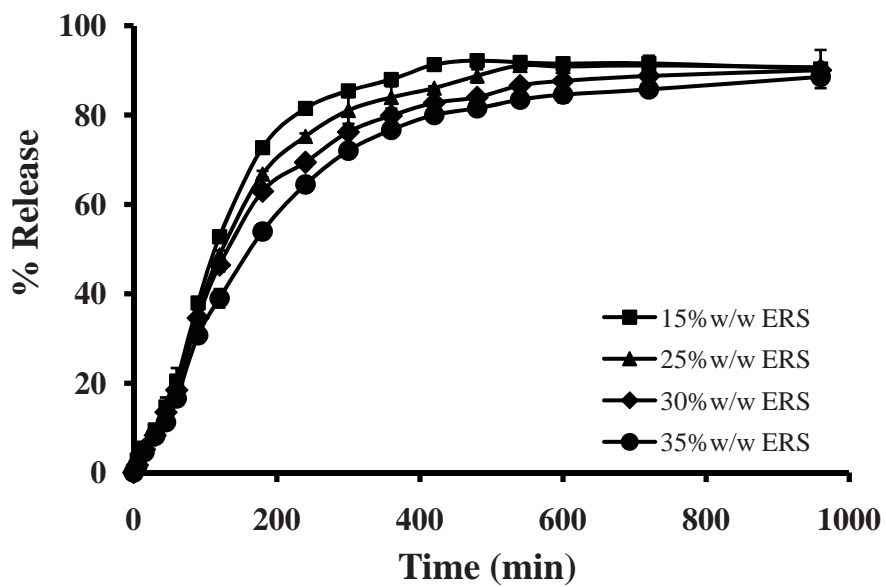


Figure 51 Effect of Eudragit RS amount on release of doxycycline hyclate using dialysis method.

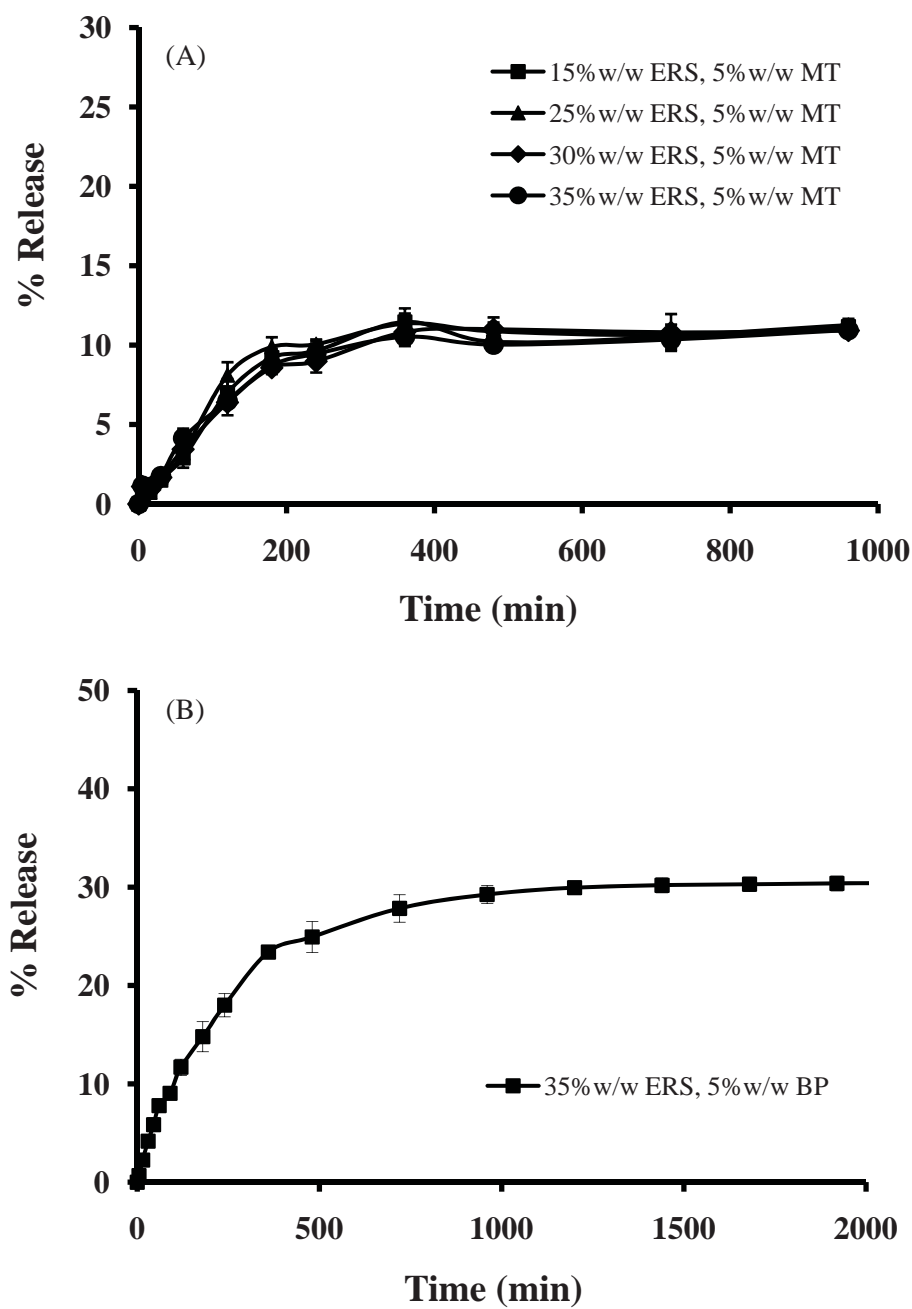


Figure 52 Effect of Eudragit RS amount on release of (A) metronidazole and (B) benzoyl peroxide using dialysis method.

4.3.7.2 Membrane-less diffusion method

The membrane-less diffusion method was studied to allow the medium solution to directly contact the gel surface and thus dissolve the gel. The drug release profiles of Ethocel, bleached shellac and Eudragit RS formula were evaluated using the membrane-less method (Figure 53-55). The drug release was tested in phosphate buffer pH 6.8 to simulating the environment of periodontitis. The doxycycline hyclate release from Ethocel systems containing 5%, 10%, 15% and 20% w/w Ethocel were 87%, 72%, 63% and 50% drug release for 24 hours, respectively (Figure 53). The doxycycline hyclate release from bleached shellac systems containing 15%, 20%, 25% and 30% w/w bleached shellac were 68%, 60%, 54% and 47% drug release for 54 hours, respectively (Figure 54). The doxycycline hyclate release from Eudragit RS systems containing 15% w/w, 25% w/w, 30% w/w and 35% w/w Eudragit RS were 91%, 79%, 71% and 68% drug release for 54 hours, respectively (Figure 55). These results indicated that the drug release from the formula decreased with an increased polymer amount. Higher polymeric content in the matrix decreased the release rate of drug because of the increased tortuosity and decreased porosity (Reza *et al.*, 2003). The drug release from bleached shellac formula was slower than that from Ethocel and Eudragit RS formula. Since the bleached shellac was hydrophobic polymer thus the drug hardly diffused pass this polymer. The specific properties of the network of polymer chains, e.g., the chain length, their flexibility and mobility, their water-uptake and swelling behavior, extent of plasticization, or potential interactions between polymer and drug would all potentially affect the diffusion rates in the polymer matrix and the drug release rate (Wischke and Schwendeman, 2008).

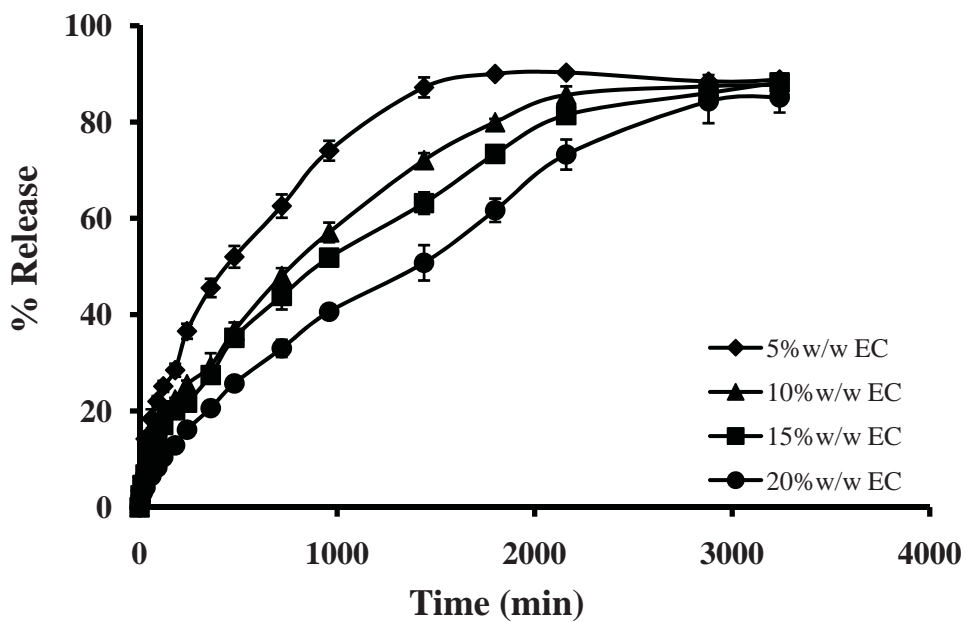


Figure 53 Effect of Ethocel amount on release of doxycycline hyclate using membrane-less method.

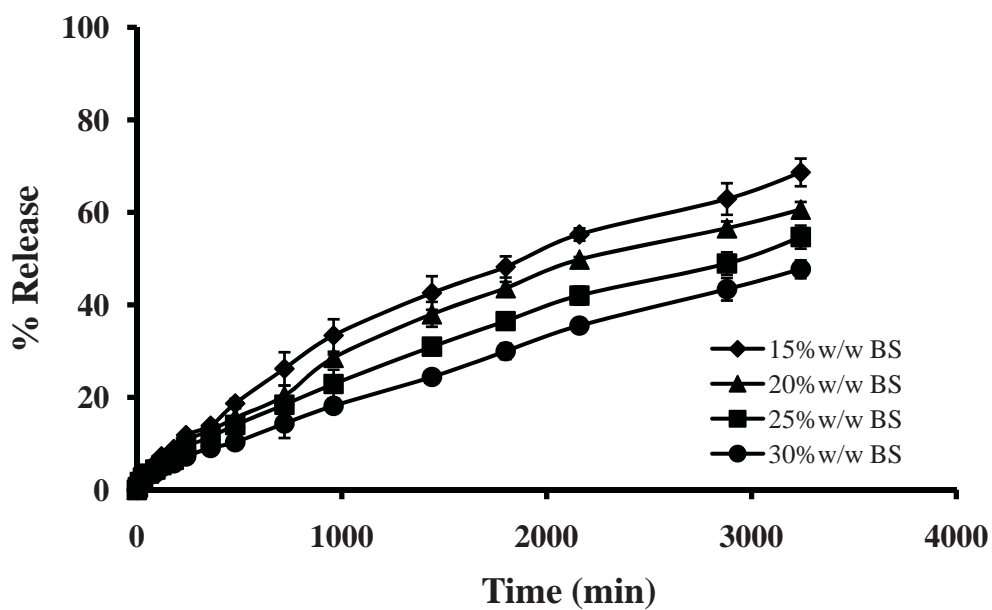


Figure 54 Effect of bleached shellac amount on release of doxycycline hyclate using membrane-less method.

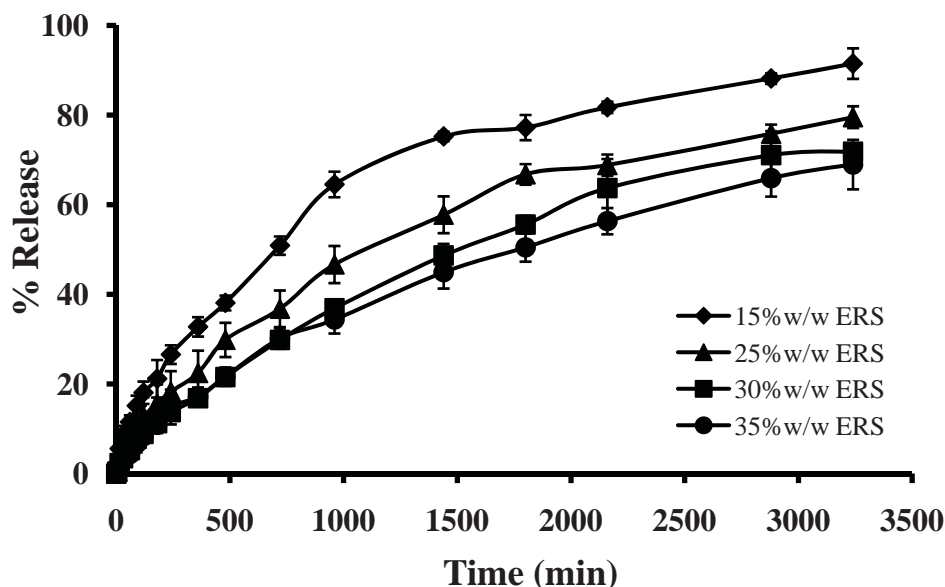


Figure 55 Effect of Eudragit RS amount on release of doxycycline hyclate using membrane-less method.

4.3.7.3 Analysis of drug release data

Release data of all formula were fitted to four different mathematical models (first order, Higuchi's, zero order and power law (Korsmeyer-Peppas) model) to characterize the mechanism of drug release. The high value of coefficient of determination (r^2) or model selection criteria (msc) indicated a superiority of the release profile fitting to mathematical equations. The r^2 and msc from curve fitting to first order, Higuchi's, zero order, and power law equations after the release studies using dialysis membrane method are shown in Table 21. The doxycycline hyclate release from all Ethocel formula using dialysis membrane method were fitted well with first order model since r^2 and msc from curve fitting were higher than Higuchi's model and zero order curve fitting. Whereas, the doxycycline hyclate release profile of all bleached shellac using dialysis membrane method were best explained by power law model, but a close relationship were also noted with first order kinetics. The doxycycline hyclate release from Eudragit RS (15% and 25%w/w) using dialysis membrane method were fitted well with first order model, whereas the doxycycline hyclate release from Eudragit RS (30% and 35%w/w) were fitted well with power law model. Whereas the doxycycline hyclate release from Eudragit RS (30% and

35% w/w) showed a close relationship were noted with first order kinetics. It has been reported that the drug release from hydrophobic polymer matrix in water provided the first order release profile (Khairuzzaman *et al.*, 2006).

The release exponent values (n) for power law of all formulations from the release studies using dialysis membrane method are shown in Table 22. The n value obtained from power law equation of the formula (Ethocel (5-10% w/w), bleached shellac (15-30% w/w) and Eudragit RS (15-35% w/w)) using dialysis membrane method ranged from 0.15-0.42. The results indicated that the formula showed drug release by Fickian diffusion mechanism. The drug release rate was decreased as a function of time due to a decrease in the concentration gradient. The drug release from system containing Ethocel (15-20% w/w) were anomalous or non-Fickian diffusion ($0.45 < n < 0.89$) indicated that the drug release was controlled by both mechanism of diffusion and polymeric chain relaxation (Pahwa *et al.*, 2011; Perioli *et al.*, 2004). The drug release rate (k) parameter after releasing studies using dialysis membrane method was investigated. The increased polymer amounts significantly decreased the drug release rate (k) ($p < 0.05$). Since the viscosity of the higher polymer amount formula is higher than that of lower polymer amount formula. The results showed the slower drug release therefore the polymer amount could sustain the doxycycline hyclate release from these systems (Table 22).

The r^2 and m sc from curve fitting to first order, Higuchi's, zero order, and power law equations after the releasing studies using membrane-less method are shown in Table 23. The doxycycline hyclate released from Ethocel (5-15% w/w), bleached shellac (15-25% w/w) and Eudragit RS (15-30% w/w) formula after release studies using membrane-less method were fitted well with first order model. Whereas the doxycycline hyclate released from Ethocel (20% w/w), bleached shellac (30% w/w) and Eudragit RS (35% w/w) formula were best explained by power law model, but a close relationship were also noted with first order kinetics. The release exponent values (n) for power law of all formula from the release studies using membrane-less method are shown in Table 24. The n value obtained from power law equation of all formula (Ethocel (5-20% w/w), bleached shellac (15-30% w/w) and Eudragit RS (25-35% w/w)) after release studies using membrane-less method ranged were

anomalous (non-Fickian) diffusion controlled release ($0.45 < n < 0.89$) except Eudragit RS (15% w/w) that showed drug release by Fickian diffusion. Considering the drug release rate (k) parameter after release studies using membrane-less method, it was indicated that the increased polymer amounts tended to decrease the drug release rate (k) ($p > 0.05$) that similar to the previous release studies using dialysis membrane method. The factors affecting release kinetic are liquid diffusion rate and polymeric chain relaxation rate. When the liquid diffusion rate is slower than the relaxation rate of the polymeric chains, the diffusion is Fickian, whereas when the relaxation process is very slow compared with the diffusion, the case II transport occurs. When liquid diffusion rate and polymer relaxation rate are of the same order of magnitude, anomalous or non-Fickian diffusion is observed (Perioli *et al.*, 2004). Solute transport from non-degradable polymeric systems is mainly considered as diffusion driven. Non-degradable polymers can be fabricated into “reservoir-” and “matrix-” type devices, which can be a rate-controlling membrane (Ful *et al.*, 2010). For matrix-type devices, drug release is more likely to be Fickian diffusion driven, which is associated with concentration gradient, diffusion distance, and the degree of swelling (Siepmann *et al.*, 2008; Lin *et al.*, 1985).

Table 21 Comparison of degree of goodness-of-fit from curve fitting of the release profiles of doxycycline hyclate in phosphate buffer pH 6.8 using dialysis membrane method to different release models.

Formula (% w/w)	First order		Higuchi's		Zero order		Power law	
	r ²	msc	r ²	msc	r ²	msc	r ²	msc
EC								
5%	0.9926	4.41	0.9621	2.70	0.9545	2.52	0.9774	2.93
10%	0.9963	5.17	0.9845	3.60	0.9398	2.37	0.9905	3.91
15%	0.9968	5.40	0.9796	3.49	0.9653	2.91	0.9826	3.45
20%	0.9980	5.86	0.9900	4.21	0.9780	3.37	0.9900	4.01
BS								
15%	0.9552	2.62	0.8798	1.67	0.9245	2.01	0.9953	4.51
20%	0.9261	2.27	0.8563	1.69	0.8705	1.60	0.9596	2.61
25%	0.9179	2.25	0.9277	2.23	0.9235	2.17	0.9727	3.23
30%	0.9244	2.32	0.9745	3.27	0.9289	2.28	0.9861	3.90
ERS								
15%	0.9766	3.49	0.9477	2.38	0.9265	2.11	0.9712	2.80
25%	0.9787	3.58	0.9429	2.36	0.9365	2.26	0.9734	2.96
30%	0.9710	3.27	0.9544	2.59	0.8798	1.67	0.9755	3.11
35%	0.9760	3.46	0.9738	3.20	0.9311	2.27	0.9864	3.70

Table 22 Estimate parameter from curve fitting of doxycycline hyclate release in phosphate buffer pH 6.8 using dialysis membrane method to power law expression.

Formula (% w/w)	k ± S.D.	tl ± S.D. (min)	n ± S.D.	Release mechanism
EC				
5%	0.1015 ± 0.0083	40.47 ± 1.85	0.40 ± 0.02	Fickian
10%	0.0784 ± 0.0101	40.35 ± 0.71	0.42 ± 0.02	Fickian
15%	0.0499 ± 0.0115	36.37 ± 2.27	0.49 ± 0.04	Anomalous
20%	0.0448 ± 0.0059	35.86 ± 1.18	0.49 ± 0.02	Anomalous
BS				
15%	0.2821 ± 0.0345	135.58 ± 33.40	0.15 ± 0.02	Fickian
20%	0.1131 ± 0.0148	110.92 ± 3.34	0.25 ± 0.02	Fickian
25%	0.0700 ± 0.0045	108.79 ± 0.61	0.30 ± 0.01	Fickian
30%	0.0507 ± 0.0074	103.05 ± 5.53	0.34 ± 0.01	Fickian
ERS				
15%	0.0774 ± 0.0037	41.86 ± 1.15	0.43 ± 0.01	Fickian
25%	0.0568 ± 0.0080	57.37 ± 1.11	0.32 ± 0.00	Fickian
30%	0.0209 ± 0.0032	59.14 ± 0.37	0.24 ± 0.00	Fickian
35%	0.0101 ± 0.0017	54.51 ± 1.22	0.38 ± 0.01	Fickian

k = release rate; tl = lag time and n = diffusional exponent

Table 23 Comparison of degree of goodness-of-fit from curve fitting of the release profiles of doxycycline hyclate in phosphate buffer pH 6.8 using membrane-less method to different release models.

Formula (% w/w)	First order		Higuchi's		Zero order		Power law	
	r ²	msc	r ²	msc	r ²	msc	r ²	msc
EC								
5%	0.9937	4.77	0.9874	3.98	0.9315	2.35	0.9927	4.33
10%	0.9956	5.16	0.9857	3.97	0.9673	3.11	0.9876	3.89
15%	0.9966	5.41	0.9933	4.71	0.9480	2.65	0.9943	4.74
20%	0.9925	4.59	0.9828	3.73	0.9740	3.34	0.9951	4.87
BS								
15%	0.9971	5.49	0.9932	4.59	0.9698	3.14	0.9970	5.20
20%	0.9956	5.05	0.9851	3.81	0.9711	3.18	0.9943	4.63
25%	0.9989	6.45	0.9809	3.56	0.9899	4.19	0.9985	5.89
30%	0.9984	6.03	0.9591	2.80	0.9970	5.42	0.9990	6.29
ERS								
15%	0.9931	4.69	0.9718	3.28	0.9398	2.45	0.9814	3.55
25%	0.9938	4.77	0.9894	4.24	0.9291	2.34	0.9895	4.09
30%	0.9965	5.32	0.9865	3.97	0.9548	2.76	0.9915	4.27
35%	0.9965	5.33	0.9954	5.06	0.9655	3.03	0.9983	5.86

Table 24 Estimate parameter from curve fitting of doxycycline hyclate release in phosphate buffer pH 6.8 using membrane-less method to power law expression.

Formula (%w/w)	k ± sd	tl ± sd (min)	n ± sd	Release mechanism
EC				
5%	0.0253 ± 0.0017	8.06 ± 7.41	0.49 ± 0.01	Anomalous
10%	0.0152 ± 0.0034	-52.41 ± 17.84	0.62 ± 0.03	Anomalous
15%	0.0093 ± 0.0014	-11.49 ± 13.20	0.58 ± 0.02	Anomalous
20%	0.0053 ± 0.0013	18.02 ± 21.74	0.64 ± 0.03	Anomalous
BS				
15%	0.0062 ± 0.0028	100.76 ± 9.84	0.59 ± 0.06	Anomalous
20%	0.0043 ± 0.0010	87.14 ± 16.44	0.62 ± 0.03	Anomalous
25%	0.0019 ± 0.0007	57.32 ± 9.29	0.70 ± 0.04	Anomalous
30%	0.0007 ± 0.0002	29.38 ± 6.37	0.82 ± 0.07	Anomalous
ERS				
15%	0.0321 ± 0.0039	47.01 ± 5.30	0.42 ± 0.01	Fickian
25%	0.0181 ± 0.0063	62.65 ± 3.14	0.48 ± 0.05	Anomalous
30%	0.0096 ± 0.0037	87.61 ± 1.77	0.55 ± 0.06	Anomalous
35%	0.0084 ± 0.0021	100.98 ± 11.66	0.55 ± 0.03	Anomalous

k = release rate; tl = lag time and n = diffusional exponent

4.3.8 Surface morphology of gels

Surface morphology and cross section area of the *in situ* gel was characterized using scanning electron microscopy. The collected samples after release test were frozen and dried by freeze-drying. The SEM micrographs of Ethocel formula with 5% w/w DH containing different amounts of Ethocel after releasing DH in PBS pH 6.8 at 37 °C at different magnifications are shown in Figure 56. The Ethocel (15-20% w/w) formula containing DH (5% w/w) showed the porous scaffold and interconnected porous structure. The pore sizes of structure were decreased with an increase of Ethocel amount. The structures were clarified at magnification 500X and 2000X which the porous structure was more enlarged. The SEM micrographs of Eudragit RS (15-35% w/w) formula with DH (5% w/w) containing different amounts of Eudragit RS after releasing DH in PBS pH 6.8 at 37 °C at different magnifications are shown in Figure 57. The structures of all Eudragit RS formula containing 5% w/w DH were spherical. These spheres were connected together and formed into the continuous phase. The particle size of Eudragit RS was decreased with increasing Eudragit RS amount. While the bleached shellac formula containing 5% w/w DH after releasing DH at 48 hours could not be formed dry structure after freeze drying since the NMP amount in the system after release studies was also high and it could not sublimate. Bleached shellac (acid) might be interacted with NMP (base) and then formed salt form of bleached shellac. NMP was stable at high temperature (boiling point 202-204°C) (Reynolds *et al.*, 2011). SEM study confirmed the diffusion mechanism which was during drug release from the *in situ* gel systems. SEM photomicrograph of *in situ* gel system taken after the release study showed that pores had been formed throughout the matrix. Hence, the formation of both pores and scaffolds structure on *in situ* gel systems indicated the involvement of both erosion and diffusion mechanisms to be responsible for controlling the release of drug from matrix. The results indicated that the solvent diffused out and water prepenetrated into the systems after release studies, indicating the highly porous structures formation. The porosity and pore-connectivity were suggested to ensure the solvent exchange and drug diffusion from *in situ* gel systems and then formed the scaffolds. The scaffolds of chitosan were prepared via *in situ* precipitation method and freeze-dried to obtain

porous scaffolds (Li *et al.*, 2010). Many studies showed the injectable biomaterials formed scaffolds *in situ* that could prove useful in injectable drug delivery for tissue engineering (Gaikwad *et al.*, 2008; Poursamar *et al.*, 2011; Rezaei and Mohammadi, 2013).

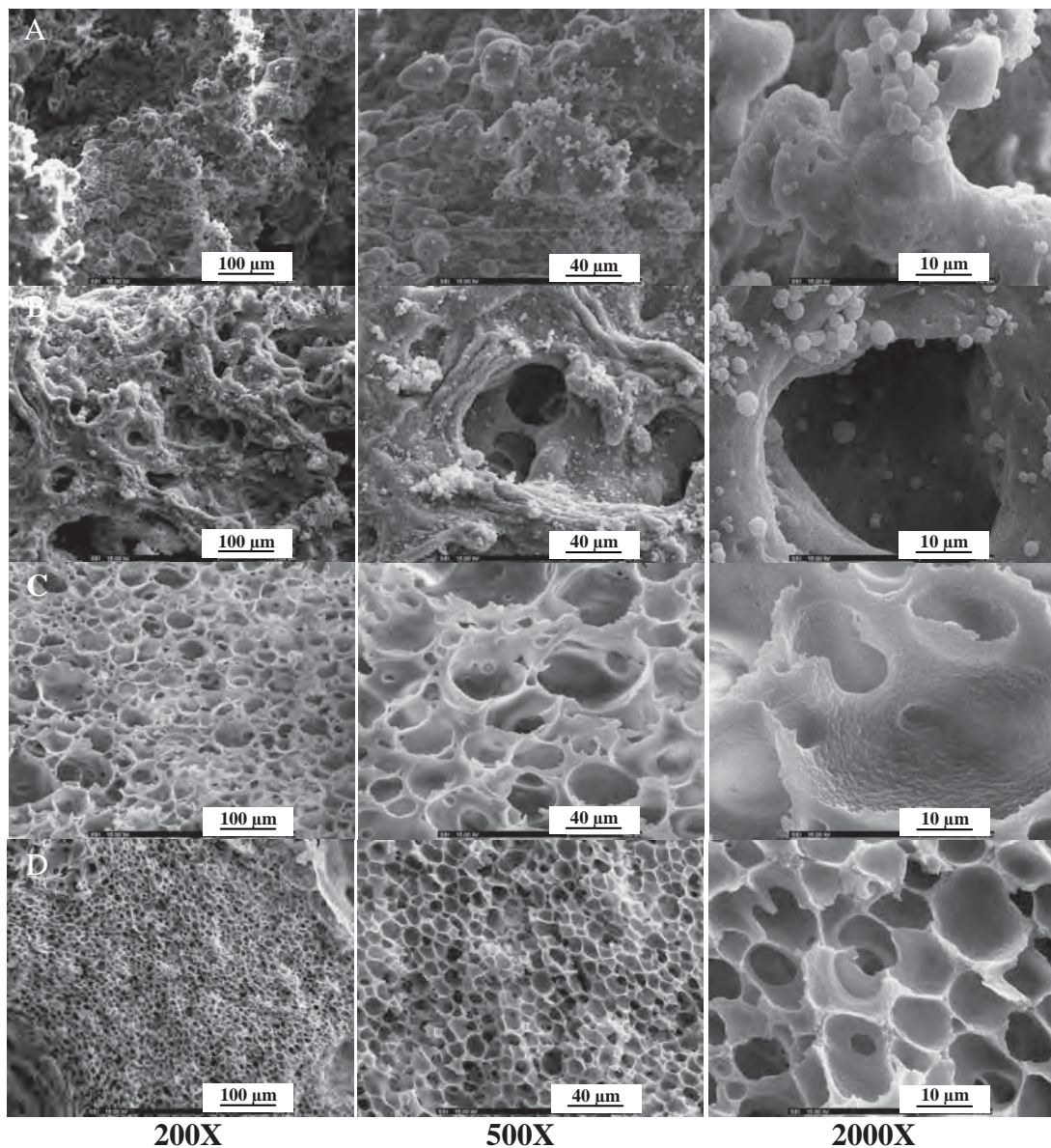


Figure 56 SEM micrograph of the dried gel systems; Doxycycline hyclate (5% w/w)-Ethocel systems containing 5% w/w Ethocel (A); 10% w/w Ethocel (B); 15% w/w Ethocel (C) and 20% w/w Ethocel (D) with different magnifications (200X, 500X and 2000X).

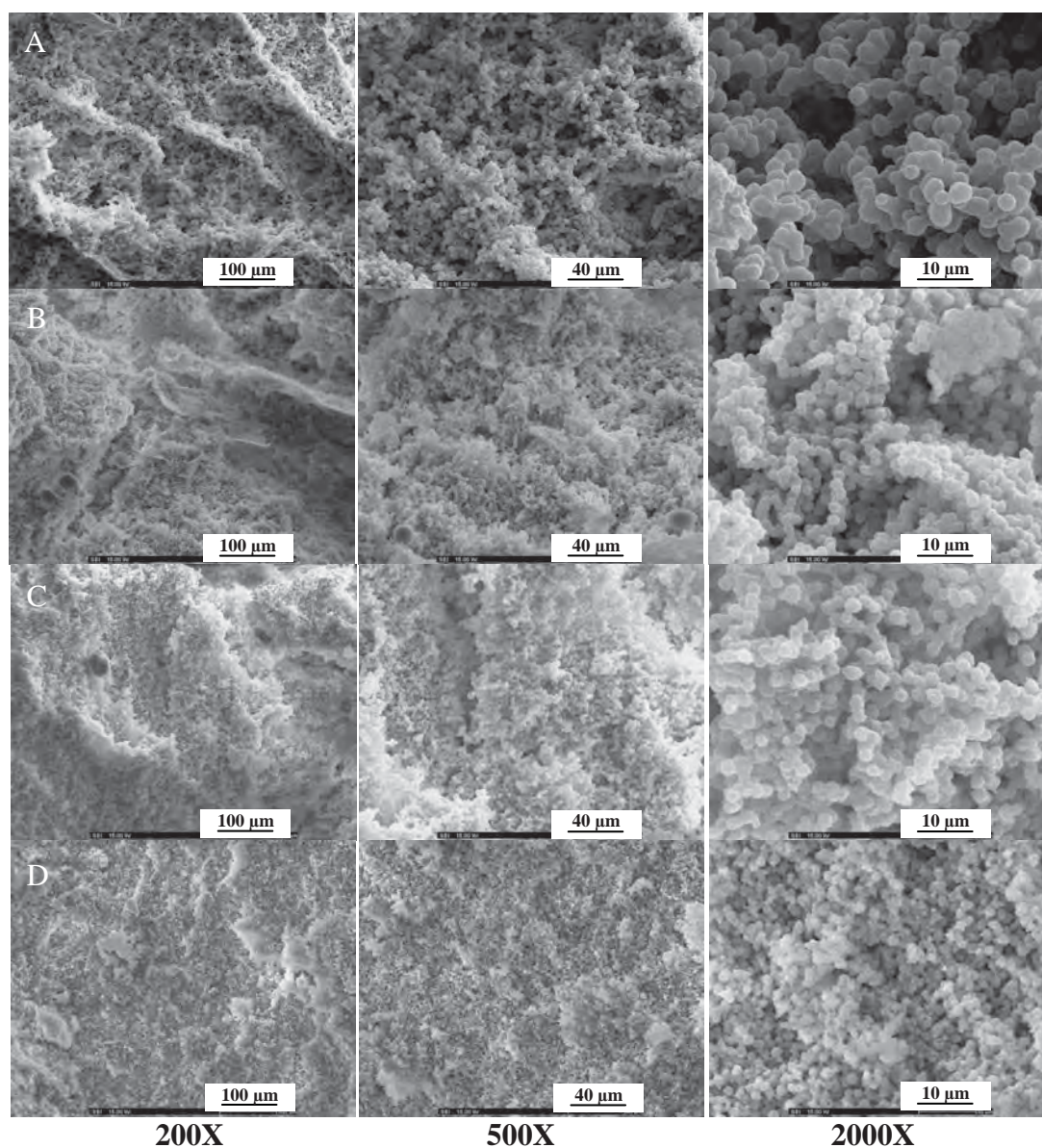


Figure 57 SEM micrograph of the dried gel systems; Doxycycline hyclate (5% w/w)-Eudragit RS systems containing 15% w/w Eudragit RS (A); 25% w/w Eudragit RS (B); 30% w/w Eudragit RS (C) and 35% w/w Eudragit RS (D) with different magnifications (200X, 500X and 2000X).

4.3.9 *In vitro* degradation studies

The weight loss of the prepared gels was evaluated in phosphate buffer pH 6.8 after 1 month. The percentage of weight loss of the Ethocel, bleached shellac and Eudragit RS formula containing different types of drugs (DH, MT and BP) are shown in Table 25. The percentage of weight loss of the Ethocel, bleached shellac and Eudragit RS formula significantly decreased with increasing polymer amounts ($p < 0.05$). In addition, the percentage of weight loss of the Ethocel, bleached shellac and Eudragit RS formula containing drugs (DH, MT and BP) was significantly decreased with increasing polymer amounts ($p < 0.05$). The results indicated that the weight loss of the prepared gel occurred by the diffusion of drug and solvent after exchange with medium. The degradation study could describe by weight remaining and molecular weight loss (Ren *et al.*, 2006).

Table 25 The percentage of weight loss of formula containing different drugs (doxycycline hyclate, metronidazole and benzoyl peroxide) (n=3)

Formula (% w/w)	% Weight loss (n=3)			
	Without drug	With drug (5% w/w)		
		DH	MT	BP
EC				
5%	94.05 ± 0.44	99.72 ± 0.31	99.33 ± 0.26	95.30 ± 0.39
10%	89.14 ± 0.37	89.12 ± 0.31	89.30 ± 0.22	89.04 ± 0.46
15%	84.22 ± 0.18	84.95 ± 0.20	84.94 ± 0.22	83.93 ± 0.57
20%	79.09 ± 0.10	79.47 ± 0.54	79.74 ± 0.14	78.68 ± 0.16
BS				
15%	90.36 ± 0.66	89.14 ± 0.07	89.89 ± 0.63	87.05 ± 0.45
20%	83.18 ± 0.86	82.59 ± 1.04	82.99 ± 1.87	81.20 ± 0.41
25%	74.82 ± 0.15	72.00 ± 3.55	71.49 ± 1.24	75.19 ± 2.77
30%	68.75 ± 1.19	64.62 ± 5.79	70.71 ± 2.53	66.32 ± 4.91
ERS				
15%	93.17 ± 0.71	86.52 ± 0.49	98.40 ± 0.31	98.33 ± 0.27
25%	88.89 ± 0.99	75.00 ± 0.25	93.21 ± 7.47	99.47 ± 0.70
30%	84.18 ± 0.11	69.46 ± 0.28	93.44 ± 2.03	99.07 ± 0.98
35%	73.18 ± 0.89	61.17 ± 2.92	73.93 ± 2.18	88.67 ± 4.81

4.3.10 Antimicrobial activity studies

The inhibition zone diameter of the Ethocel (5-20% w/w), bleached shellac (15-30% w/w) and Eudragit RS (15-35% w/w) formula containing different types of drugs (DH, MT and BP) against *S. aureus*, *E. coli*, *C. albicans*, *S. mutans* and *P. gingivalis* using agar diffusion method are shown in Figures 58-60. The inhibition zone diameter against *S. aureus*, *E. coli*, *S. mutans* and *P. gingivalis* of the Ethocel (5-20% w/w) systems containing DH (5% w/w) were significantly higher than that of the gel base (without drugs) ($p < 0.05$). On the other hand, the inhibition zone diameter against *S. aureus*, *S. mutans* and *P. gingivalis* of the Ethocel (5-20% w/w) systems containing MT (5% w/w) were significantly higher than that of the gel based ($p < 0.05$). The inhibition zone diameter against *P. gingivalis* of the Ethocel, bleached shellac and Eudragit RS systems containing BP (5% w/w) were significantly higher than that of the gel base ($p < 0.05$). The increased Ethocel (5-20% w/w) amount in the systems containing 5% w/w drugs (DH, MT and BP) did not affect the antimicrobial activity against all microbes ($p > 0.05$). However the Ethocel systems containing 5% w/w MT, the increased amount of Ethocel in preparation significantly decreased inhibition zone diameter against *C. albicans* ($p < 0.05$) (Figure 58). The inhibition zone diameter against *S. aureus*, *E. coli*, *S. mutans* and *P. gingivalis* of the bleached shellac (15-30% w/w) and Eudragit RS (15-35% w/w) systems containing DH and MT (5% w/w) were significantly higher than that of the gel base (without drugs) ($p < 0.05$). When the bleached shellac amount was increased from 15% w/w to 30% w/w, the inhibition zone diameter against all microbes significantly decreased ($p < 0.05$) (Figure 59). The Eudragit RS amount was increased from 15% w/w to 35% w/w, the inhibition zone diameter against all microbes significantly decreased ($p < 0.05$) (Figure 60). The results supported that the increased bleached shellac and Eudragit RS amount could sustain release drugs. Doxycycline hyclate displayed a variable degree of antimicrobial activity against different tested strains. Doxycycline hyclate showed the highest activity was significant in broad spectrum against all tested microorganisms. Gram-positive bacteria are more sensitive to the presence of doxycycline hyclate than Gram-negative bacteria. This is possibly because Gram-negative bacteria possess an outer membrane surrounding the cell wall, which restricts diffusion of hydrophobic

compounds through its lipopolysaccharide covering (Beveridge, 1999). In the addition, metronidazole displayed a well antimicrobial activity against both *S. mutans* and *P. gingivalis*. The results suggested that Ethocel, bleached shellac and Eudragit RS systems containing doxycycline hyclate, metronidazole and benzoyl peroxide (5%w/w) showed the antimicrobial activities against *S. mutans* and *P. gingivalis*. However, the antimicrobial activities were decreased as the amount of polymer was increased. Because of the higher polymeric content in the matrix the release rate of drug was decreased regarding the increasing of tortuosity and decreasing porosity (Reza *et al.*, 2003).

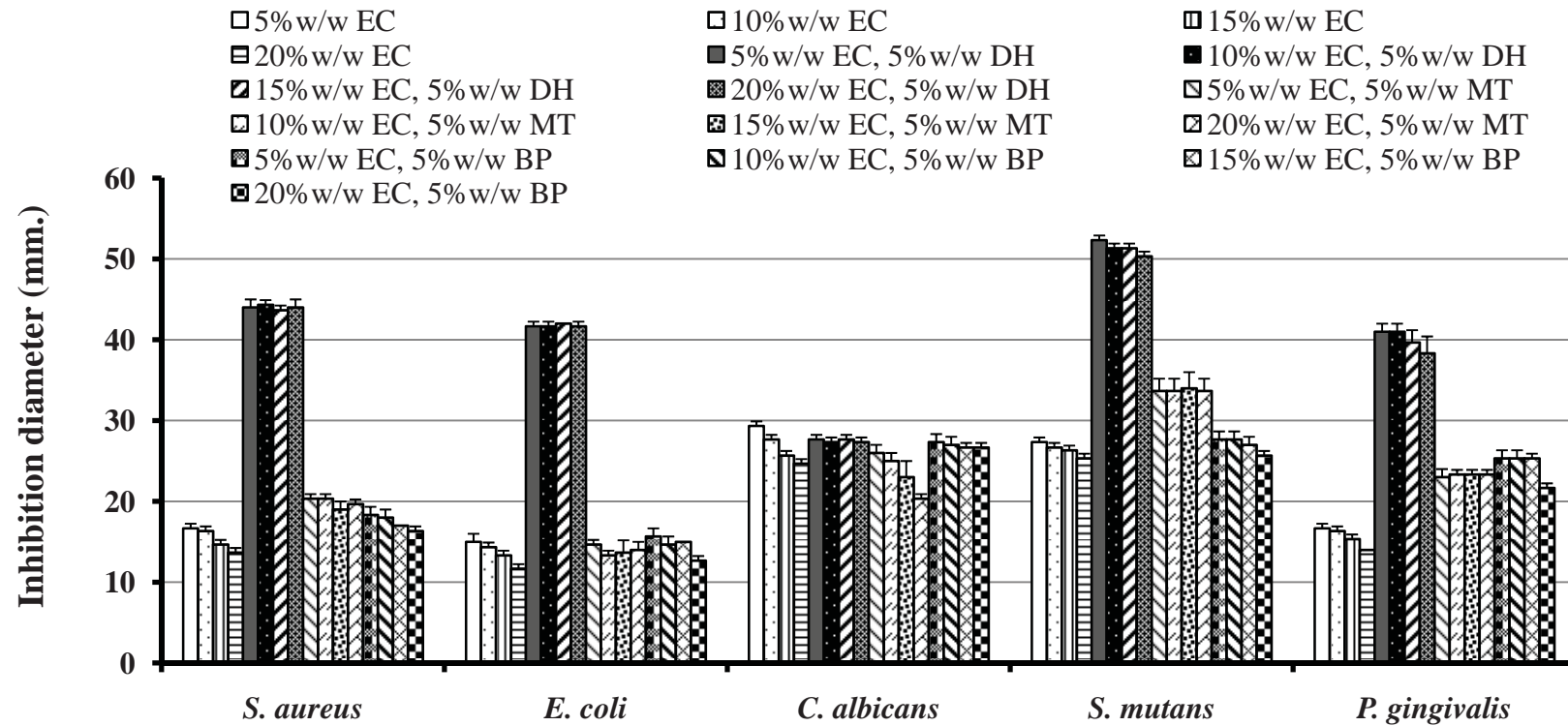


Figure 58 Inhibition zone diameter of the Ethocel formula containing different types of drugs (doxycycline hyclate, metronidazole and benzoyl peroxide) against *S. aureus*, *E. coli*, *C. albicans*, *S. mutans* and *P. gingivalis*.

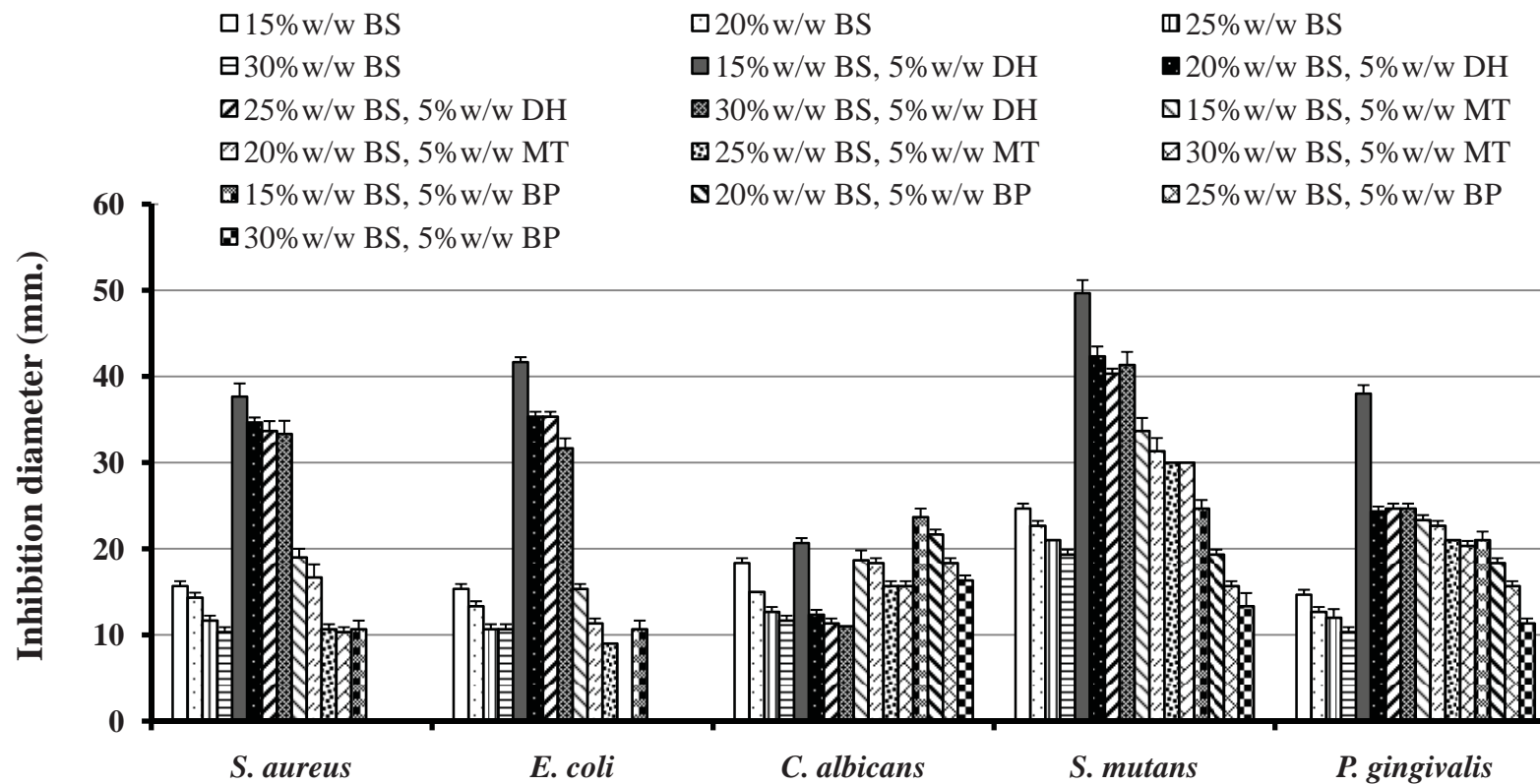


Figure 59 Inhibition zone diameter of the bleached shellac formula containing different types of drugs (doxycycline hyclate, metronidazole and benzoyl peroxide) against *S. aureus*, *E. coli*, *C. albicans*, *S. mutans* and *P. gingivalis*.

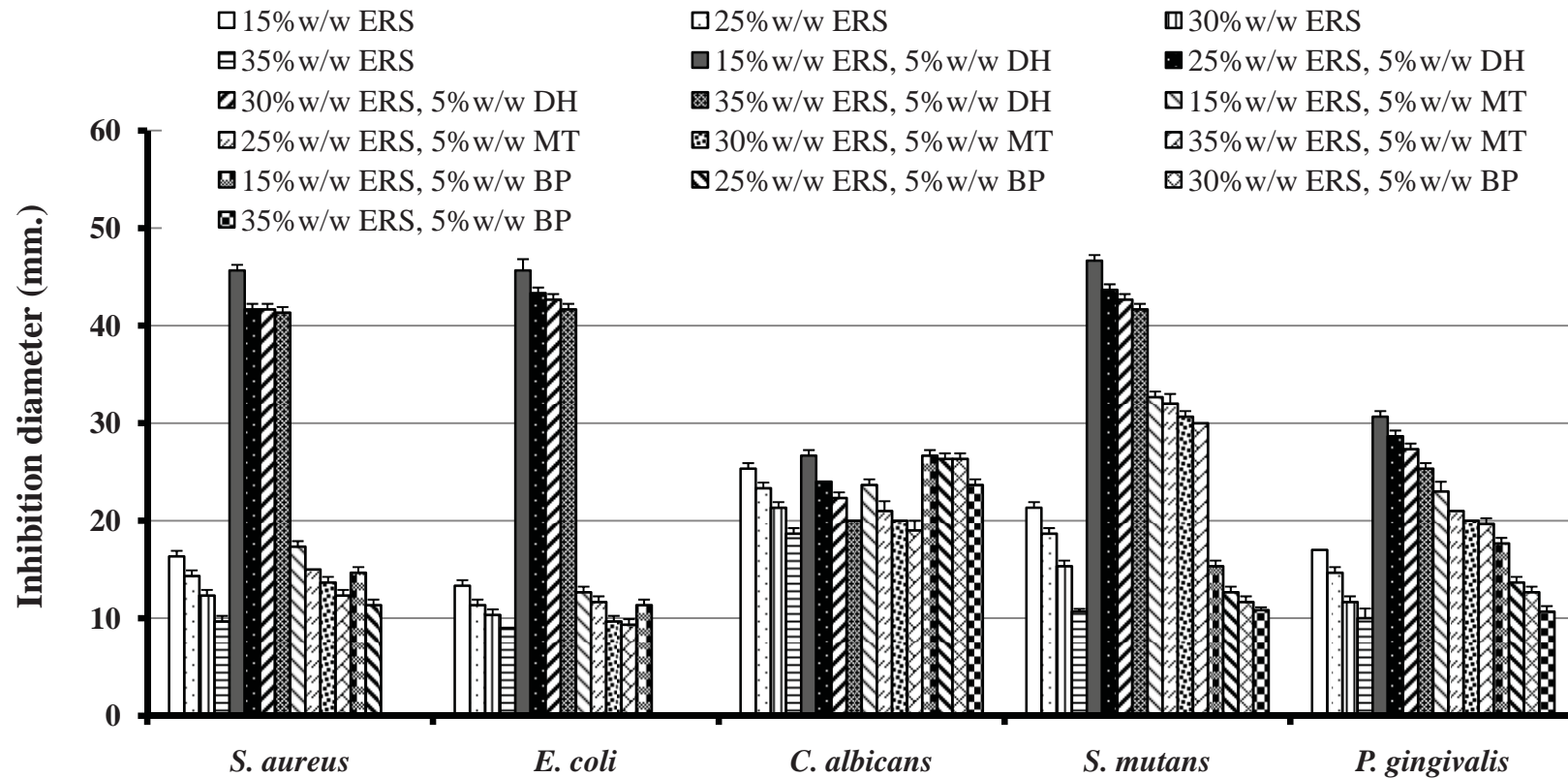


Figure 60 Inhibition zone diameter of the Eudragit RS formula containing different types of drugs (doxycycline hyclate, metronidazole and benzoyl peroxide) against *S. aureus*, *E. coli*, *C. albicans*, *S. mutans* and *P. gingivalis*.

4.4 Investigation of an influence of hydrophilic (polyethylene glycol 1500) and hydrophobic (peppermint oil) agents on physicochemical properties of the *in situ* forming gel systems

It is known that the addition of plasticizer could change the physicochemical properties of the polymer (Snejdrova and Dittrich, 2012). Two plasticizers represented as hydrophilic and hydrophobic plasticizer, respectively were investigated in this study. The influence of hydrophilic (polyethylene glycol 1500) and hydrophobic (peppermint oil) agents were examined on physicochemical properties of the selected formulations. The 35% w/w Eudragit RS system was concerned and suitably selected in the further studies. Since the 35%w/w of Eudragit RS systems could reduce the burst drug release and its viscosity could be sufficiently low for good injectability via an injection needle.

4.4.1 Influence of hydrophilic substance (polyethylene glycol 1500)

The appearances and pH values of the 35% w/w Eudragit RS formula containing different amounts of polyethylene glycol 1500 without and with doxycycline hyclate (5%w/w) is shown in Table 26. The gel base systems and containing polyethylene glycol 1500 (without drug) were clear and colorless, whereas all systems with doxycycline hyclate were yellow and clear. The pH values of 35% w/w Eudragit RS formula containing PEG1500 (2.5-10%w/w) without and with DH (5%w/w) were in the range of 8.25 ± 0.02 to 8.54 ± 0.02 and 3.70 ± 0.02 to 3.77 ± 0.01 , respectively, whereas the pH values of 35%w/w Eudragit RS system without and with DH (5%w/w) were 8.69 ± 0.09 and 3.77 ± 0.05 , respectively. The effects of polyethylene glycol 1500 (PEG1500) amounts on viscosity of Eudragit RS (35%w/w) formula without and with doxycycline hyclate were investigated. The relationships between shear rate and apparent viscosity of PEG1500 (2.5-10%w/w) in Eudragit RS (35%w/w) formula without and with doxycycline hyclate (5%w/w) are presented in Figures 61 and 62, respectively. The apparent viscosity of 35%w/w Eudragit RS formulation without and with 5%w/w DH was increased as the PEG1500 amount was increased. The apparent viscosities of 35%w/w Eudragit RS formula without and with 5%w/w DH containing PEG1500 (2.5-10%w/w) were constant when the shear rate was increased indicating

Newtonian. The apparent viscosities of 35%w/w Eudragit RS formula without and with 5% w/w DH containing PEG1500 at 37°C were lower than that of formula at 25°C. Eudragit RS (35%w/w) formula without and with 5%w/w DH containing PEG1500 (2.5-10%) exhibited a decrease in viscosity with increasing temperature.

Table 26 Gel appearance and pH value of 35% w/w Eudragit RS formula containing different amounts of polyethylene glycol 1500 and without and with doxycycline hyclate (5% w/w)

Amount of PEG1500 (% w/w)	Appearance	Clarity	pH \pm S.D. (n=3)
<i>Without drug</i>			
2.5%	Colorless	Clear	8.25 \pm 0.02
5%	Colorless	Clear	8.44 \pm 0.02
7.5%	Colorless	Clear	8.47 \pm 0.02
10%	Colorless	Clear	8.54 \pm 0.02
<i>With DH (5%w/w)</i>			
2.5%	Yellow	Clear	3.77 \pm 0.01
5%	Yellow	Clear	3.70 \pm 0.02
7.5%	Yellow	Clear	3.74 \pm 0.05
10%	Yellow	Clear	3.75 \pm 0.02

The rheological behaviors of 35%w/w Eudragit RS formula without and with 5% w/w DH containing PEG1500 (2.5-10% w/w) were investigated. The shear stress versus shear rate flow curves of 35%w/w Eudragit RS formula without and with 5% w/w DH containing PEG 1500 (2.5-10% w/w) at different temperature are shown in Figures 63 and 64, respectively. The shear stress of 35%w/w Eudragit RS systems without and with 5% w/w DH was increased as the amount of PEG1500 was increased. However, the shear stress of 35%w/w Eudragit RS systems without and with 5% w/w DH containing PEG1500 (2.5-10% w/w) was decreased with an increasing temperature. All formulations showed Newtonian behavior and the up curve did coincide with the down curve. The curves of 35%w/w Eudragit RS formula without and with 5%w/w DH moved to a higher shear stress value with increasing PEG1500 amounts indicating compact structure of the gels. On the other hand, the curves of 35%w/w Eudragit RS formula without and with 5% w/w DH containing PEG1500 (2.5-10% w/w) moved to a lower shear stress value with increasing temperature indicating loose structure of the gels.

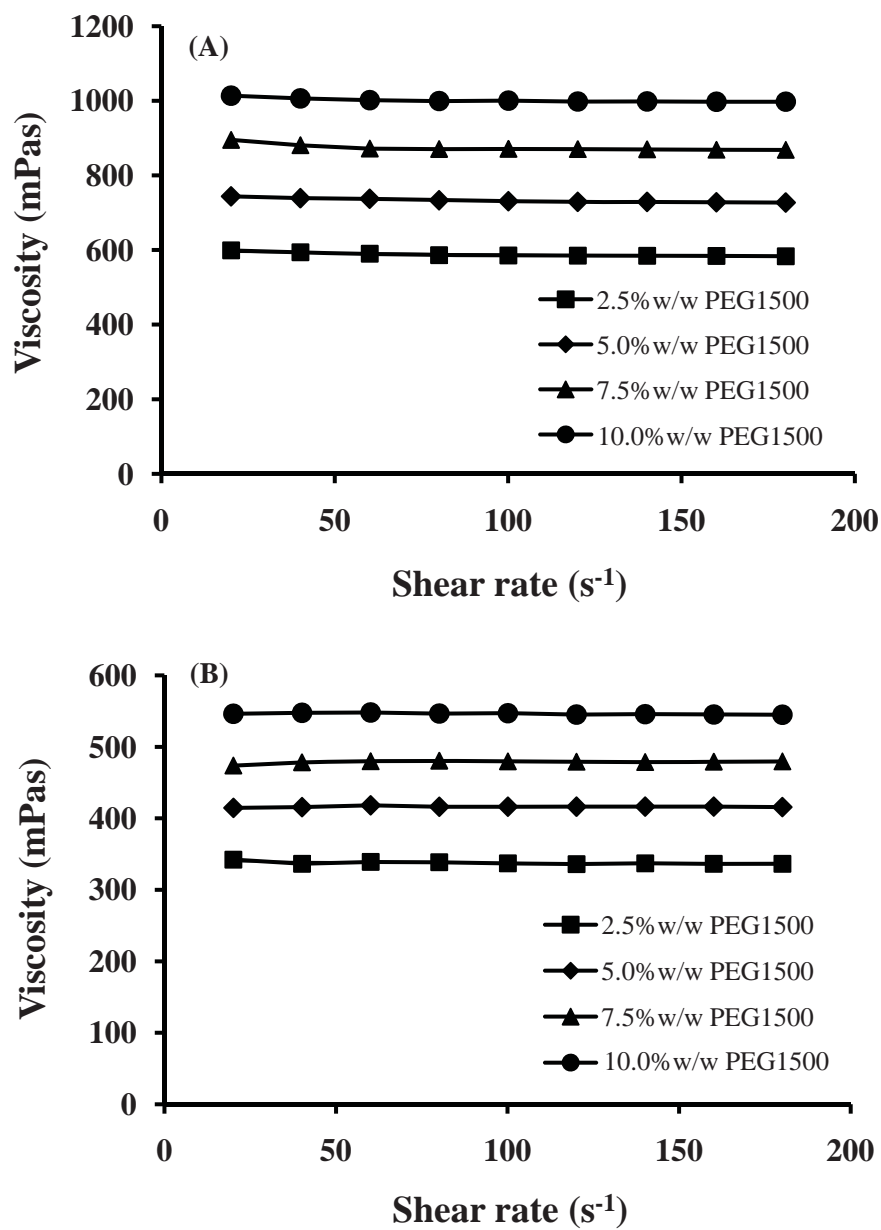


Figure 61 Viscosity curves of Eudragit RS formula containing different amount of polyethylene glycol 1500 (2.5%, 5%, 7.5% and 10% w/w) at (A) 25°C and (B) 37°C

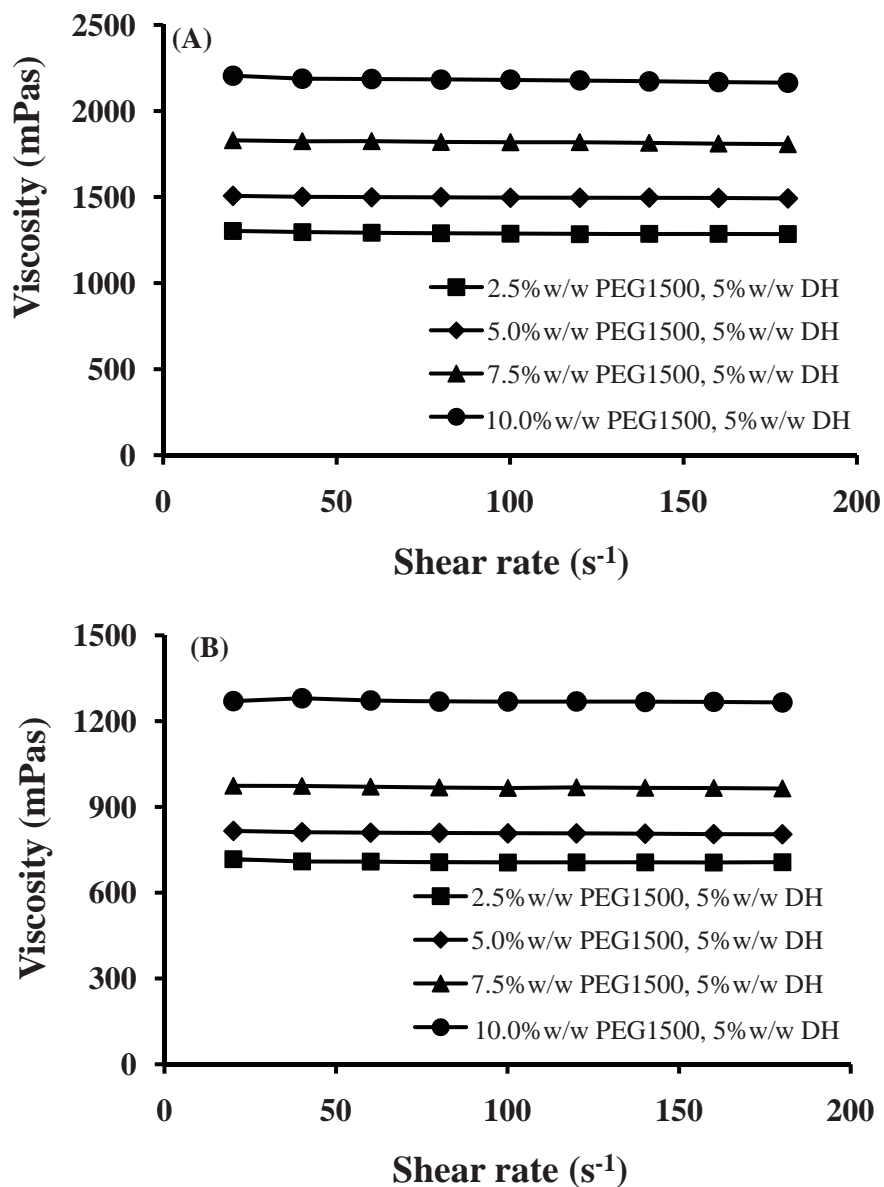


Figure 62 Viscosity curves of Eudragit RS (35% w/w)-doxycycline hyclate (5% w/w) formula containing different amount of polyethylene glycol 1500 (2.5%, 5%, 7.5% and 10% w/w) at (A) 25°C and (B) 37°C

N value of 35%w/w Eudragit RS formula without and with 5%w/w DH containing PEG1500 (2.5-10%w/w) was close to 1, indicating that the flow type was Newtonian. However, the increasing temperature did not change the flow types of the systems. In the case of the viscosity coefficient (or consistency index, η), as the PEG1500 amount of 35%w/w Eudragit RS formula without and with 5%w/w DH was higher, the viscosity coefficient was also significantly greater ($p<0.05$). In contrast, the viscosity coefficient of 35%w/w Eudragit RS formula without and with 5%w/w DH containing PEG1500 (2.5-10%w/w) was significantly decreased when the temperature was increased ($p<0.05$). These results indicated Newtonian behavior of 35%w/w Eudragit RS formula without and with 5%w/w DH containing PEG 1500, which confirmed the previous studies.

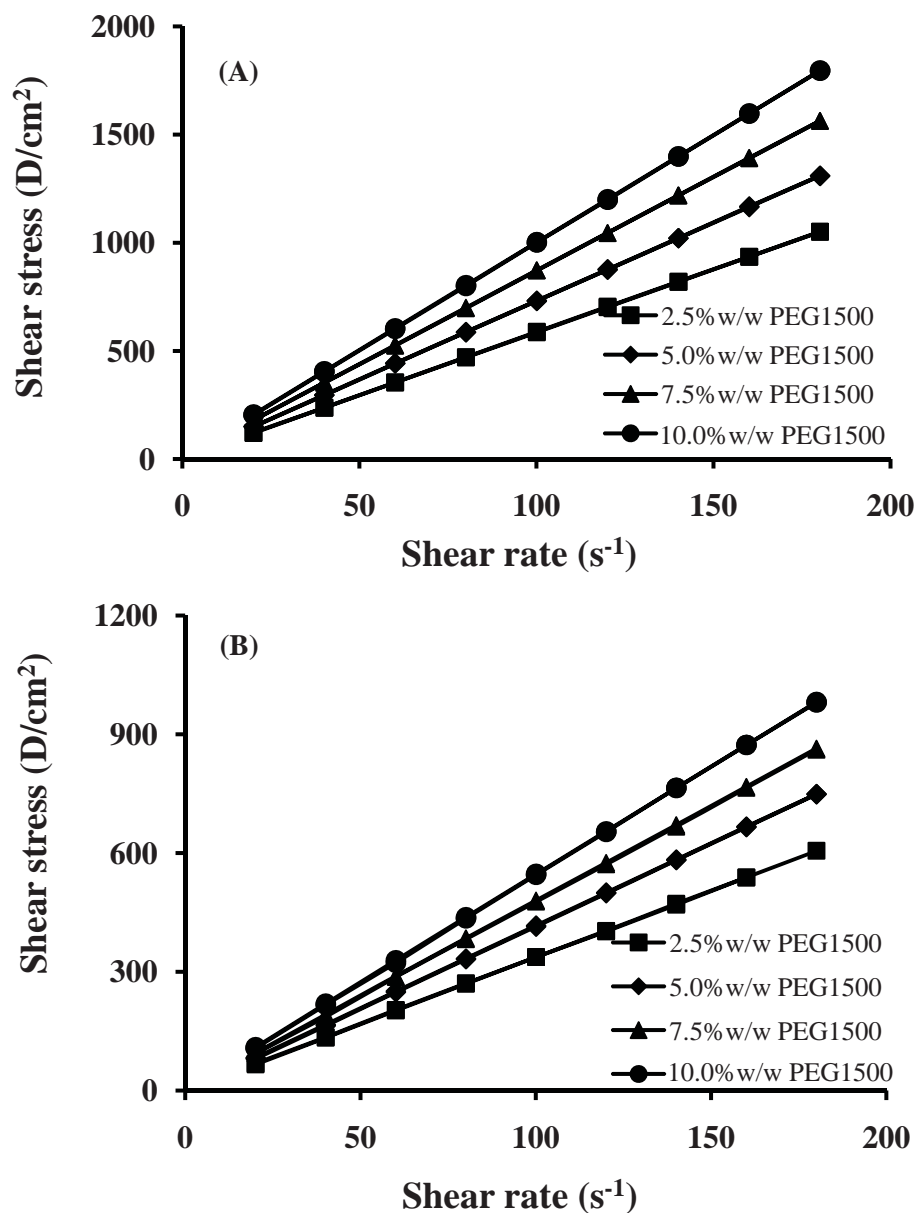


Figure 63 Flow curve of Eudragit RS formula containing different amount of polyethylene glycol 1500 (2.5%, 5%, 7.5% and 10%w/w) at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

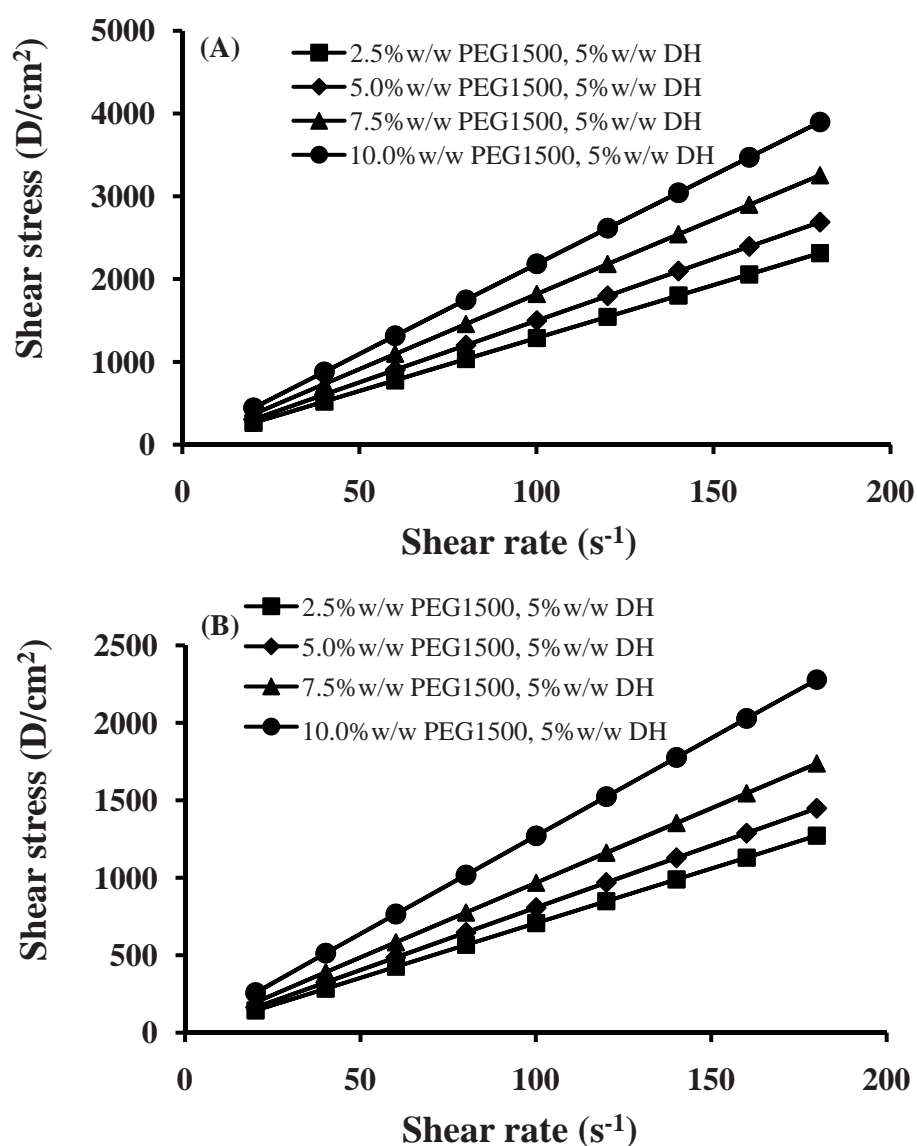


Figure 64 Flow curve of Eudragit RS (35% w/w)-doxycycline hyclate (5% w/w) formula containing different amount of polyethylene glycol 1500 (2.5%, 5%, 7.5% and 10% w/w) at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

Table 27 Flow parameters of Eudragit RS (35% w/w) formula containing different amount of polyethylene glycol 1500 without and with doxycycline hyclate (5% w/w) at 25°C and 37°C (n=3)

Concentration of PEG1500 (% w/w)	25°C		37°C	
	Flow index (N) (mean ± S.D.)	Consistency index (η) (mean ± S.D., [D/cm ²] ^N s)	Flow index (N) (mean ± S.D.)	Consistency index (η) (mean ± S.D., [D/cm ²] ^N s)
Without drug				
2.5%	0.98 ± 0.01	629.40 ± 4.89	1.00 ± 0.01	334.77 ± 4.95
5%	0.99 ± 0.00	771.53 ± 10.00	1.01 ± 0.00	400.07 ± 4.81
7.5%	0.99 ± 0.00	925.17 ± 6.20	1.01 ± 0.01	440.37 ± 31.23
10%	0.99 ± 0.00	1048.33 ± 12.74	1.01 ± 0.01	534.20 ± 16.17
With DH (5% w/w)				
2.5%	0.99 ± 0.00	1335.00 ± 18.68	0.99 ± 0.01	681.90 ± 16.30
5%	0.99 ± 0.01	1545.67 ± 45.35	1.00 ± 0.00	822.83 ± 8.03
7.5%	0.99 ± 0.00	1869.00 ± 30.64	1.00 ± 0.01	989.27 ± 14.10
10%	0.99 ± 0.00	2269.67 ± 18.88	0.99 ± 0.00	1309.33 ± 16.26

The syringeability of 35% w/w Eudragit RS formula containing without and with 5% w/w DH containing different amounts of PEG1500 (2.5-10% w/w) is shown in Tables 28. The work of syringeability of 35% w/w Eudragit RS formula containing without and with 5% w/w DH was increased ($p > 0.05$) with the increasing of PEG1500 amounts. However, the work of the 35% w/w Eudragit RS formula with 5% w/w DH containing PEG1500 (2.5%-10% w/w) was significantly higher than that of the 35% w/w Eudragit RS formula without drug containing PEG1500 ($p < 0.05$).

The *in vitro* gel formation of 35% w/w Eudragit RS formula without and with DH (5% w/w) containing PEG1500 (2.5-10% w/w) in phosphate buffer pH 6.8 are shown in Figures 65 and 66, respectively. The effect of amounts of PEG1500 on the *in vitro* gel formation was demonstrated. All formula could form the gel after injected into PBS pH 6.8. The results suggested that the addition of PEG1500 in Eudragit RS systems could be easily injected into the periodontal pockets and formed a gel *in situ* immediately.

The rate of water diffusion into the gels of the 35% w/w Eudragit RS formulations comprising different amounts of polyethylene glycol 1500 (2.5-10% w/w) without and with doxycycline hyclate (5% w/w) is shown in Tables 29. The rate of water diffusion into the gels of all Eudragit RS systems containing different amounts of PEG1500 (2.5-10% w/w) without and with DH (5% w/w) at 4 hours were in the range of

0.0042±0.0021 to 0.0049±0.0012 and 0.0042±0.0000 to 0.0056±0.0012 mm/min, respectively, whereas that of the 35%w/w Eudragit RS systems without PEG1500 were 0.0056±0.0024 mm/min. On the other hands, the rate of water diffusion into the gels of all Eudragit RS systems containing different amounts of PEG1500 (2.5-10% w/w) without and with DH (5%w/w) at 24 hours were in the range of 0.0031±0.0006 to 0.0038±0.0006 and 0.0034±0.0002 to 0.0038±0.0007 mm/min, respectively, whereas that of the 35%w/w Eudragit RS-DH (5%w/w) systems without PEG1500 were 0.0035±0.0012 mm/min. The results suggested that the increased PEG1500 (2.5-10% w/w) amounts in the Eudragit RS systems without and with DH (5%w/w) was not different ($p>0.05$). It has been reported that the hydrophilic substances such as PEG enhanced the water uptake of PEO-based matrix tablets. Thus, PEG did not facilitate the drug release rate of highly water-soluble drug, due to the significant diffusion of the drug (Kojima *et al.*, 2008).

Table 28 Syringeability of Eudragit RS (35% w/w) formula without and with 5% w/w doxycycline hyclate containing different amount of polyethylene glycol 1500 (2.5%, 5%, 7.5% and 10% w/w)

Concentration of PEG1500 (% w/w)	Work (N.mm) (n=3)	
	Without drug	DH (5% w/w)
2.5%	24.10 ± 1.74	52.06 ± 0.21
5%	28.79 ± 1.07	55.93 ± 1.68
7.5%	32.11 ± 0.72	57.78 ± 2.24
10%	37.08 ± 2.27	65.17 ± 0.79

Table 29 Effect of polyethylene glycol 1500 amount in the 35%w/w Eudragit RS formula without and with doxycycline hyclate (5% w/w) on the rate of water diffusion into gels.

Concentration of PEG1500 (% w/w)	Rate of water diffusion in to gels (mm/min) (mean ± S.D.)			
	without drug		with DH (5% w/w)	
	at 4 hours	at 24 hours	at 4 hours	at 24 hours
2.5%	0.0049 ± 0.0012	0.0038 ± 0.0006	0.0049 ± 0.0012	0.0038 ± 0.0007
5%	0.0042 ± 0.0021	0.0031 ± 0.0006	0.0056 ± 0.0012	0.0034 ± 0.0002
7.5%	0.0042 ± 0.0036	0.0034 ± 0.0007	0.0042 ± 0.0000	0.0035 ± 0.0003
10%	0.0042 ± 0.0021	0.0032 ± 0.0004	0.0049 ± 0.0012	0.0034 ± 0.0002

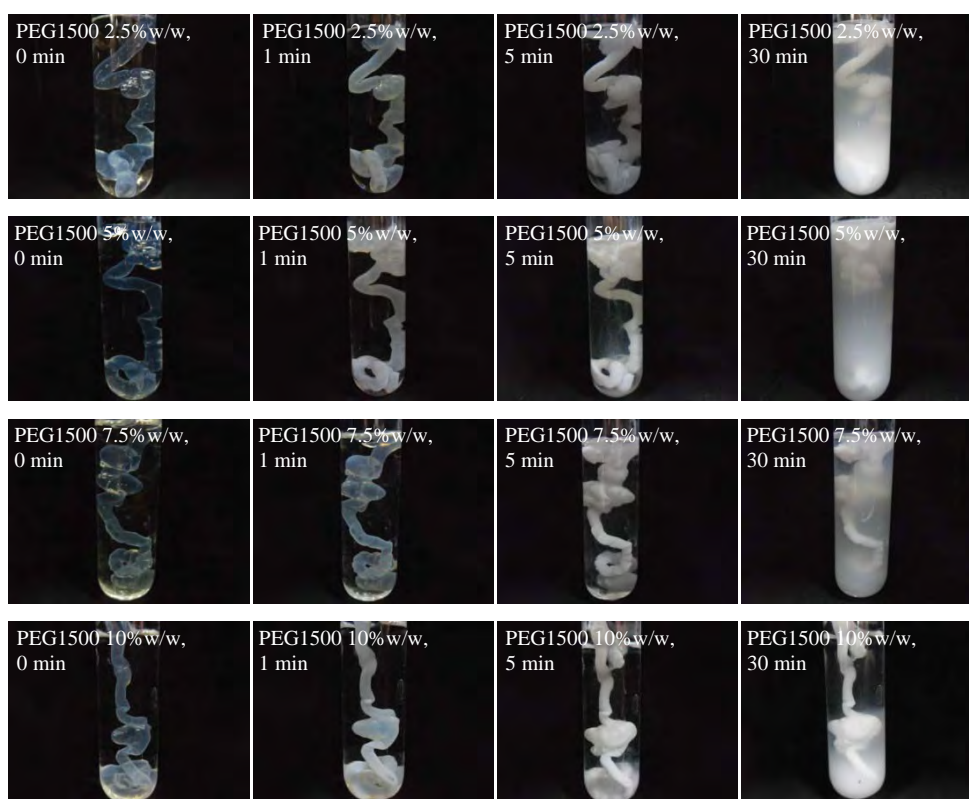


Figure 65 *In vitro* gel formation of Eudragit RS (35% w/w) formula containing different amount of polyethylene glycol 1500 (2.5%, 5%, 7.5% and 10% w/w) at various times (0, 1, 5 and 30 min).

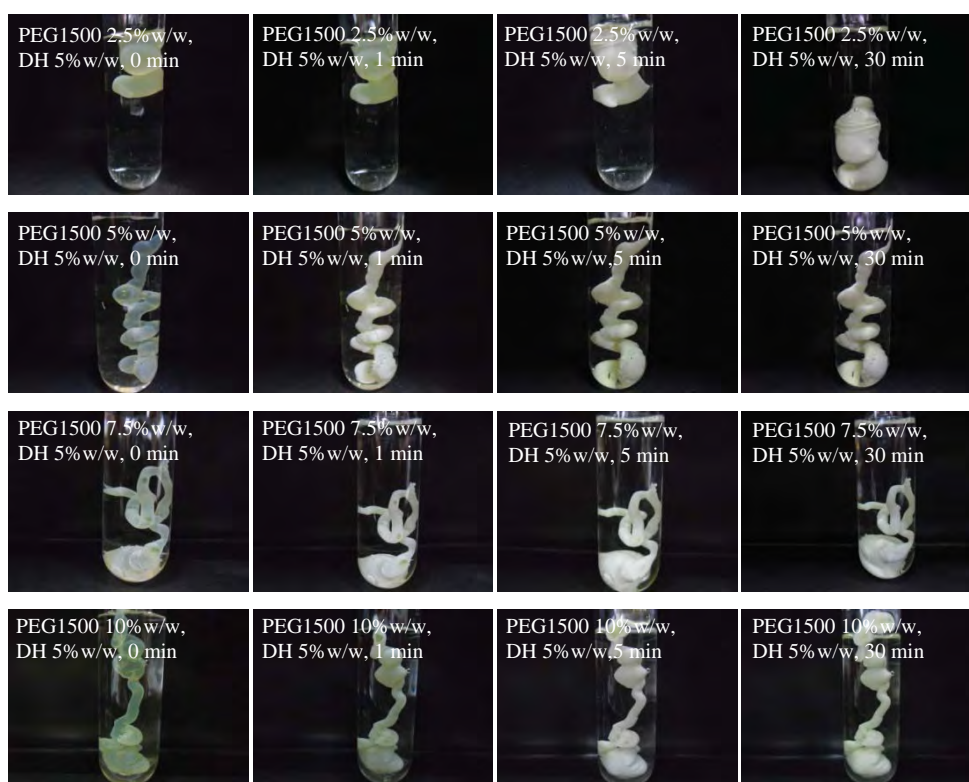


Figure 66 *In vitro* gel formation of Eudragit RS (35% w/w) -doxycycline hyclate (5% w/w) formula containing different amount of polyethylene glycol 1500 (2.5%, 5%, 7.5% and 10% w/w) at various times (0, 1, 5 and 30 min)

Drug release profiles were compared between control formulation (35% w/w Eudragit RS formula without PEG1500) and formula containing different amounts of PEG1500 in *in situ* gelling systems. The drug release profiles of 35% w/w Eudragit RS-doxycycline hyclate (5% w/w) formula containing PEG1500 (2.5-10% w/w) in phosphate buffer pH 6.8 were evaluated using dialysis membrane and membrane-less method as shown in Figure 67 and 68, respectively. The drug release profile of systems containing DH (5% w/w) in 35% w/w Eudragit RS systems containing PEG 2.5%, 5%, 7.5% and 10% w/w by dialysis membrane method were about 75%, 79%, 77% and 76% drug release at 24 hours, whereas that of systems without PEG1500 was about 90% drug release at the same time. In the addition, the drug release profile of systems containing DH (5% w/w) in 35% w/w Eudragit RS systems containing PEG 2.5%, 5%, 7.5% and 10% w/w by membrane-less method were about 45%, 44%, 42%

and 40% drug release at 24 hours, whereas that of systems without PEG1500 was about 88% drug release. Both dialysis membrane and membrane-less methods, the doxycycline hyclate release from all Eudragit RS systems containing PEG1500 (2.5-10% w/w) was always significantly slower than that of the systems without PEG1500 ($p < 0.05$). However, the increased PEG1500 (2.5-10% w/w) amounts in the Eudragit RS systems did not change the drug release profiles. The highly water soluble drugs dissolve easily when water penetrates into a matrix tablet, the dissolution of a drug cannot be a rate-limiting step in the release process (Vazquez *et al.*, 1992; Velasco *et al.*, 1999). The hydrophilic substances such as PEG enhanced the water uptake of PEO-based matrix tablets. Drugs with higher water solubilities were released faster from the PEO/PEG matrices thus the drug-release rate also increased as the amount of drug content increased. Thus, PEG did not facilitate the drug release rate of highly water-soluble drug, due to the significant diffusion of the drug (Kojima *et al.*, 2008).

In order to investigate the release kinetics of the 35% w/w Eudragit RS formula containing different amounts of PEG1500, the release data were analyzed with the following mathematical models: first order, Higuchi's, zero order and power law equation as shown in Table 30. The doxycycline hyclate release from the 35% w/w Eudragit RS formula containing different amounts of PEG1500 using dialysis membrane method were best explained by power law model, but a close relationship of the 35% w/w Eudragit RS systems containing PEG1500 (2.5-5% w/w) were also noted with first order kinetics, indicating the drug release depended on the drug concentration in the systems. Whereas, the 35% w/w Eudragit RS systems containing PEG1500 (7.5-10% w/w) showed a close relationship were noted with Higuchi's equation, indicating the drug release depended on the drug diffusion. The doxycycline hyclate release from the 35% w/w Eudragit RS formula containing different amounts of PEG1500 using membrane-less method were fitted well with Higuchi's model since r^2 and msc from curve fitting were higher than first order and zero order curve fitting.

The release exponent values (n) for power law of all 35% w/w Eudragit RS formula containing different amounts of PEG1500 (2.5-10% w/w) from the release studies using dialysis membrane and membrane-less method are shown in Table 31. The n value obtained from power law equation of all formula using dialysis membrane

and membrane-less method were between ranged from 0.21-0.32. The results indicated that all formula showed drug release by Fickian diffusion mechanism, which a rate of drug release decreased as a function of time, due to a decrease in the concentration gradient. On considering the drug release rate (k) parameter of the 35% w/w Eudragit RS formula containing PEG1500 (2.5-10% w/w) after release studies using dialysis membrane and membrane-less method, the results indicated that the increased PEG1500 amounts did not significant alter the drug release rate ($p > 0.05$). Therefore, the doxycycline hyclate was released by diffusion and the release rate did not decrease, even in the *in situ* gel with the highest viscosity. However, It has been reported that the type of plasticizer added to the polymer blend can play an important role for the resulting coating properties and drug release kinetics. The hydrophilic plasticizer can release from polymer when contact with water and thus conditions for the incorporated drug release are changed, whereas hydrophobic plasticizer remains in the system and ensures standard conditions during the process of drug release (Siepmann *et al.*, 2008; Bodmier and Paeratakul, 1997). The drug release from the Eudragit RS 30D systems with hydrophilic plasticizer (PEG 400 and propylene glycol) was faster than that with hydrophilic plasticizer (tributyl citrate) (Okarter and Singla, 2000).

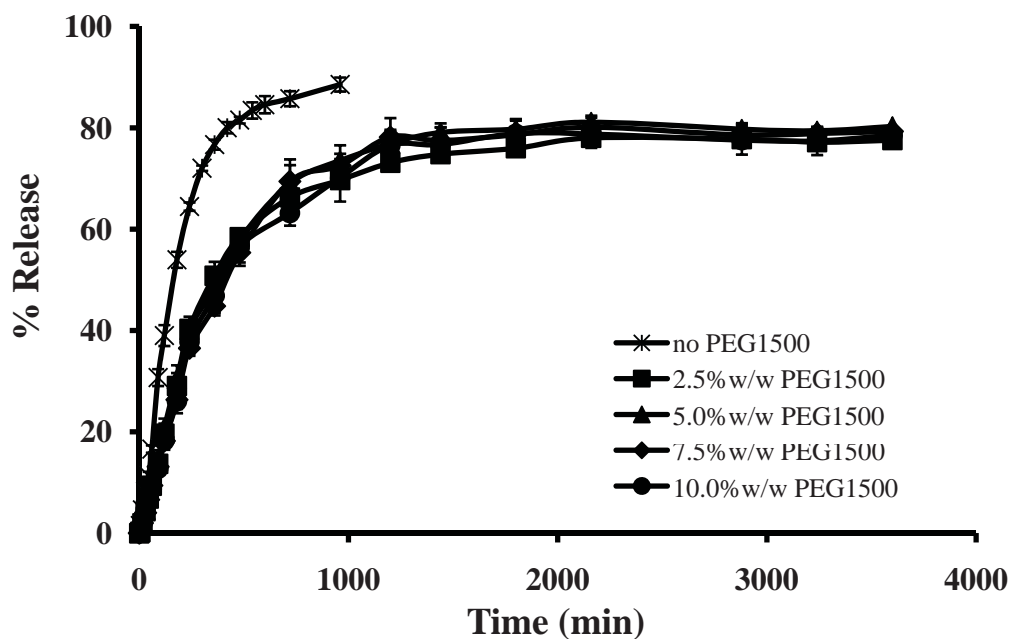


Figure 67 Effect of polyethylene glycol 1500 amount in 35%w/w Eudragit RS formula on release of doxycycline hyclate using dialysis method.

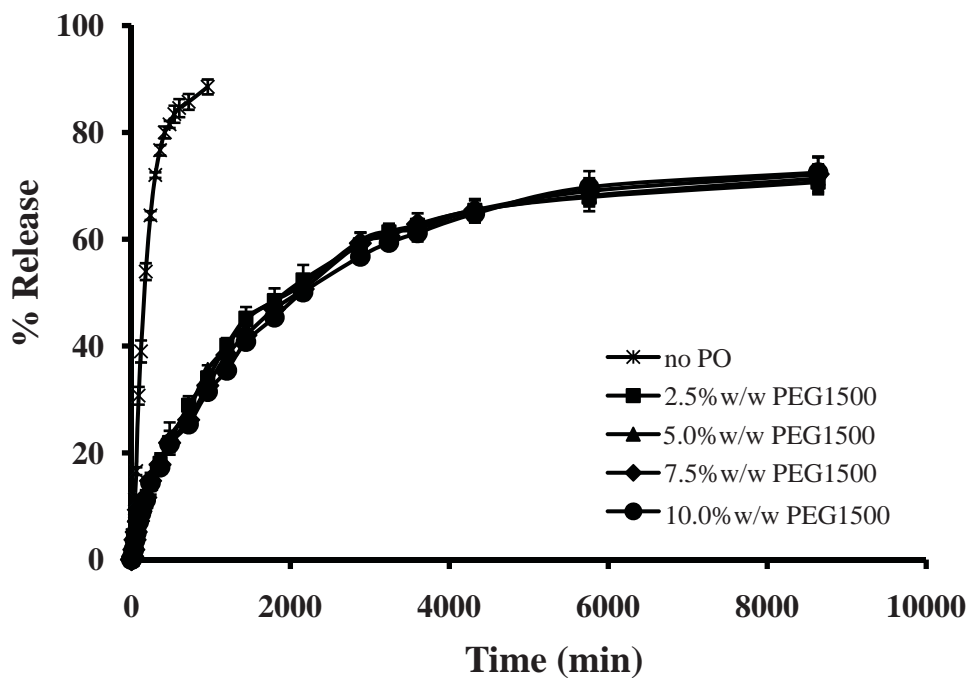


Figure 68 Effect of polyethylene glycol 1500 amount in 35%w/w Eudragit RS formula on release of doxycycline hyclate using membrane-less method.

Table 30 Comparison of degree of goodness-of-fit from curve fitting of the release profiles of doxycycline hyclate from 35%w/w Eudragit RS formula containing different amounts of polyethylene glycol 1500 (PEG1500) (2.5-10%w/w) in phosphate buffer pH 6.8 using dialysis membrane and membrane-less method to different release models.

Formula	First order		Higuchi's		Zero order		Power law	
	r ²	msc	r ²	msc	r ²	msc	r ²	msc
<i>Dialysis membrane method</i>								
PEG 2.5%	0.9180	2.10	0.9073	1.98	0.8008	1.21	0.9737	3.04
PEG 5%	0.9624	2.91	0.9531	2.70	0.8447	1.5	0.9846	3.57
PEG 7.5%	0.9672	3.02	0.9782	3.38	0.8570	1.55	0.9773	3.19
PEG 10%	0.9561	2.73	0.9694	3.04	0.8548	1.53	0.9830	3.48
<i>Membrane-less method</i>								
PEG 2.5%	0.7818	1.29	0.9884	4.14	0.9188	2.20	0.9469	2.58
PEG 5%	0.7840	1.30	0.9871	4.04	0.9165	2.17	0.9497	2.64
PEG 7.5%	0.8237	1.50	0.9952	5.04	0.9462	2.61	0.9481	2.61
PEG 10%	0.8481	1.65	0.9962	5.27	0.9510	2.71	0.9551	2.75

Table 31 Estimate parameter from curve fitting of doxycycline hyclate release from 35%w/w Eudragit RS formula containing different amounts of polyethylene glycol 1500 (PEG1500) (2.5-10%w/w) in phosphate buffer pH 6.8 using dialysis membrane and membrane-less method to power law expression.

Formula	k ± S.D.	tl ± S.D. (min)	n ± S.D.	Release mechanism
<i>Dialysis membrane method</i>				
PEG 2.5%	0.1715 ± 0.0157	167.04 ± 7.09	0.21 ± 0.01	Fickian
PEG 5%	0.1319 ± 0.0262	153.17 ± 10.96	0.26 ± 0.03	Fickian
PEG 7.5%	0.1073 ± 0.0068	157.98 ± 2.62	0.28 ± 0.01	Fickian
PEG 10%	0.1244 ± 0.0338	161.93 ± 6.64	0.26 ± 0.04	Fickian
<i>Membrane-less method</i>				
PEG 2.5%	0.0526 ± 0.0109	172.83 ± 2.63	0.30 ± 0.03	Fickian
PEG 5%	0.0525 ± 0.0083	173.26 ± 0.96	0.30 ± 0.02	Fickian
PEG 7.5%	0.0453 ± 0.0039	168.55 ± 2.79	0.31 ± 0.02	Fickian
PEG 10%	0.0401 ± 0.0039	167.09 ± 0.48	0.32 ± 0.02	Fickian

k = release rate; tl = lag time and n = diffusional exponent

The SEM micrographs of 35%w/w Eudragit RS formula with DH (5%w/w) containing different amounts of PEG1500 after releasing DH in PBS pH 6.8 at 37 °C at different magnifications are shown in Figure 69. The structures of all Eudragit RS formula containing PEG1500 (2.5-10%w/w) were continuous phase. The pore sizes of structure after releasing test were increased with an increase of PEG1500 amount. Whereas the wall surface of the systems with the high PEG1500 amount was more compact than that of the systems with the low PEG1500 amount. The structures were clarified at magnification 500X and 2000X which the porous structure was more enlarged. The results indicated that the drug in the NMP solvent was exchanged with medium, the formation of pore structure was occurred.

The percentage of weight loss of the 35%w/w Eudragit RS formula without and with doxycycline hyclate (5%w/w) containing different amounts of polyethylene glycol 1500 (2.5-10% w/w) in phosphate buffer pH 6.8 after 1 month is shown in Table 32. The percentage of weight loss of the 35%w/w Eudragit RS formula without and with DH (5%w/w) did not significantly change when the increased PEG1500 amounts ($p>0.05$). The results suggested that PEG did not affect the drug and solvent diffusion.

The 35%w/w Eudragit RS-doxycycline hyclate (5%w/w) formula containing different amounts of PEG1500 were tested for the antimicrobial activity against *S. aureus*, *E. coli*, *C. albicans*, *S. mutans* and *P. gingivalis* using agar diffusion method as shown in Figure 70. The inhibition zone diameter against *S. aureus*, *E. coli*, *S. mutans* and *P. gingivalis* of the systems with DH (5%w/w) containing PEG1500 (2.5-10% w/w) was significantly higher than that of the gel base (without DH) ($p<0.05$). On the other hand, the inhibition zone diameter against *C. albicans* of the systems containing 5%w/w DH was significantly lower than that of the gel base ($p<0.05$). The addition of PEG1500 in 35%w/w Eudragit RS systems containing DH (5%w/w) significantly decreased the inhibition zone diameter against *S. aureus*, *E. coli* and *S. mutans* ($p<0.05$). The increasing PEG1500 amount did not significantly change the antimicrobial activity against *S. aureus*, *E. coli*, *C. albicans*, *S. mutans* and *P. gingivalis* ($p>0.05$). The results indicated that the increased PEG1500 amounts in the Eudragit systems did not affect the antimicrobial activity against microbes, since the increasing PEG1500 amount did not change the rate of drug diffusion. However, the

antimicrobial activities against all microbes were decreased when the addition of PEG1500, which supported the previous release studies.

It has been reported that the silver nanoparticles coated with polyethylene glycol was most effective in killing various bacterial strains including *Escherichia coli* DH5 α , *Bacillus subtilis*, *Micrococcus luteus*, and *Staphylococcus aureus*, which the minimum inhibitory concentration of polyethylene glycol coated silver nanoparticles were also less compared to the tween 80 or sodium dodecyl sulphate of nanoparticles (Bhattacharya *et al.*, 2012). Consistence with that polyethylene glycol coated nanoparticles produced more intracellular reactive oxygen species in bacteria. Moreover, when human cell lines MCF7 and Chang Liver were incubated in presence of these nanoparticles for 18 h with same concentrations as used for bacteria, no toxicity was observed. But significant increase in cell killing was observed with longer incubation time. Thus this investigation implicates the potential therapeutic use of silver nanoparticles as antibacterial agent particularly the polyethylene glycol coated one.

Table 32 The percentage of weight loss of 35% w/w Eudragit RS formula containing different amounts of polyethylene glycol 1500 without and with 5% w/w doxycycline hyclate (n=3)

Concentration of PEG1500 (% w/w)	% Weight loss (n=3)	
	Without drug	DH (5% w/w)
2.5%	74.15 \pm 2.69	63.56 \pm 0.04
5%	73.12 \pm 1.57	63.58 \pm 0.34
7.5%	74.41 \pm 0.57	63.35 \pm 0.60
10%	75.61 \pm 1.38	63.59 \pm 0.22

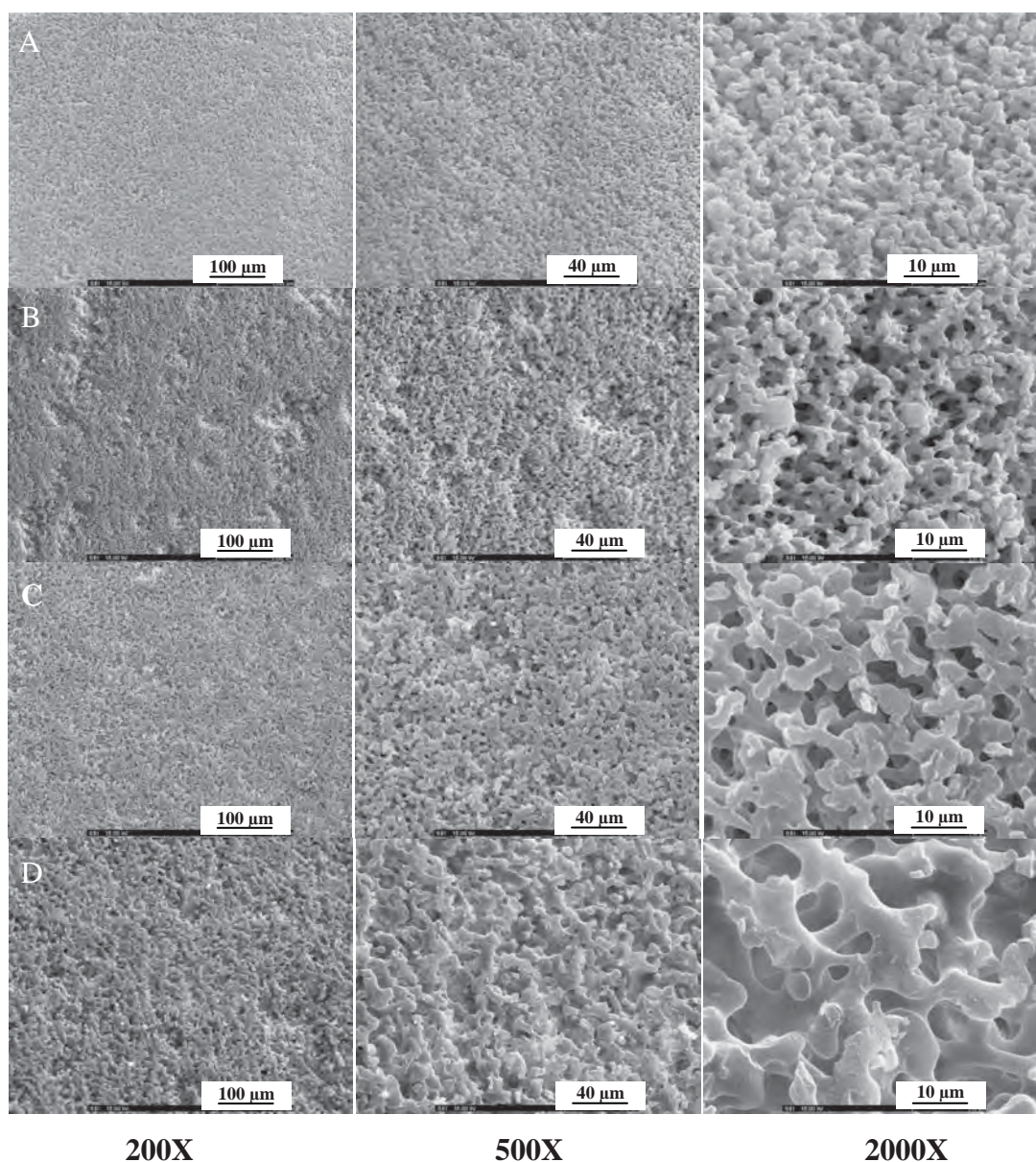


Figure 69 SEM micrograph of the dried gel systems; Doxycycline hyclate (5%w/w)-Eudragit RS (35%w/w) systems containing 2.5%w/w PEG1500 (A); 5%w/w PEG1500 (B); 7.5%w/w PEG1500 (C) and 10%w/w PEG1500 (D) with different magnifications (200X, 500X and 2000X).

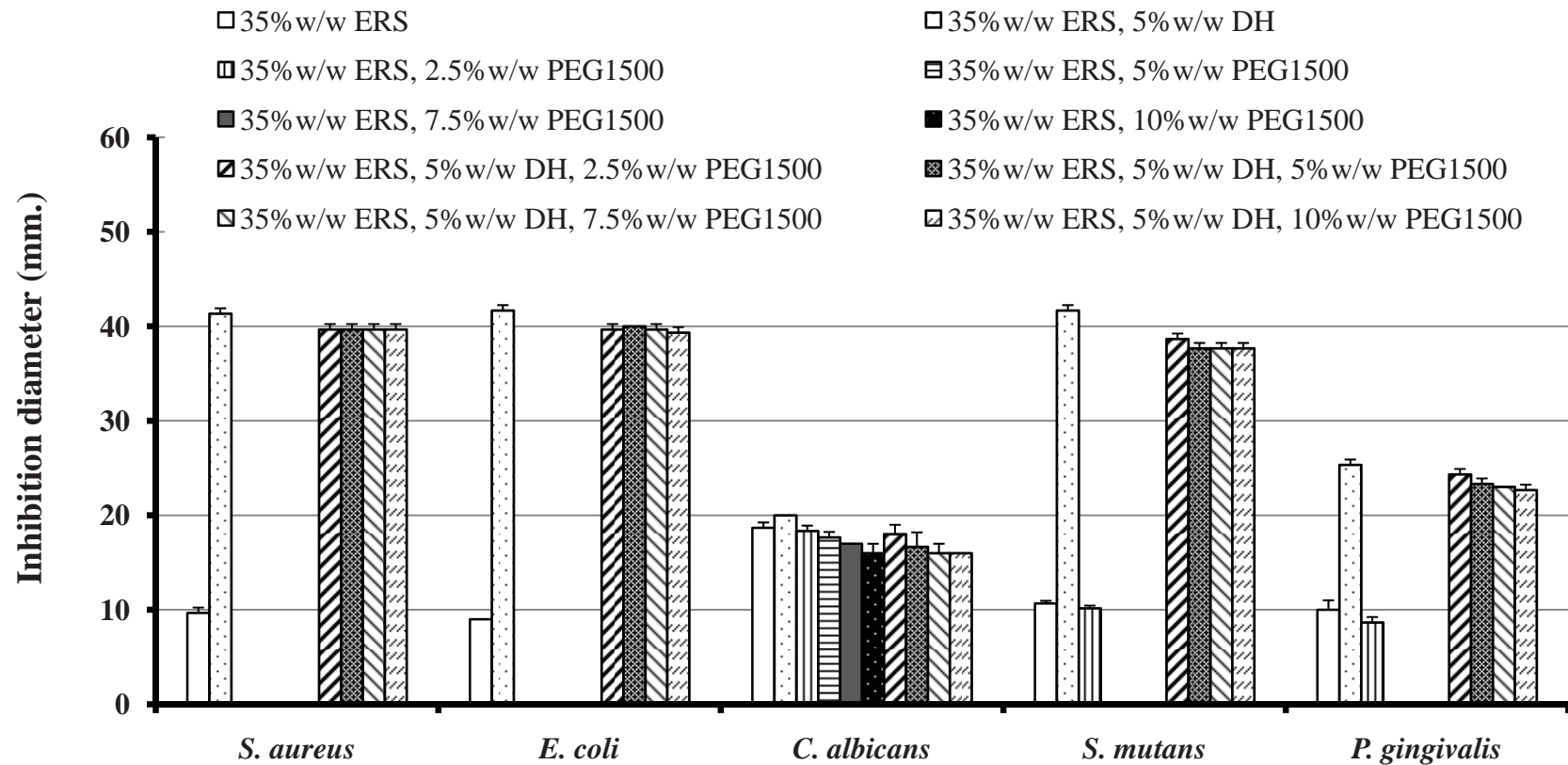


Figure 70 Inhibition zone diameter of the Eudragit RS (35% w/w)-doxycycline hyclate (5% w/w) formula containing different amounts of polyethylene glycol 1500 against *S. aureus*, *E. coli*, *C. albicans*, *S. mutans* and *P. gingivalis*.

4.4.2 Influence of hydrophobic (peppermint oil) agents

The appearances and pH values of the 35% w/w Eudragit RS formula containing different amounts of peppermint oil without and with doxycycline hyclate (5% w/w) is shown in Table 33. The gel base systems and systems containing peppermint oil (without drug) were clear and colorless, whereas all systems comprising doxycycline hyclate were yellow and clear. The pH values of 35% w/w Eudragit RS formula containing PO (2.5-10% w/w) without and with DH (5% w/w) were in the range of 7.74 ± 0.03 to 8.21 ± 0.01 and 3.44 ± 0.02 to 3.64 ± 0.02 , respectively, whereas the pH values of 35% w/w Eudragit RS system without and with DH (5% w/w) were 8.69 ± 0.09 and 3.77 ± 0.05 , respectively. The effects of peppermint oil amounts on viscosity of Eudragit RS (35% w/w) formula without and with DH were investigated. The relationships between shear rate and apparent viscosity of PO (2.5-10% w/w) in Eudragit RS (35% w/w) formula without and with DH (5% w/w) are shown in Figures 71 and 72, respectively. The apparent viscosities of 35% w/w Eudragit RS formula without and with 5% w/w DH containing PO (2.5-10% w/w) were constant when the shear rate was increased indicating Newtonian. The apparent viscosities of 35% w/w Eudragit RS formula without and with 5% w/w DH containing PO (2.5-10% w/w) at 37°C were lower than that of formula at 25°C. Eudragit RS (35% w/w) formula without and with 5% w/w DH containing PO (2.5-10%) exhibited a decrease in viscosity with increasing temperature.

The rheological behaviors of 35% w/w Eudragit RS formula without and with 5% w/w DH containing different amounts of PO (2.5-10% w/w) were investigated. The shear stress versus shear rate flow curves of 35% w/w Eudragit RS formula without and with 5% w/w DH containing PO (2.5-10% w/w) at different temperature are shown in Figures 73 and 74, respectively. The shear stress of 35% w/w Eudragit RS systems without and with 5% w/w DH was increased as the amount of PO was increased. However, the shear stress of 35% w/w Eudragit RS systems without and with 5% w/w DH containing PO (2.5-10% w/w) was decreased with an increasing temperature. All formulations showed Newtonian behavior. The curves of 35% w/w Eudragit RS formula without and with 5% w/w DH moved to a higher shear stress value with

increasing PO amounts indicating compact structure of the gels. On the other hand, the curves of 35%w/w Eudragit RS formula without and with 5%w/w DH containing PO (2.5-10%w/w) moved to a lower shear stress value with increasing temperature indicating loose structure of the gels.

Table 33 Gel appearance and pH value of 35%w/w Eudragit RS formula containing different amounts of peppermint oil and without and with doxycycline hyclate (5%w/w)

Amount of PO (% w/w)	Appearance	Clarity	pH \pm S.D. (n=3)
Without drug			
2.5%	Colorless	Clear	8.21 \pm 0.01
5%	Colorless	Clear	8.04 \pm 0.02
7.5%	Colorless	Clear	7.97 \pm 0.01
10%	Colorless	Clear	7.74 \pm 0.03
With DH (5% w/w)			
2.5%	Yellow	Clear	3.64 \pm 0.02
5%	Yellow	Clear	3.46 \pm 0.04
7.5%	Yellow	Clear	3.44 \pm 0.02
10%	Yellow	Clear	3.55 \pm 0.01

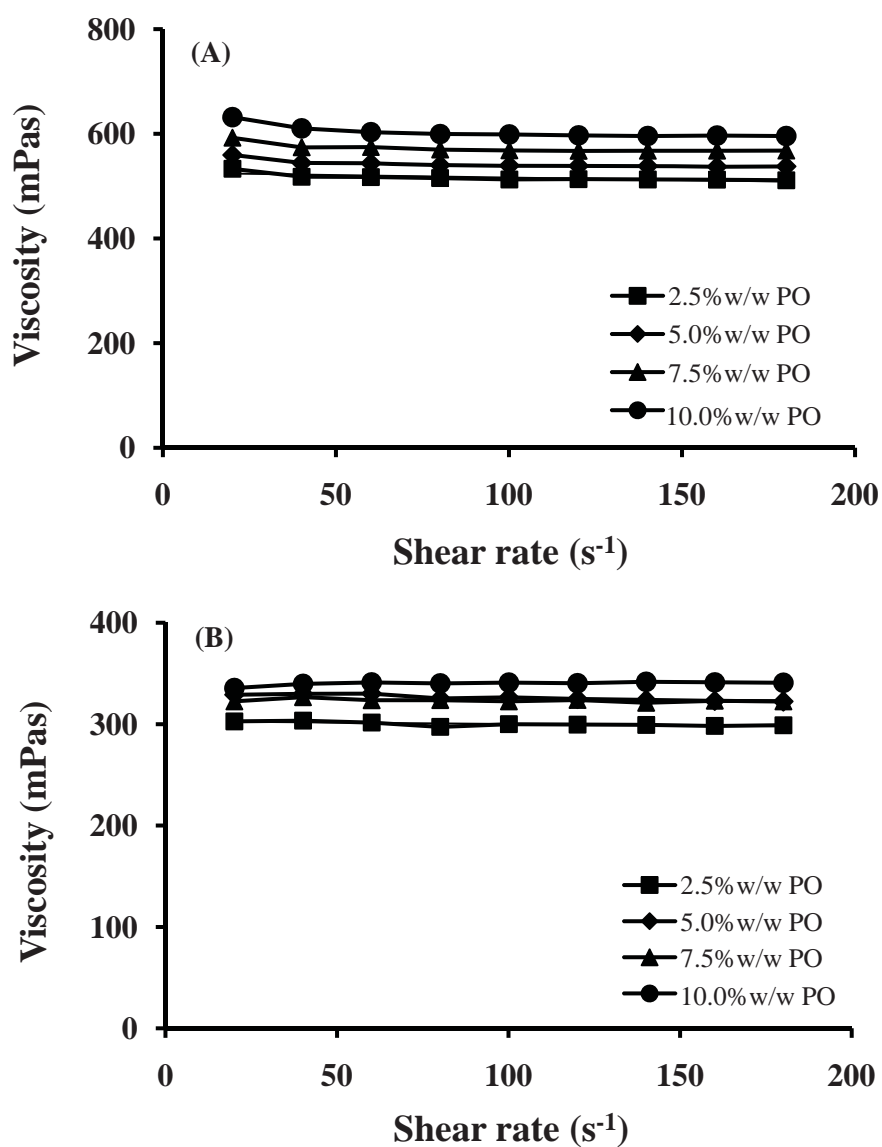


Figure 71 Viscosity curves of Eudragit RS (35% w/w) formula containing different amount of peppermint oil (2.5%, 5%, 7.5% and 10% w/w) at (A) 25°C and (B) 37°C

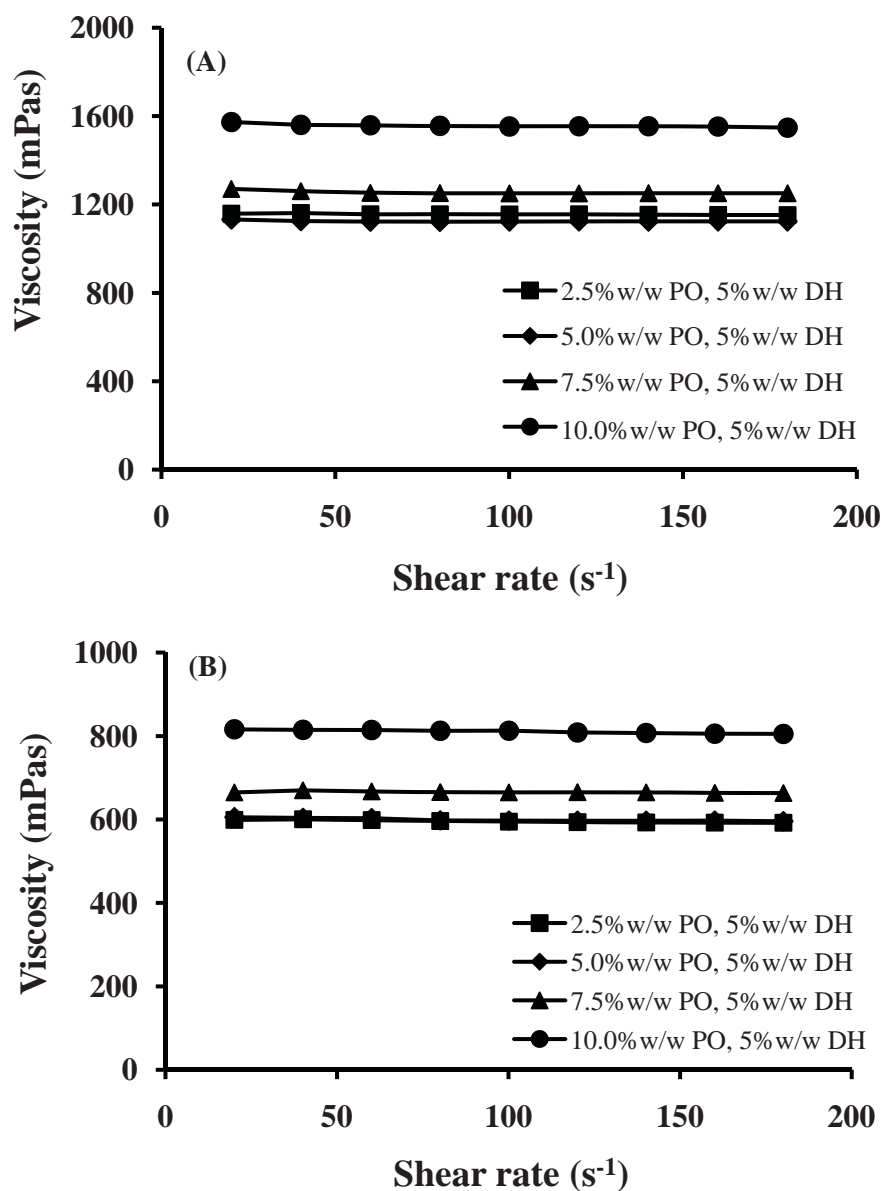


Figure 72 Viscosity curves of Eudragit RS (35% w/w)-doxycycline hyclate (5% w/w) formula containing different amount of peppermint oil (2.5%, 5%, 7.5% and 10% w/w) at (A) 25°C and (B) 37°C

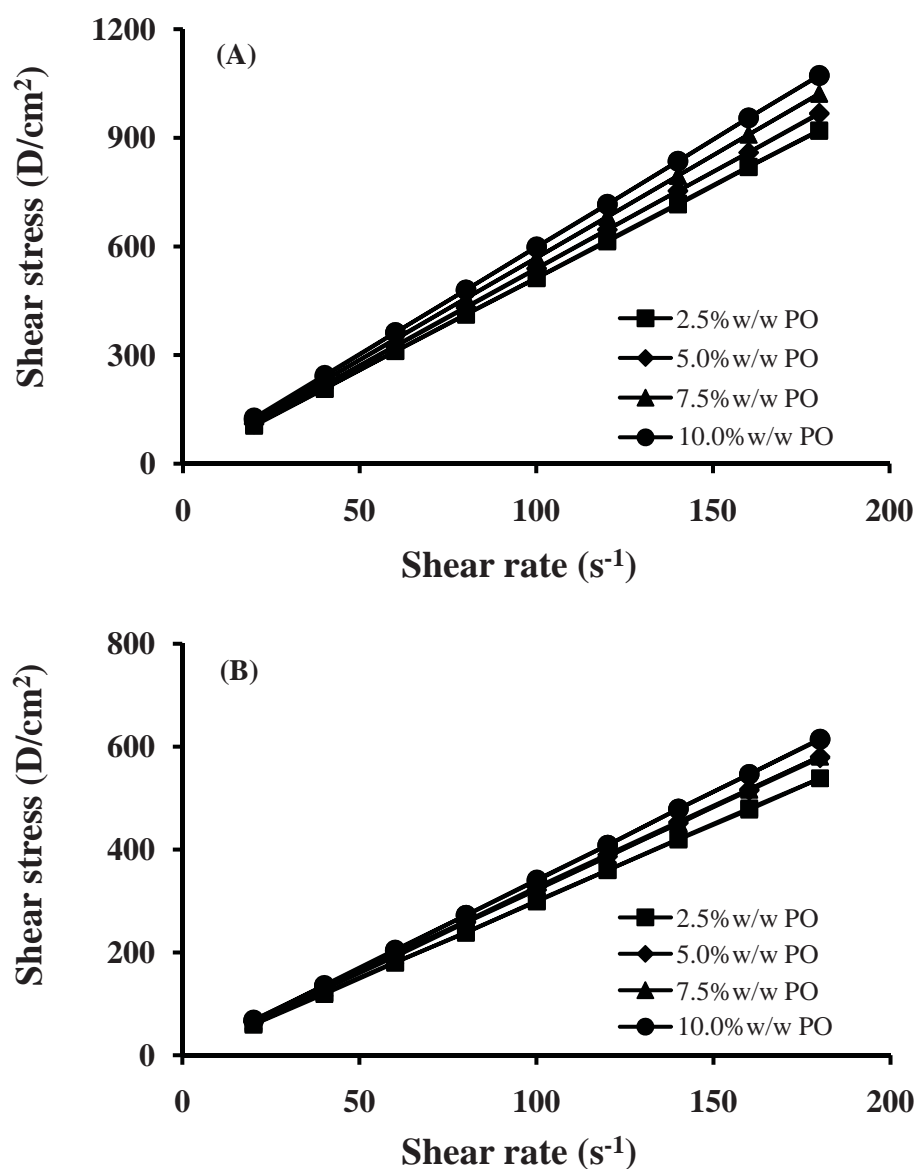


Figure 73 Flow curve of Eudragit RS (35% w/w) formula containing different amount of peppermint oil (2.5%, 5%, 7.5% and 10% w/w) at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

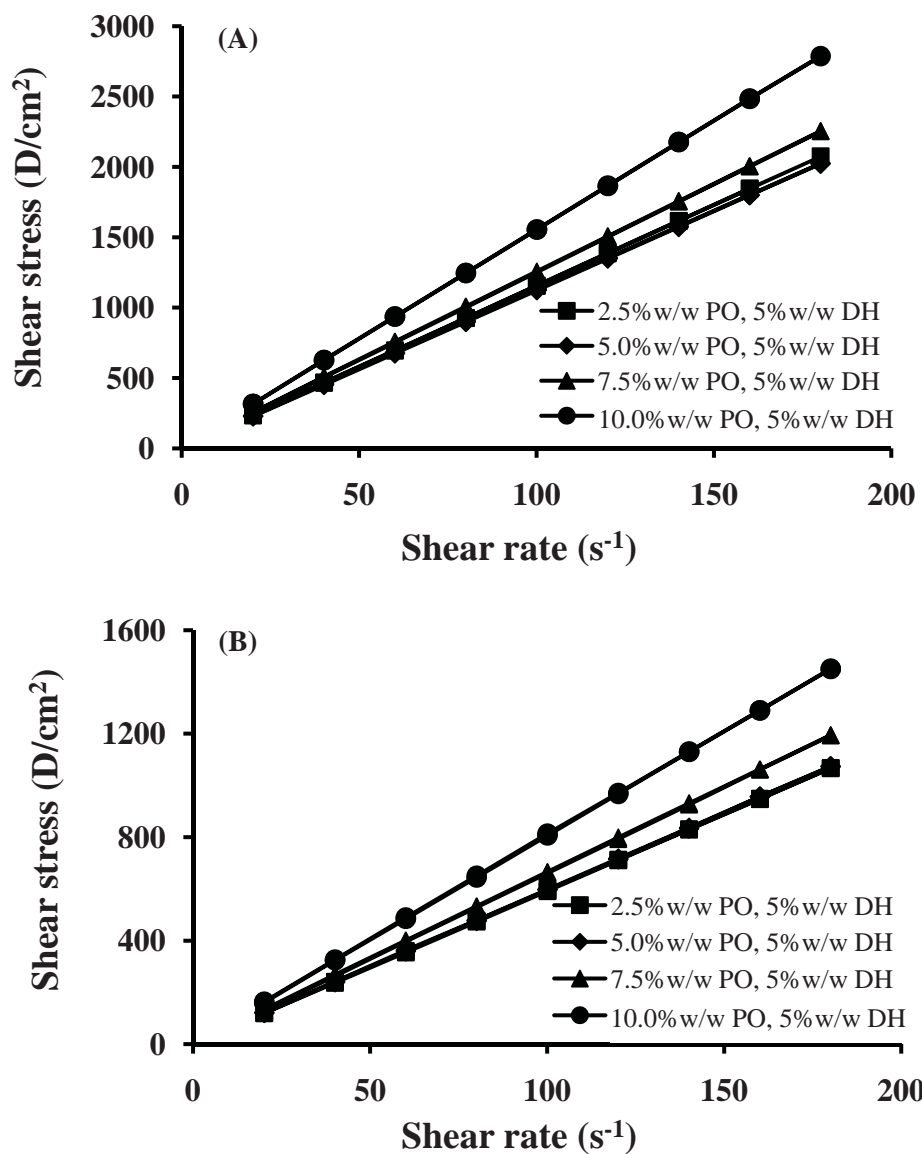


Figure 74 Flow curve of Eudragit RS (35%w/w)-doxycycline hyclate (5%w/w) formula containing different amount of peppermint oil (2.5%, 5%, 7.5% and 10%w/w) at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

The flow parameters of 35%w/w Eudragit RS formula without and with DH (5%w/w) containing different amounts of PO (2.5-10%w/w) are shown in Tables 34. N value of 35%w/w Eudragit RS formula without and with 5%w/w DH containing PO (2.5-10%w/w) was close to 1, indicating that the flow type was Newtonian. However, the increasing temperature did not change the flow types of the systems. In the case of the viscosity coefficient (η), as the PO amount of 35%w/w Eudragit RS formula without and with DH (5%w/w) was higher, the viscosity coefficient was also significantly greater ($p<0.05$). In contrast, the viscosity coefficient of 35%w/w Eudragit RS formula without and with DH (5%w/w) containing PO (2.5-10%w/w) was significantly decreased when the temperature was increased ($p<0.05$). These results indicated Newtonian behavior of 35%w/w Eudragit RS formula without and with DH (5%w/w) containing PO (2.5-10%w/w), which confirmed the previous studies.

Table 34 Flow parameters of Eudragit RS (35%w/w) formula containing different amount of peppermint oil without and with doxycycline hyclate (5%w/w) at 25°C and 37°C (n=3)

Concentration of PO (% w/w)	25°C		37°C	
	Flow index (N) (mean \pm S.D.)	Consistency index (η) (mean \pm S.D., [D/cm ²] ^N s)	Flow index (N) (mean \pm S.D.)	Consistency index (η) (mean \pm S.D., [D/cm ²] ^N s)
Without drug				
2.5%	0.99 \pm 0.01	543.73 \pm 9.02	1.00 \pm 0.01	300.67 \pm 10.25
5%	0.99 \pm 0.01	570.00 \pm 5.00	0.99 \pm 0.01	330.97 \pm 18.00
7.5%	0.98 \pm 0.01	610.13 \pm 11.37	0.99 \pm 0.00	342.30 \pm 7.88
10%	0.98 \pm 0.01	645.77 \pm 11.67	1.01 \pm 0.01	334.17 \pm 9.94
With 5%w/w DH				
2.5%	1.00 \pm 0.01	1190.33 \pm 35.28	1.00 \pm 0.01	601.50 \pm 11.16
5%	0.99 \pm 0.01	1157.67 \pm 8.02	0.99 \pm 0.01	610.90 \pm 13.95
7.5%	0.99 \pm 0.00	1297.33 \pm 12.74	1.00 \pm 0.00	655.53 \pm 13.35
10%	0.99 \pm 0.00	1603.00 \pm 23.81	1.00 \pm 0.00	813.10 \pm 21.17

The syringeability of 35%w/w Eudragit RS formula without and with DH (5%w/w) containing different amounts of PO (2.5-10%w/w) is shown in Tables 35. The work of syringeability of 35%w/w Eudragit RS formula without and with DH (5%w/w) containing PO (2.5-10%w/w) was significantly higher than that of the systems without PO ($p<0.05$). However, the work of syringeability of 35%w/w Eudragit RS formula without DH containing different amounts of PO (2.5-10%w/w)

did not significantly different ($p>0.05$), whereas the work of syringeability of 35%w/w Eudragit RS formula with DH (5%w/w) containing PO was significantly increased when the increased PO amounts ($p<0.05$).

The *in vitro* gel formation of 35%w/w Eudragit RS formula without and with DH (5%w/w) containing PO (2.5-10%w/w) in phosphate buffer pH 6.8 are shown in Figures 75 and 76, respectively. The effect of amounts of PO on the *in vitro* gel formation was demonstrated. All formula containing PO formed soft and elastic gel after injected into PBS pH 6.8, whereas the Eudragit RS formula without PO formed a dense and hard structure. The results suggested that the addition of PO in Eudragit RS systems could improve the physical properties of Eudragit RS system and it could be easily injected into the periodontal pocket and formed a gel *in situ* immediately. It has been reported that the triacetin-hydrophobic solvent (30%) at which depot formation could be transformed a solid state to a rubber state (Liu and Venkatraman, 2012). The addition of peppermint oil (hydrophobic plasticizer) in the Eudragit RS systems could transform from the polymer from a solid state to an elastic state. The addition castor oil enhanced the film flexibility of poly(ϵ -caprolactone) via acted as a lubricant among polymer chains has been reported (Lin *et al.*, 2004)).

The rate of water diffusion into the gels of the 35%w/w Eudragit RS formula comprising different amounts of peppermint oil (2.5-10%w/w) without and with doxycycline hyclate (5%w/w) is shown in Tables 36. The rate of water diffusion into the gels of all Eudragit RS systems containing different amounts of PO (2.5-10%w/w) without and with DH (5%w/w) at 4 hours was decreased with the PO amount increased ($p>0.05$). The rate of water diffusion into the gels of all Eudragit RS systems containing different amounts of PO (2.5-10%w/w) without and with DH (5%w/w) at 24 hours did not significantly differ ($p>0.05$). It has been reported that the added hydrophobic plasticizer in the systems slowed down the water and solvent exchange rate (Liu and Venkatraman, 2012).

Table 35 Syringeability of Eudragit RS (35% w/w) formula without and with doxycycline hyclate (5% w/w) containing different amounts of peppermint oil (2.5%, 5%, 7.5% and 10% w/w).

Concentration of PO (% w/w)	Work (N.mm) (n=3)	
	Without drug	DH (5% w/w)
2.5%	26.96 ± 2.31	27.20 ± 2.35
5%	26.42 ± 2.44	28.93 ± 3.27
7.5%	26.25 ± 2.52	33.78 ± 3.30
10%	27.13 ± 0.97	40.37 ± 3.79

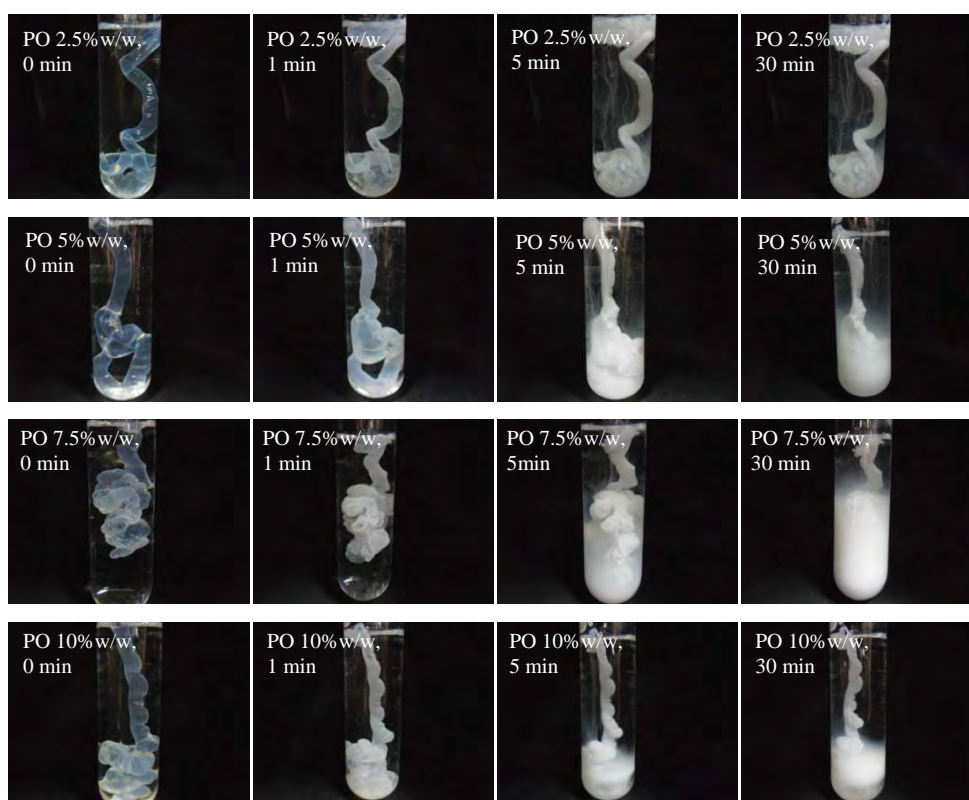


Figure 75 *In vitro* gel formation of Eudragit RS (35% w/w) formula containing different amount of peppermint oil (2.5%, 5%, 7.5% and 10% w/w) at various times (0, 1, 5 and 30 min).

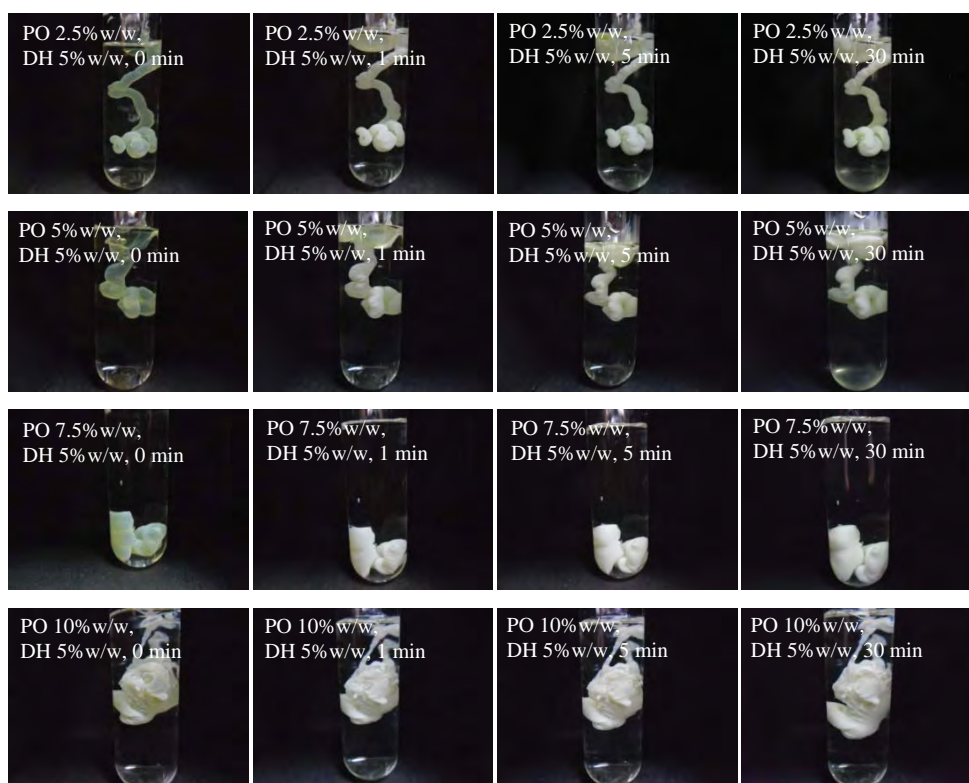


Figure 76 *In vitro* gel formation of Eudragit RS (35%w/w)-doxycycline hyclate (5%w/w) formula containing different amount of peppermint oil (2.5%, 5%, 7.5% and 10%w/w) at various times (0, 1, 5 and 30 min).

Table 36 Effect of peppermint oil amount in the 35%w/w Eudragit RS formula without and with doxycycline hyclate (5%w/w) on rate of water diffusion into gels.

Concentration of PO (%w/w)	Rate of water diffusion in to gels (mm/min) (mean \pm S.D.)			
	without drug		with DH (5%w/w)	
	at 4 hours	at 24 hours	at 4 hours	at 24 hours
2.5%	0.0063 \pm 0.0021	0.0037 \pm 0.0008	0.0056 \pm 0.0012	0.0039 \pm 0.0002
5%	0.0049 \pm 0.0024	0.0035 \pm 0.0006	0.0049 \pm 0.0012	0.0037 \pm 0.0005
7.5%	0.0035 \pm 0.0012	0.0031 \pm 0.0005	0.0028 \pm 0.0012	0.0034 \pm 0.0005
10%	0.0028 \pm 0.0012	0.0031 \pm 0.0006	0.0021 \pm 0.0000	0.0029 \pm 0.0002

Drug release profiles were compared between control formulation (35%w/w Eudragit RS formula without PO) and formula containing different amounts of PO in *in situ* gelling systems. The drug release profiles of 35%w/w Eudragit RS-doxycycline hyclate (5%w/w) formula containing PO (2.5-10%w/w) in phosphate buffer pH 6.8 were evaluated using dialysis membrane and membrane-less method as shown in Figure 77 and 78, respectively. The drug release profile of systems containing DH (5%w/w) in 35%w/w Eudragit RS systems containing PO 2.5%, 5%, 7.5% and 10%w/w by dialysis membrane method were about 81%, 77%, 72% and 63% drug release at 24 hours, whereas that no PO was about 88% drug release at the same time. In the addition, the drug release profile of systems containing DH (5%w/w) in 35%w/w Eudragit RS systems containing PO 2.5%, 5%, 7.5% and 10%w/w by membrane-less method were about 61%, 55%, 48% and 32% at 24 hours, whereas that of systems without PO was about 88% at the same time. Both dialysis membrane and membrane-less methods, the doxycycline hyclate release from all Eudragit RS systems containing PO (2.5-10%w/w) was always significantly slower than that of the systems without PO ($p < 0.05$). The doxycycline hyclate release from the systems was decreased when the PO amount was increased. The doxycycline hyclate release from the 35%w/w Eudragit RS formula containing PO (2.5-10%w/w) using dialysis membrane method were best explained by power law model, but a close relationship were also noted with Higuchi's model, indicating the release mechanism to be diffusion based. Whereas the doxycycline hyclate release from the 35%w/w Eudragit RS formula containing PO (2.5-5%w/w) using membrane-less method were best explained by power law model, but the doxycycline hyclate release from all Eudragit RS formula containing PO (2.5-10%w/w) were fitted well with zero order model. Which the drug delivery system was desirable developed to achieve to zero order release kinetics since, the drug is delivered at the same rate over the release of the systems. Therefore the addition of hydrophobic nature of hydrophobic plasticizer could contribute toward reduction in the penetration of the solvent molecules in the matrix since the drug release decreased as previously reported (Ganesh *et al.*, 2008).

The release exponent values (n) for power law of the 35%w/w Eudragit RS formula containing different amounts of PO (2.5-10%w/w) from the release studies using dialysis membrane and membrane-less method are shown in Table 37. The n

values of all systems containing PO (2.5-10%w/w) using dialysis membrane and membrane-less method ranged from 0.13-0.40, indicating all formula showed drug release by Fickian diffusion mechanism. It suggested that of drug release decreased as a function of time due to a decrease in the concentration gradient. However, the n value of only systems containing 10%w/w PO using membrane-less method was 0.52, indicating anomalous (non-Fickian) diffusion ($0.43 < n < 0.83$). The results indicated that the drug release was controlled by both mechanism of diffusion and polymeric chain relaxation. The drug diffusion through most types of polymeric systems was often best described by Fickian diffusion, but there was also a relaxation of the polymer chains, which influenced the drug release mechanism. This process was described as non-Fickian diffusion (Baumgartner *et al.*, 2000; Nagarwal *et al.*, 2009)

The release rate (k) parameter of the 35%w/w Eudragit RS formula containing different amounts of PO after release studies using dialysis membrane and membrane-less methods is shown in Table 38. The drug release rate of the 35%w/w Eudragit RS formula containing PO (2.5-10%w/w) was decreased with the increasing PO amount, therefore PO significantly decreased the drug release rate ($p < 0.05$). It has been reported that the incorporation of hydrophobic agents could reduce the burst release. When the prepared gel mixed with the hydrophobic substance was injected into the buffer solution, the hydrophilic solvent (NMP) near the surface left the polymer solution quickly to form the *in situ* gel almost immediately, whereas the hydrophobic substance limited the fast water entry into the matrix, delayed phase inversion and ultimately suppressed the burst release by slowing down the solvent released (Lie and Venkatraman, 2012). Therefore the solvent and drug release could be controlled by regulating the solvent miscibility with water through mixing the hydrophilic and hydrophobic substances. The addition of a hydrophobic substance could overcome the defect of the fast phase inversion caused by pure hydrophobic solvent, and enhanced the subsequent release by allowing the formation of a more “rubbery” (plasticized) structure because of solvent retention inside the *in situ* gel (Liu and Venkatraman, 2012).

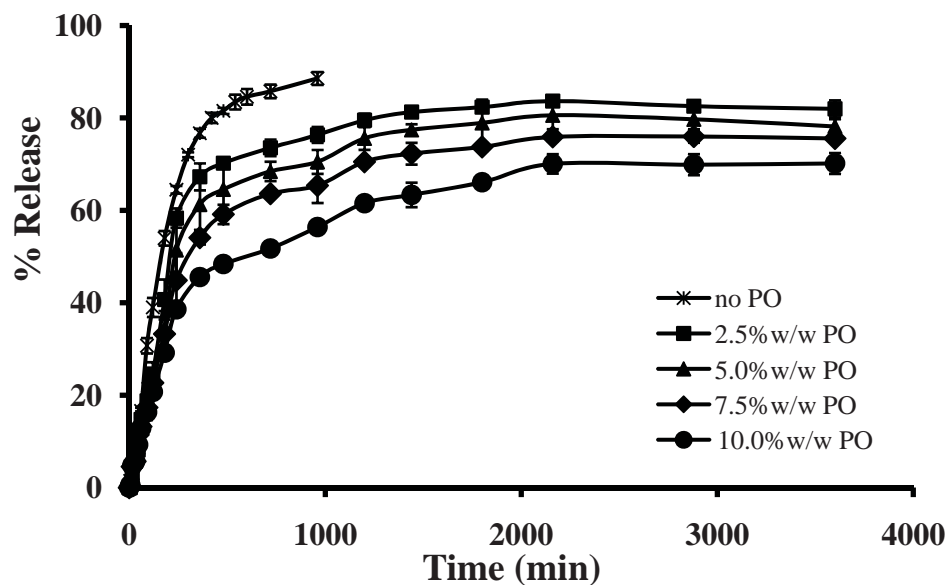


Figure 77 Effect of peppermint oil amount in 35% w/w Eudragit RS formula on release of doxycycline hyclate using dialysis method.

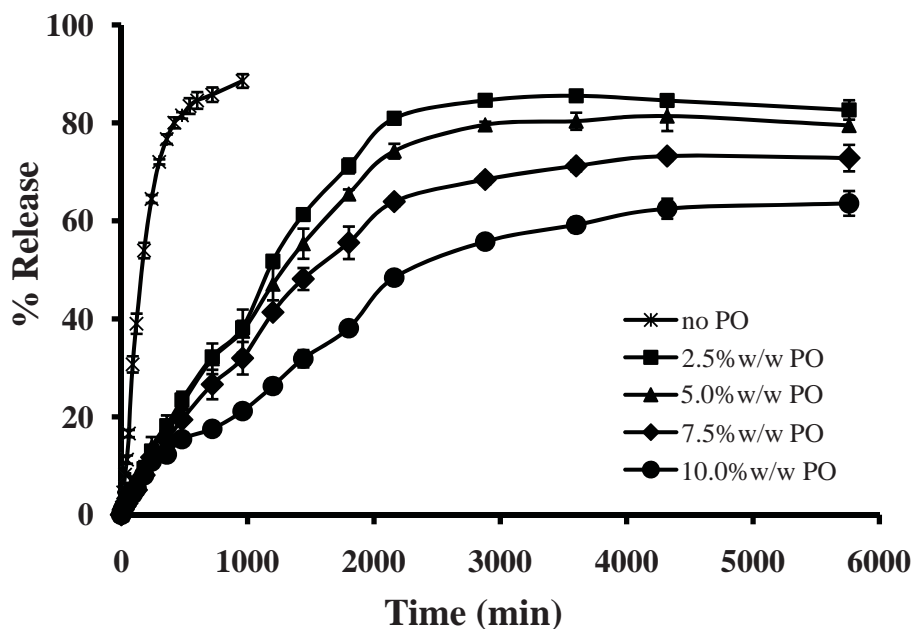


Figure 78 Effect of peppermint oil amount in 35% w/w Eudragit RS formula on release of doxycycline hyclate using membrane-less method.

The SEM micrographs of Eudragit RS formula with 5%w/w DH containing different amounts of PO (2.5-10%w/w) after releasing DH in PBS pH 6.8 at 37 °C at different magnifications are shown in Figure 79. The structures of all Eudragit RS formula containing PO (2.5-10%w/w) were continuous cells. When increasing PO amount in the Eudragit RS systems, the cell structures were more fused and thickened and the porous was more pronounced. This study suggested that the increased of PO amount decreased the solvent exchange thus the drug release was decreased as previously studied (Ganesh *et al.*, 2008). The hydrophobic plasticizer could contribute toward reduction in the penetration of the solvent molecules in the matrix.

The percentage of weight loss of the 35%w/w Eudragit RS formula containing different amounts of peppermint oil (2.5-10%w/w) without and with doxycycline hyclate (5% w/w) in phosphate buffer pH 6.8 after 1 month is shown in Table 39. The percentage of weight loss of the 35%w/w Eudragit RS formula containing different amounts of PO (2.5-10%w/w) without DH was significantly decreased when increasing PO amounts ($p < 0.05$). The addition of PO in the Eudragit RS-DH (5%w/w) systems showed the decreased percentage of weight loss of the systems. The 35%w/w Eudragit RS-DH (5%w/w) formula containing PO (10%w/w) was significantly decreased the percentage of weight loss of the 35%w/w Eudragit RS ($p < 0.05$) when the increased PO amount. The results suggested that the higher PO amount added into the Eudragit RS systems decreased the percentage of weight loss of the systems since the drug and solvent exchange with medium was difficult to occur (Ganesh *et al.*, 2008; Huang *et al.*, 1994). SEM micrographs also confirmed the formation of *in situ* forming gel systems indicating the cell structure were more fused and thickened and the porous was more pronounced, thus supporting the decreased weight loss.

Table 37 Comparison of degree of goodness-of-fit from curve fitting of the release profiles of doxycycline hyclate from 35%w/w Eudragit RS formula containing different amounts of peppermint oil (PO) (2.5-10%w/w) in phosphate buffer pH 6.8 using dialysis membrane and membrane-less method to different release models.

Formula	First order		Higuchi's		Zero order		Power law	
	r ²	msc	r ²	msc	r ²	msc	r ²	msc
<i>Dialysis membrane method</i>								
PO 2.5%	0.8615	1.61	0.9018	1.82	0.6795	0.77	0.9654	2.36
PO 5%	0.8567	1.58	0.9237	2.07	0.7288	0.94	0.9594	2.35
PO 7.5%	0.8814	1.79	0.9244	2.18	0.6432	0.70	0.9623	2.61
PO 10%	0.8733	1.70	0.9061	2.00	0.6210	0.70	0.9710	2.99
<i>Membrane-less method</i>								
PO 2.5%	0.9331	2.40	0.9663	2.99	0.9925	4.44	0.9949	4.60
PO 5%	0.9059	2.08	0.9689	3.11	0.9951	4.91	0.9986	5.94
PO 7.5%	0.9068	2.04	0.9640	2.99	0.9973	5.33	0.9853	3.62
PO 10%	0.9529	2.61	0.9523	2.74	0.9882	4.04	0.9527	2.59

Table 38 Estimate parameter from curve fitting of doxycycline hyclate release from 35%w/w Eudragit RS formula containing different amounts of peppermint oil (PO) (2.5-10%w/w) in phosphate buffer pH 6.8 using dialysis membrane and membrane-less method to power law expression.

Formula	k ± S.D.	tl ± S.D. (min)	n ± S.D.	Release mechanism
<i>Dialysis membrane method</i>				
PO 2.5%	0.3117 ± 0.0249	119.82 ± 0.13	0.13 ± 0.01	Fickain
PO 5%	0.2482 ± 0.0449	119.31 ± 0.16	0.15 ± 0.02	Fickain
PO 7.5%	0.2088 ± 0.0276	118.43 ± 0.54	0.17 ± 0.02	Fickain
PO 10%	0.1483 ± 0.0062	115.14 ± 0.63	0.20 ± 0.01	Fickain
<i>Membrane-less method</i>				
PO 2.5%	0.0413 ± 0.0018	228.11 ± 2.22	0.37 ± 0.01	Fickain
PO 5%	0.0372 ± 0.0052	220.76 ± 7.28	0.38 ± 0.02	Fickain
PO 7.5%	0.0274 ± 0.0059	218.94 ± 4.30	0.40 ± 0.03	Fickain
PO 10%	0.0082 ± 0.0010	155.99 ± 23.30	0.52 ± 0.02	Anomalous

k = release rate; tl = lag time and n = diffusional exponent

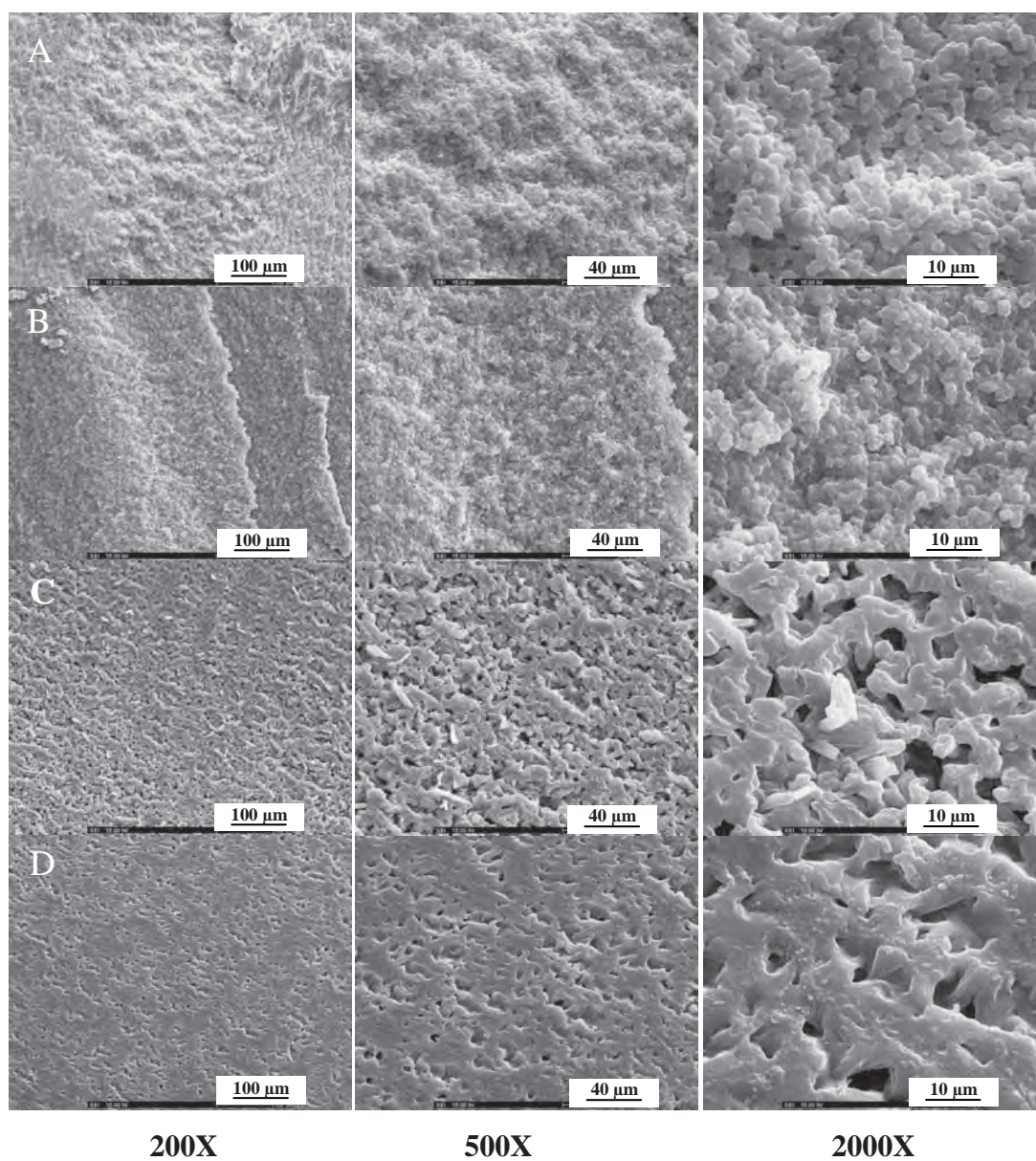


Figure 79 SEM micrograph of the dried gel systems; Doxycycline hyclate (5%w/w)-Eudragit RS (35%w/w) systems containing 2.5%w/w PO (A); 5%w/w PO (B); 7.5%w/w PO (C) and 10%w/w PO (D) with different magnifications (200X, 500X and 2000X).

Table 39 The percentage of weight loss of 35% w/w Eudragit RS formula containing different amounts of peppermint oil without and with 5% w/w doxycycline hyclate (n=3).

Concentration of PO (% w/w)	% Weight loss (n=3)	
	Without drug	DH (5% w/w)
2.5%	94.80 ± 1.71	63.12 ± 0.83
5%	92.78 ± 0.97	63.12 ± 0.10
7.5%	88.30 ± 0.79	61.84 ± 0.27
10%	84.92 ± 0.76	59.65 ± 0.35

The 35% w/w Eudragit RS-doxycycline hyclate (5% w/w) formula containing different amounts of PO were tested for the antimicrobial activity against *S. aureus*, *E. coli*, *C. albicans*, *S. mutans* and *P. gingivalis* using agar diffusion method as shown in Figure 80. The inhibition zone diameter against *S. aureus*, *E. coli*, *S. mutans* and *P. gingivalis* of the Eudragit RS-DH (5% w/w) systems containing PO (2.5-10% w/w) was significantly higher than that of the gel base (without DH) ($p < 0.05$). On the other hand, the inhibition zone diameter against *C. albicans* of the systems containing DH (5% w/w) was significantly lower than that of the gel base ($p < 0.05$). PO in 35% w/w Eudragit RS-DH (5% w/w) significantly decreased the inhibition zone diameter against *S. aureus*, *E. coli* and *S. mutans* ($p < 0.05$). The increasing PO amount did not significantly alter the antimicrobial activity against *S. aureus*, *E. coli* and *P. gingivalis* ($p > 0.05$), whereas the antimicrobial activity against *C. albicans* and *S. mutans* of the 35% w/w Eudragit RS systems with DH (5% w/w) were significantly decreased with increasing PO amount ($p < 0.05$). It has been reported that peppermint oil had the additive effect with oxytetracycline against *E. coli* by inhibiting replication of the F' lac metabolic plasmid (Schelz *et al.*, 2006). An important characteristic of essential oils and their components is their hydrophobicity, which enables them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structure and rendering them more permeable (Jeyakumar *et al.*, 2011; Sikkema *et al.*, 1994). Extensive leakage from bacterial cells or the exit of critical molecules and ions will lead to death (Denver and Hugo, 1991). The previous studies showed the incorporating hydrophobic plasticizer in *in situ* forming gel systems reduced the drug release, release rate (k) and rate of water diffusion into the gel, resulting the decreased antimicrobial activity.

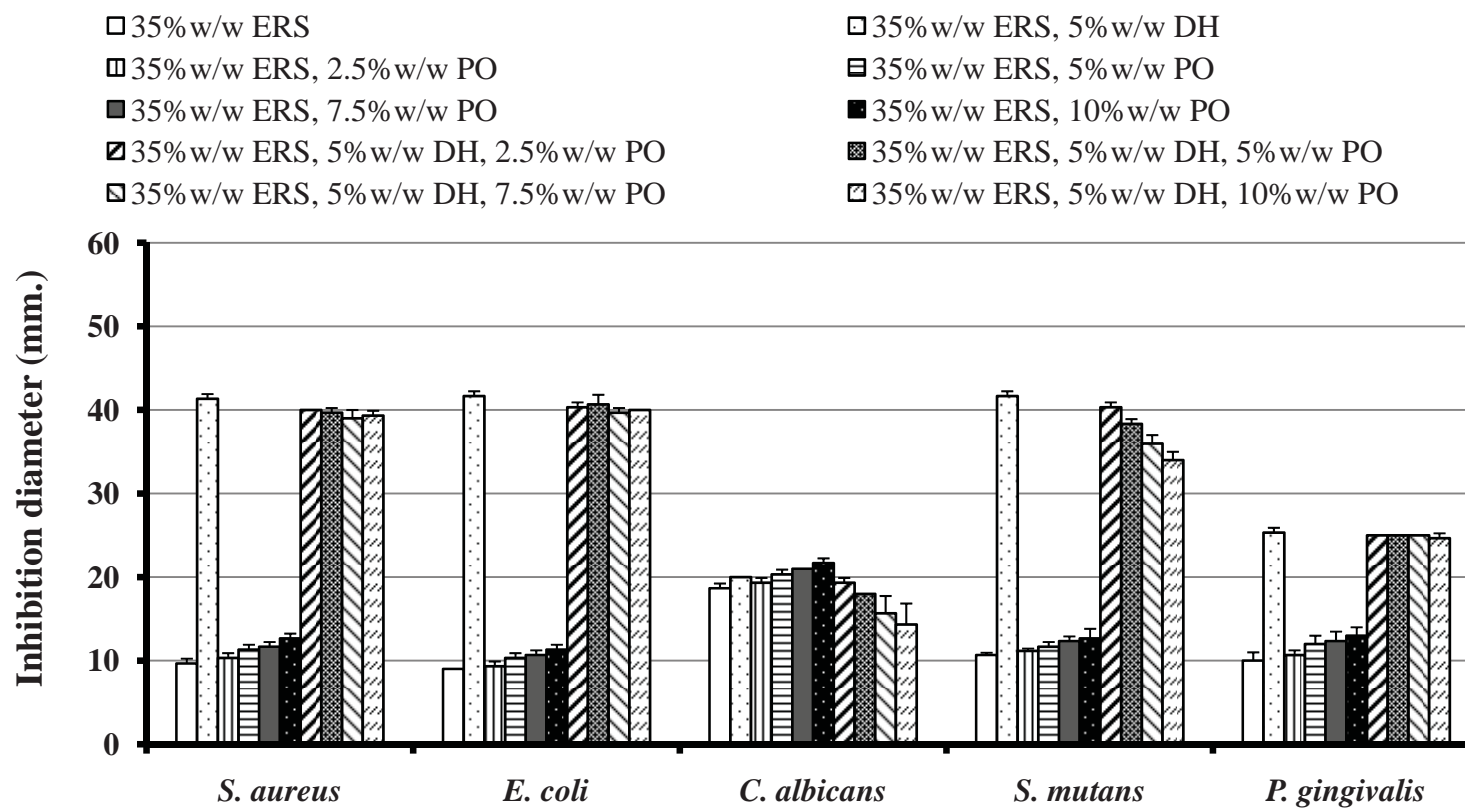


Figure 80 Inhibition zone diameter of the Eudragit RS (35% w/w)-doxycycline hyclate (5% w/w) formula containing different amounts of peppermint oil against *S. aureus*, *E. coli*, *C. albicans*, *S. mutans* and *P. gingivalis*

In the addition, benzoyl peroxide *in situ* forming gels were developed using Eudragit RS as polymer dispersed in *N*-methyl-pyrrolidone (NMP) (Mahadlek *et al.*, 2013). Peppermint oil and polyethylene glycol 1500 were also incorporated as the excipients. The prepared systems were evaluated for rheology, syringeability, *in situ* gel formation, antimicrobial activity and drug release (with dialysis method in PBS pH 6.8 at 50 rpm, 37 °C). When the amount of PO and PEG 1500 was increased, the shear stress was increased (data not shown). The shear stress of all systems was decreased as the temperature was increased. The rheological data of all prepared gels are fitted to power law model. Based on the values of *N*, it was apparent that all prepared gels exhibited nearly Newtonian behavior. All prepared gels showed a non-thixotropic behavior that did not depend on time. Increasing the amount of PO or PEG 1500 increased the viscosity of the ERS systems as shown in Table 40. Whereas the viscosity was decreased as temperature was increased. It is suggested that the incorporation of each excipient caused no change in the rheological properties of the ERS systems. Increasing the amount of PO or PEG 1500 increased the syringeability of the ERS systems (Table 40). The prepared gels should have an optimal viscosity that it will be easy for injectability as a sol, then contacted with physiological fluid and it transformed into gel due to the gradual insolubility of polymer in the new environment since the solvent in the prepared gels exchanged with the physiological fluid. All prepared gels comprising BP, PO and PEG 1500 could form *in situ* gel in the used medium which the pH was close to the environment pH of periodontal pocket (Figure 82). Antimicrobial activity against *S. mutans* was increased as the amount of BP was increased from 1-20% w/w (data not shown). The inhibition zone against *S. mutans* of the prepared system was decreased when PO and PEG 1500 was incorporated owing to the higher viscous environment and thereafter retardation of drug diffusion was occurred. The systems containing 5%w/w BP in 35%w/w ERS was about 30% drug release in 20 hrs, whereas that of systems containing 15%w/w PO was about 45% drug release in 96 hrs. The drug release was increased as the amount of PO was increased. The drug release profile of systems was not different as the amount of PEG1500 was increased (Figure 81). However, all prepared gels could sustain the BP release. The PEG1500 amount did not affect on the drug release. Release kinetic

obtained from curve fitting with various release equations using least square fit technique indicated that the release patterns corresponded with Higuchi's model (Table 41) therefore the release of BP was performed with diffusion control.

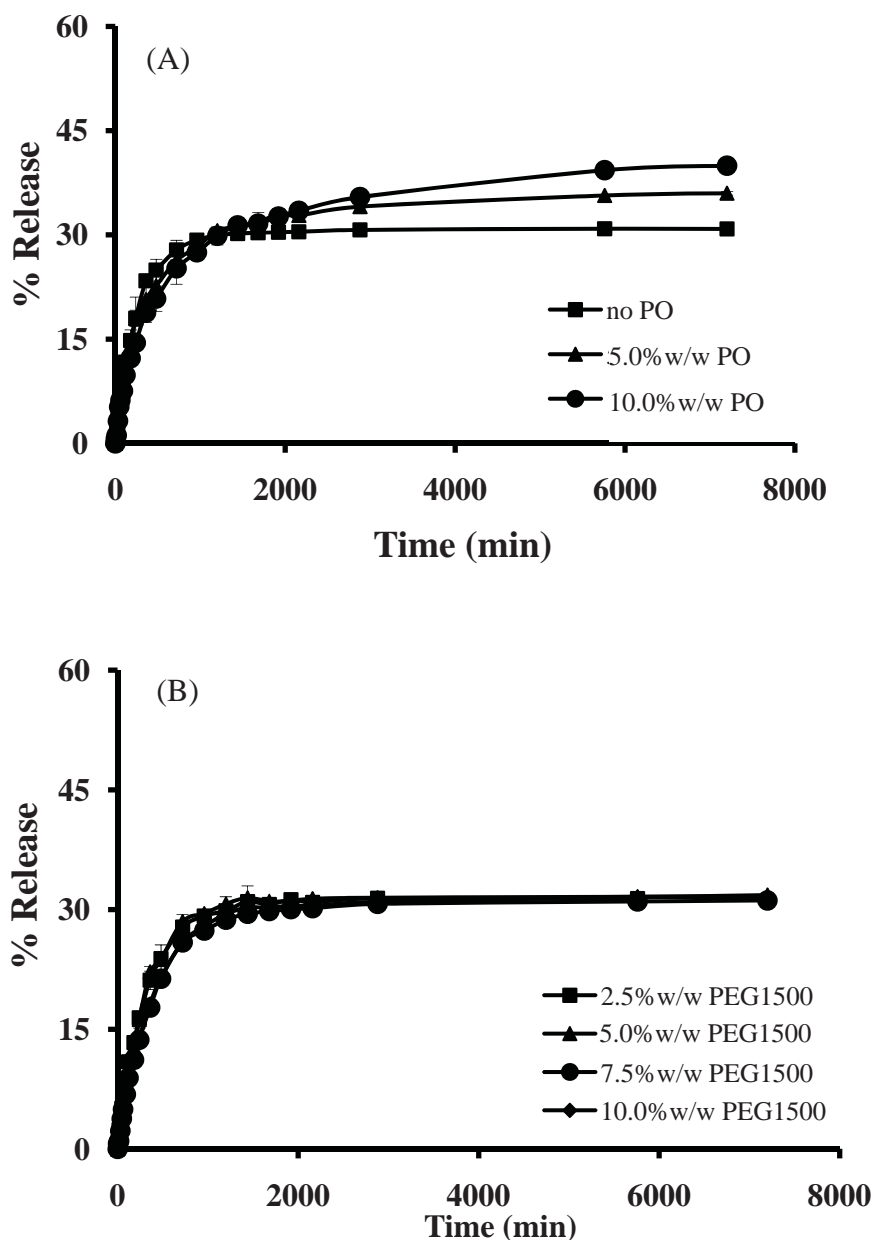


Figure 81 Release profiles of benzoyl peroxide in the Eudragit RS systems containing different amounts of (A) peppermint oil and (B) polyethylene glycol 1500.

Table 40 Flow parameters, syringeability and antimicrobial activity against *S. mutans* of Eudragit RS (35% w/w) formula containing different amount of peppermint oil and polyethylene glycol 1500 without and with benzoyl peroxide (5% w/w).

Formula (% w/w)	25°C		37°C		Syringeability (N)	Inhibition zone (mm)
	Flow index (N) (mean ± S.D.)	Consistency index (η) (mean ± S.D., [D/cm ²] ^N s)	Flow index (N) (mean ± S.D.)	Consistency index (η) (mean ± S.D., [D/cm ²] ^N s)		
35%ERS	1.00 ± 0.00	500.70 ± 5.37	1.01 ± 0.00	271.57 ± 1.29	4.57 ± 0.36	10.7 ± 0.3
35%ERS, 5%BP	1.00 ± 0.01	734.67 ± 13.48	1.02 ± 0.01	403.20 ± 13.40	5.74 ± 0.40	11.5 ± 0.5
35%ERS, 5%BP, 5%PO	0.99 ± 0.01	808.00 ± 36.61	1.00 ± 0.01	497.30 ± 2.88	7.77 ± 0.92	10.8 ± 0.3
35%ERS, 5%BP, 10%PO	1.00 ± 0.01	849.93 ± 12.22	1.01 ± 0.00	460.47 ± 30.37	10.37 ± 1.00	10.8 ± 0.3
35%ERS, 5%BP, 2.5%PEG1500	1.00 ± 0.01	878.27 ± 22.00	1.01 ± 0.00	520.43 ± 4.08	9.58 ± 0.23	11.0 ± 0.5
35%ERS, 5%BP, 5%PEG1500	1.00 ± 0.00	1069.67 ± 32.87	1.00 ± 0.00	625.70 ± 8.38	10.63 ± 0.30	11.0 ± 0.5
35%ERS, 5%BP, 7.5%PEG1500	1.00 ± 0.00	1190.67 ± 21.96	1.00 ± 0.01	687.27 ± 25.42	11.48 ± 0.43	10.7 ± 0.6
35%ERS, 5%BP, 10%PEG1500	1.00 ± 0.00	1413.67 ± 31.56	1.00 ± 0.02	823.00 ± 41.51	12.73 ± 0.95	10.3 ± 0.6

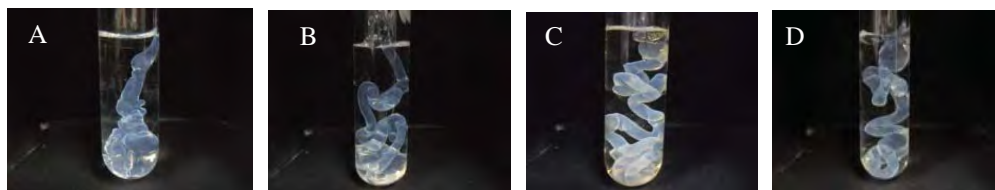


Figure 82 Appearance of gel formed in phosphate buffer pH 6.8; (A) 35 %w/w ERS gels, (B) 35 %w/w ERS gels containing BP (5 %w/w), (C) 35 %w/w ERS gels containing BP (5 %w/w) and PO (5 %w/w), and (D) 35 %w/w ERS gels containing BP (5 %w/w) and PEG1500 (2.5 %w/w).

Table 41 Comparison of degree of goodness-of-fit from curve fitting of drug release from various systems

System	Power law		First order		Higuchi's		Zero order	
	r ²	msc	r ²	msc	r ²	msc	r ²	msc
35%ERS, 5%BP	0.9790	3.0056	0.8423	1.5397	0.9346	2.3942	0.8405	1.5280
35%ERS, 5%BP, 5%PO	0.9950	4.7077	0.5890	0.4448	0.9434	2.4269	0.8870	1.7361
35%ERS, 5%BP, 10%PO	0.9674	3.0228	0.7436	0.9611	0.9627	2.8889	0.8674	1.7705
35%ERS, 5%BP, 2.5%PEG1500	0.9769	3.1005	0.6010	0.4743	0.8741	1.6720	0.8367	1.3678
35%ERS, 5%BP, 5.0%PEG1500	0.9713	2.8856	0.6540	0.6169	0.9013	1.8716	0.8222	1.2829
35%ERS, 5%BP, 7.5%PEG1500	0.9739	3.0440	0.8943	1.9391	0.9734	3.3210	0.8001	1.3875
35%ERS, 5%BP, 10.0%PEG1500	0.9978	2.9073	0.9001	1.9957	0.9770	3.4651	0.8703	1.7571

4.5 Stability study

The stability of the *in situ* gel systems at 4°C, 25°C and 45°C after 3 months were conducted for their appearance, pH value, viscosity studies, rheological behaviors, drug release and antimicrobial activities. The Eudragit RS (35% w/w) system without and with DH (5% w/w) and containing PO and PEG1500 (5% w/w) were selected for this study. The appearances of the gel base systems and systems containing PEG1500 and PO after stability test at 4°C and 25°C were clear and colorless similar to the systems before stability test, whereas the appearances of the systems after stability test at 45°C were changed to a slightly clear yellow. However, the 35% w/w Eudragit RS-doxycycline hydrochloride (5% w/w) systems containing PEG and PO (5% w/w) after stability test at 4°C were slightly yellow when compared with the systems before stability test, whereas that of systems at 25°C and 45°C were black. The appearance results indicated that the *in situ* gel systems should be kept in 4°C, which obtained the best suitable appearance. The manufacturing as the freshly prepared formulation might be the proper technique to prepare the *in situ* gel forming system for this drug. The pH values of various Eudragit RS systems containing PEG1500, PO and without/with DH (5% w/w) after stability test at 4°C, 25°C and 45°C are shown in Table 42. The pH values of the gel base systems and systems containing PEG1500 and PO (5% w/w) after stability test at 4°C, 25°C and 45°C were in the range between 8.15 to 9.23 whereas, the pH values of the 35% w/w Eudragit RS-doxycycline hydrochloride (5% w/w) systems containing PEG and PO (5% w/w) after stability test at 4°C, 25°C and 45°C were in the range of 3.22 to 4.16.

The effects of polyethylene glycol 1500 and peppermint oil (5% w/w) on viscosity of Eudragit RS (35% w/w) formula without and with doxycycline hydrochloride after stability test at 4°C, 25°C and 45°C were investigated. The relationships between shear rate and apparent viscosity of PEG1500 and PO (5% w/w) in Eudragit RS (35% w/w) formula without and with doxycycline hydrochloride (5% w/w) at 4°C, 25°C and 45°C are presented in Figures 83, 84 and 85, respectively. The apparent viscosity of all formula after stability test at 4°C, 25°C and 45°C did not differ from that of before stability test ($p > 0.05$).

Table 42 The pH values of various systems after stability test (3 months) at 4°C, 25°C and 45°C.

Formula (%w/w)	pH \pm S.D. (n=3)			
	before stability test	after stability test (3 months)		
		at 4°C	at 25°C	at 45°C
<i>Without drug</i>				
ERS 35%	8.69 \pm 0.09	9.14 \pm 0.02	9.12 \pm 0.03	8.67 \pm 0.04
ERS 35%, PEG1500 5%	8.44 \pm 0.02	9.23 \pm 0.03	8.65 \pm 0.04	8.72 \pm 0.03
ERS 35%, PO 5%	8.04 \pm 0.02	9.06 \pm 0.02	8.02 \pm 0.02	8.15 \pm 0.06
<i>with 5%w/w DH</i>				
ERS 35%, DH 5%	3.77 \pm 0.05	4.16 \pm 0.04	3.90 \pm 0.11	3.57 \pm 0.02
ERS 35%, PEG1500 5%, DH 5%	3.70 \pm 0.02	4.04 \pm 0.03	3.61 \pm 0.02	3.40 \pm 0.01
ERS 35%, PO 5%, DH 5%	3.46 \pm 0.04	3.97 \pm 0.02	3.41 \pm 0.03	3.22 \pm 0.02

In the addition, the rheological behaviors of various systems after stability test at 4°C, 25°C and 45°C were investigated as a function of amount and temperature. The shear stress versus shear rate flow curves of various systems after stability test at 4°C, 25°C and 45°C are shown in Figures 86, 87 and 88, respectively. All formula showed Newtonian behavior, indicating the up curve did coincide with the down curve. The shear stress of all formula decreased with an increasing temperature. The flow parameters of various systems after stability test at 4°C, 25°C and 45°C are shown in Tables 43, 44 and 45, respectively. N value of all formulations was close to 1, indicating that the flow type was Newtonian. Temperature did not change the flow types of the systems. In the case of the viscosity coefficient (η), as the viscosity coefficient of various systems after stability test at 4°C, 25°C and 45°C did not significantly differ from that of various systems before stability test ($p > 0.05$).

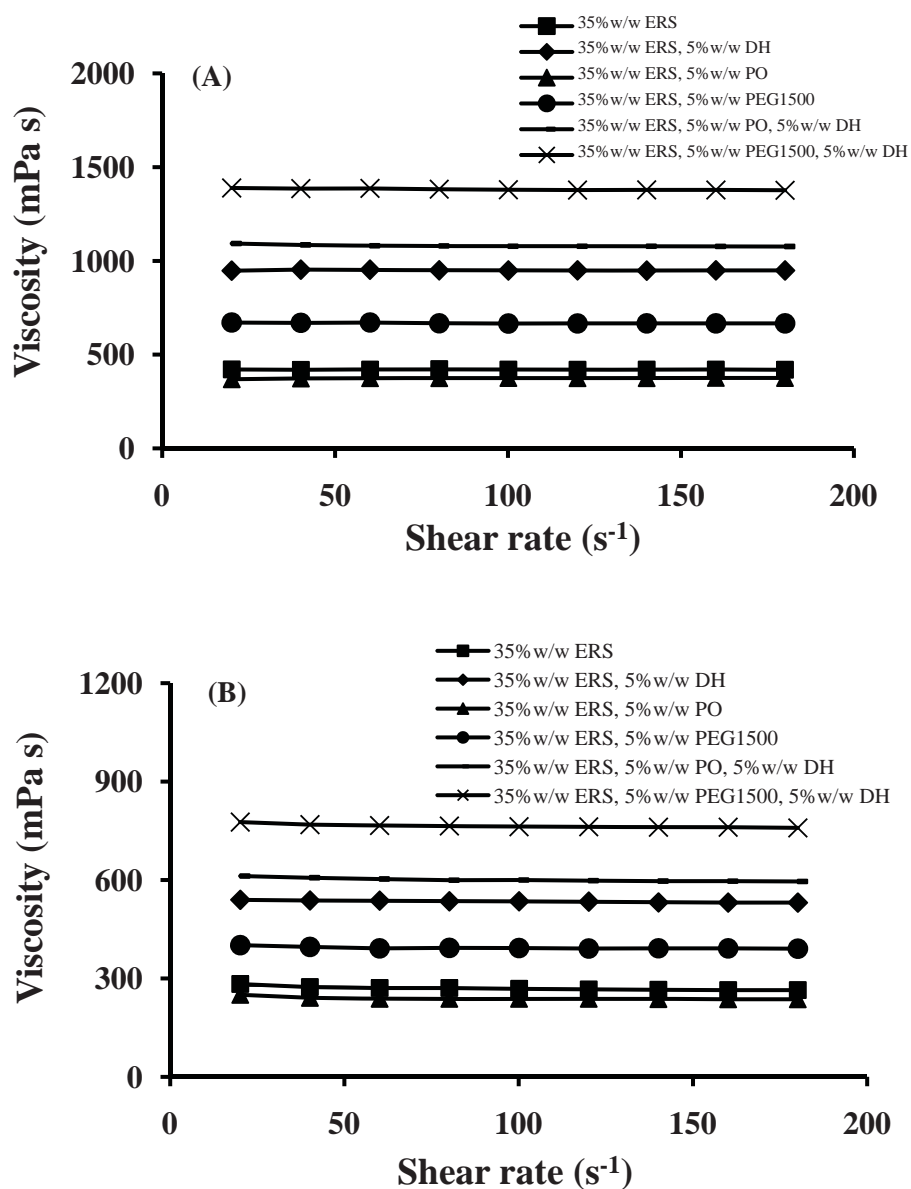


Figure 83 Viscosity curves of 35% w/w Eudragit RS formula containing polyethylene glycol, peppermint oil and doxycycline hyclate after stability test (3 months) at 4°C at (A) 25°C and (B) 37°C

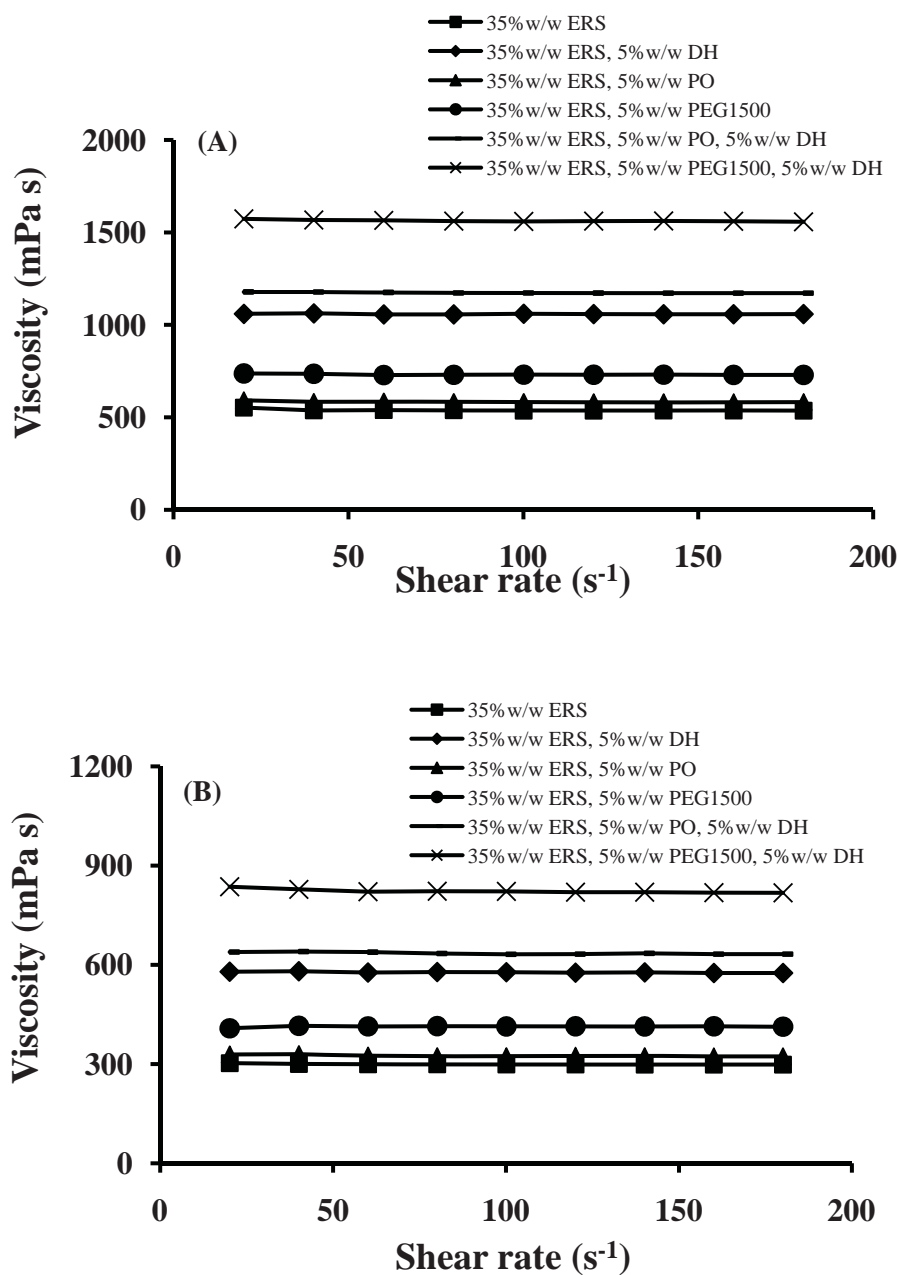


Figure 84 Viscosity curves of 35%w/w Eudragit RS formula containing polyethylene glycol, peppermint oil and doxycycline hyclate after stability test (3 months) at 25°C at (A) 25°C and (B) 37°C

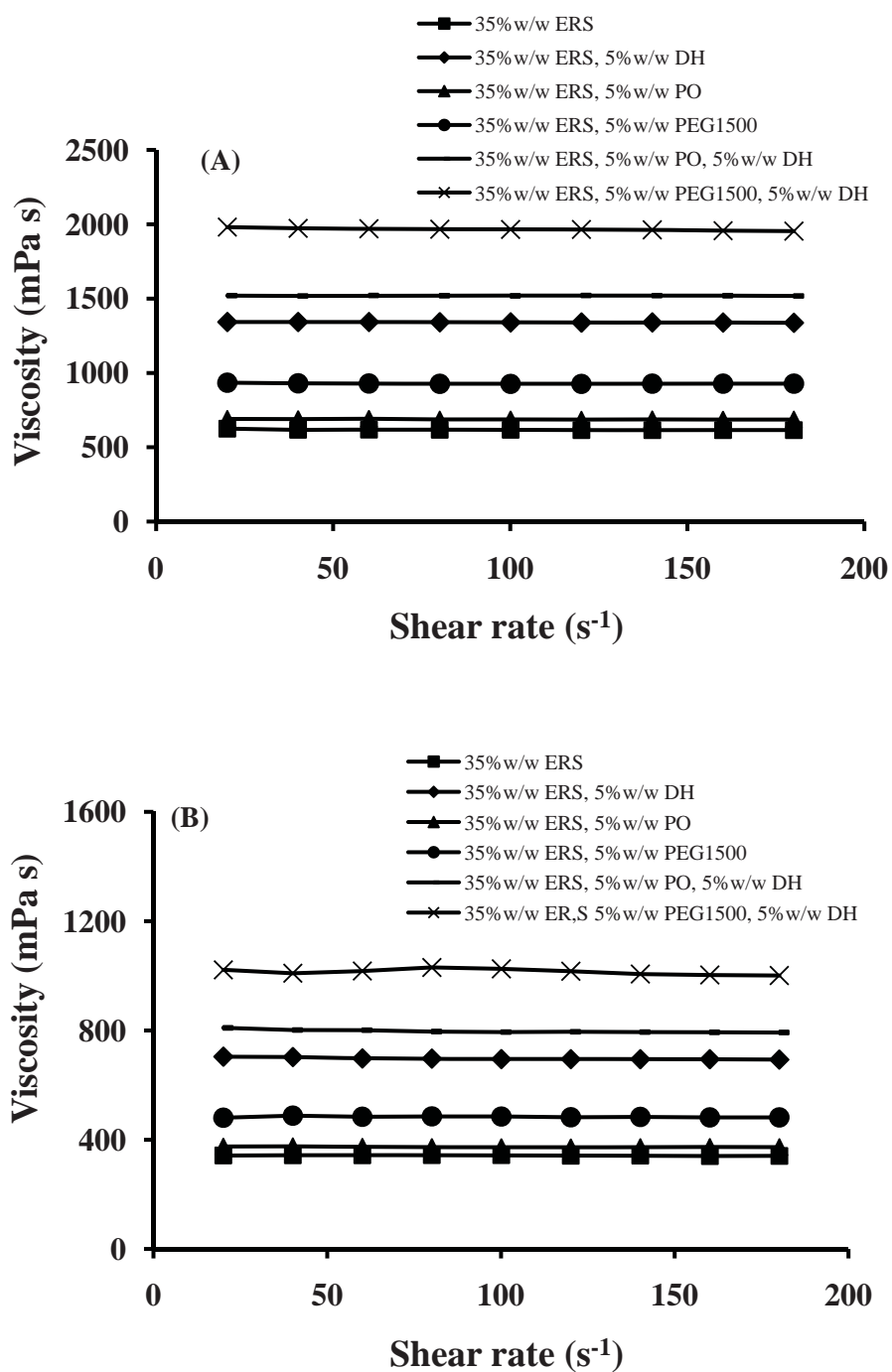


Figure 85 Viscosity curves of 35% w/w Eudragit RS formula containing polyethylene glycol, peppermint oil and doxycycline hyclate after stability test (3 months) at 45°C at (A) 25°C and (B) 37°C

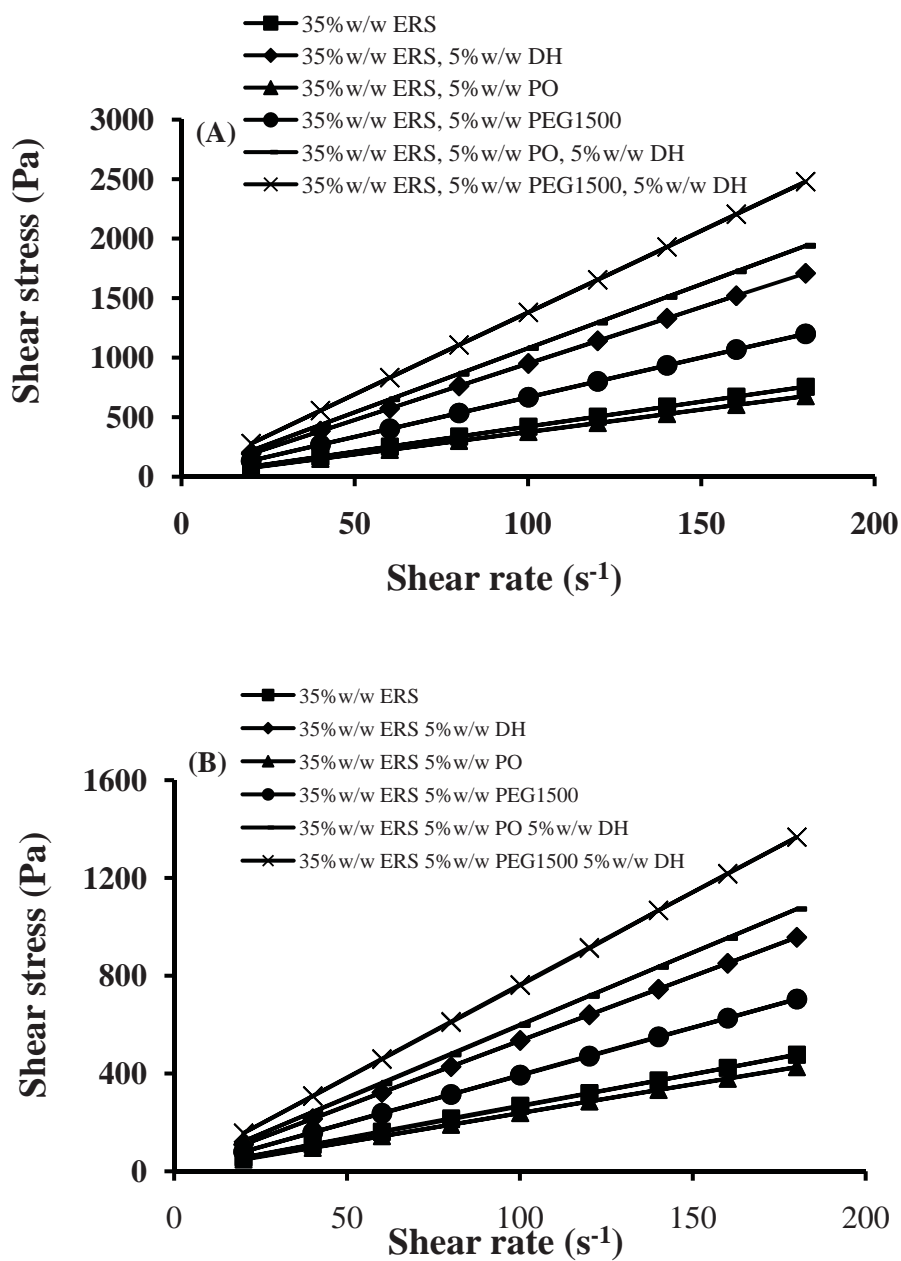


Figure 86 Flow curve of 35% w/w Eudragit RS formula containing polyethylene glycol, peppermint oil and doxycycline hyclate after stability test (3 months) at 4°C at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

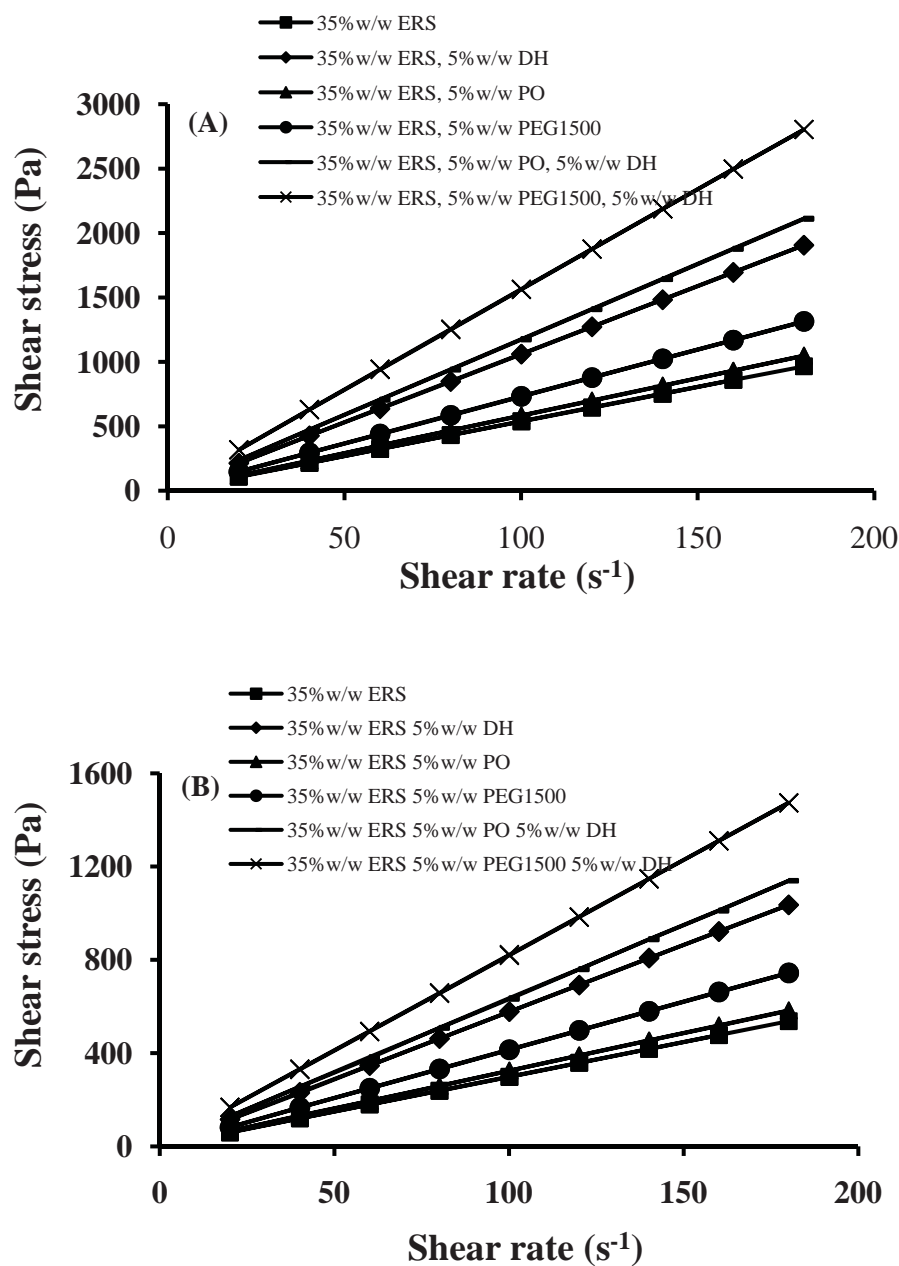


Figure 87 Flow curve of 35% w/w Eudragit RS formula containing polyethylene glycol, peppermint oil and doxycycline hyclate after stability test (3 months) at 25°C at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

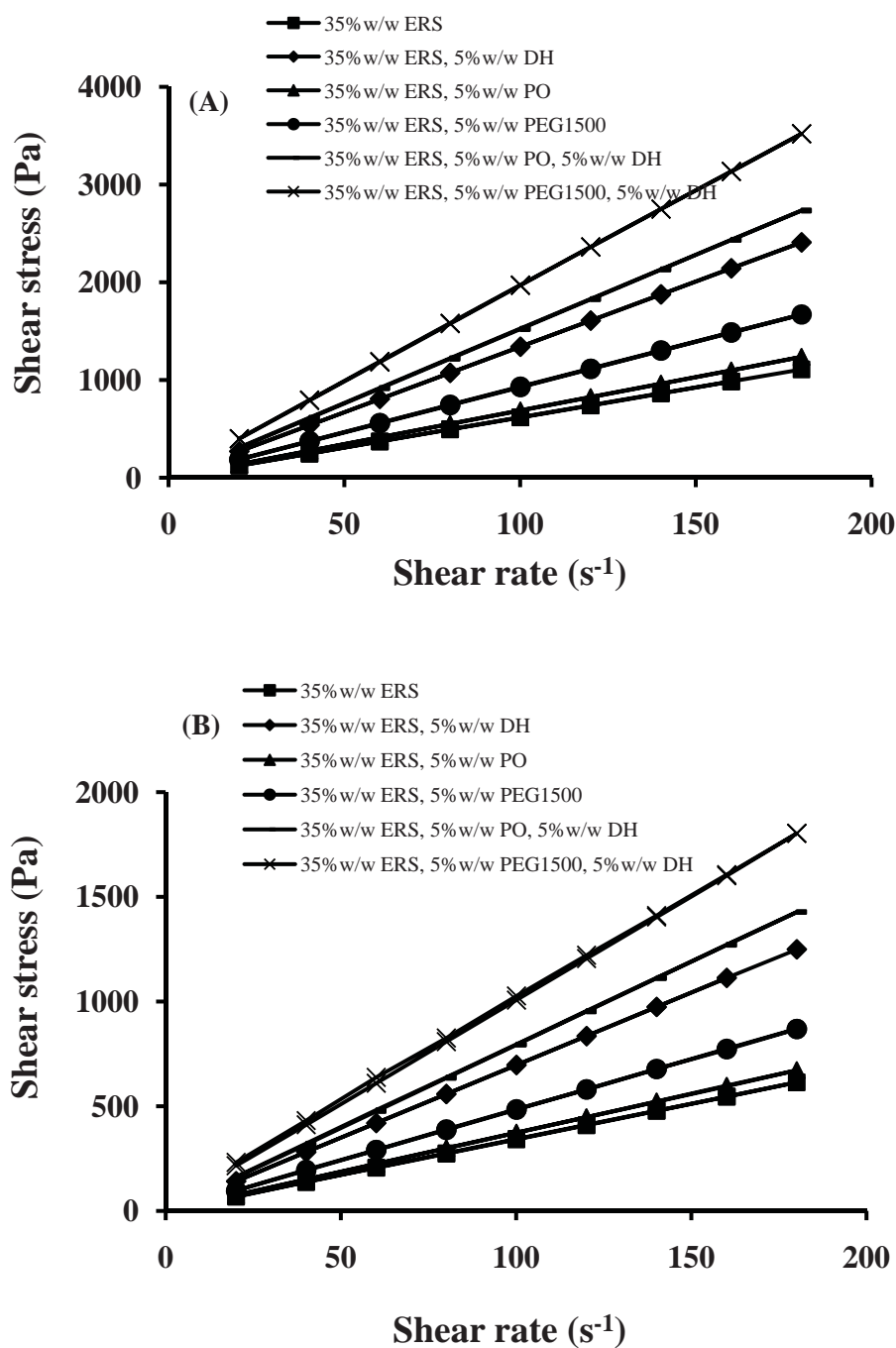


Figure 88 Flow curve of 35% w/w Eudragit RS formula containing polyethylene glycol, peppermint oil and doxycycline hyclate after stability test (3 months) at 45°C at (A) 25°C and (B) 37°C. Open symbols represent the up-curve, and closed symbols represent the down-curve.

Table 43 Flow parameters of Eudragit RS (35% w/w) formula containing polyethylene glycol 1500 and peppermint oil and with and without doxycycline hyclate after stability test (3 months) at 4°C (n=3).

Formula (% w/w)	25°C		37°C	
	Flow index (N) (mean ± S.D.)	Consistency index (η) (mean ± S.D., [D/cm ²] ^N s)	Flow index (N) (mean ± S.D.)	Consistency index (η) (mean ± S.D., [D/cm ²] ^N s)
35% ERS	1.00 ± 0.00	423.10 ± 8.95	0.98 ± 0.01	298.67 ± 9.07
35% ERS, 5% DH	1.00 ± 0.01	970.30 ± 4.32	0.99 ± 0.01	556.53 ± 17.51
35% ERS, 5% PO	1.00 ± 0.01	373.23 ± 16.31	1.00 ± 0.03	249.03 ± 10.19
35% ERS, 5% PO, 5% DH	1.00 ± 0.01	1104.33 ± 14.43	0.99 ± 0.01	407.43 ± 8.01
35% ERS, 5% PEG1500	1.00 ± 0.01	678.43 ± 6.83	0.99 ± 0.01	632.30 ± 17.10
35% ERS, 5% PEG1500, 5% DH	1.00 ± 0.01	1411.33 ± 36.96	0.99 ± 0.00	795.80 ± 16.37

Table 44 Flow parameters of Eudragit RS (35% w/w) formula containing polyethylene glycol 1500 and peppermint oil and with and without doxycycline hyclate after stability test (3 months) at 25°C (n=3).

Formula (% w/w)	25°C		37°C	
	Flow index (N) (mean ± S.D.)	Consistency index (η) (mean ± S.D., [D/cm ²] ^N s)	Flow index (N) (mean ± S.D.)	Consistency index (η) (mean ± S.D., [D/cm ²] ^N s)
35% ERS	1.00 ± 0.01	548.80 ± 5.38	1.00 ± 0.01	302.37 ± 13.28
35% ERS, 5% DH	1.00 ± 0.01	1077.00 ± 160.9	1.00 ± 0.00	576.50 ± 10.93
35% ERS, 5% PO	1.00 ± 0.00	584.10 ± 0.46	0.99 ± 0.02	337.03 ± 19.60
35% ERS, 5% PO, 5% DH	1.00 ± 0.00	1196.33 ± 8.96	0.99 ± 0.00	653.87 ± 2.01
35% ERS, 5% PEG1500	1.00 ± 0.01	747.33 ± 20.65	1.00 ± 0.01	412.60 ± 10.81
35% ERS, 5% PEG1500, 5% DH	0.99 ± 0.01	1607.00 ± 16.82	0.99 ± 0.00	848.23 ± 8.72

Table 45 Flow parameters of Eudragit RS (35% w/w) formula containing polyethylene glycol 1500 and peppermint oil and with and without doxycycline hyclate after stability test (3 months) at 45°C (n=3).

Formula (%w/w)	25°C		37°C	
	Flow index (N) (mean ± S.D.)	Consistency index (η) (mean ± S.D., [D/cm ²] ^N s)	Flow index (N) (mean ± S.D.)	Consistency index (η) (mean ± S.D., [D/cm ²] ^N s)
35% ERS	1.00 ± 0.00	628.27 ± 19.86	1.00 ± 0.02	342.17 ± 15.83
35% ERS, 5% DH	1.00 ± 0.00	1357.33 ± 16.44	0.99 ± 0.01	718.37 ± 10.80
35% ERS, 5% PO	1.00 ± 0.00	700.80 ± 698.10	1.00 ± 0.01	380.37 ± 1.76
35% ERS, 5% PO, 5% DH	1.00 ± 0.00	1552.00 ± 52.31	1.00 ± 0.01	813.83 ± 18.70
35% ERS, 5% PEG1500	1.00 ± 0.01	950.40 ± 49.08	1.00 ± 0.01	481.93 ± 19.15
35% ERS, 5% PEG1500, 5% DH	0.99 ± 0.00	2035 ± 16.09	0.96 ± 0.01	1255.67 ± 54.17

Drug release profiles of various formulations after stability test (3 months) at 4°C, 25°C and 45°C were evaluated. The drug release profiles of the 35%w/w Eudragit RS-DH (5%w/w), the 35%w/w Eudragit RS-DH (5%w/w) containing PEG1500 (5%w/w) and the 35%w/w Eudragit RS DH (5%w/w) containing PO (5%w/w) formula in phosphate buffer pH 6.8 were evaluated using dialysis membrane method after stability test (3 months) at 4°C, 25°C and 45°C are shown in Figure 89, 90 and 91, respectively. The doxycycline hyclate release at 24 hours from the 35%w/w Eudragit RS-DH (5%w/w) systems before stability test and after stability test at 4°C, 25°C and 45°C were 77.4%, 77.0%, 69.9% and 64.8%, respectively. The doxycycline hyclate release at 24 hours from the 35%w/w Eudragit RS-DH (5%w/w) systems containing PEG1500 (5%w/w) before stability test and after stability test at 4°C, 25°C and 45°C were 79.1%, 83.0%, 73.6% and 65.8%, respectively. The doxycycline hyclate release at 24 hours from the 35%w/w Eudragit RS-DH (5%w/w) systems containing PO (5%w/w) before stability test and after stability test at 4°C, 25°C and 45°C were 77.4%, 68.2%, 65.3% and 57.6%, respectively.

The coefficient of determination (r^2) and msc from curve fitting to first order, Higuchi's, zero order, and power law equations after the release studies using dialysis membrane method are shown in Table 46. The doxycycline hyclate release from the 35%w/w Eudragit RS-DH (5%w/w) systems after stability test (3 months) at 4°C, 25°C and 45°C were best explained by power law model, but a close relationship were first order, Higuchi's and Higuchi's model, respectively. The doxycycline hyclate release from the 35%w/w Eudragit RS-DH (5%w/w) systems containing PEG1500 (5%w/w) after stability test (3 months) at 4°C, 25°C and 45°C were best explained by power law model, but a close relationship were first order, zero order and Higuchi's model, respectively. Whereas the doxycycline hyclate release from the 35%w/w Eudragit RS-DH (5%w/w) systems containing PO (5%w/w) after stability test (3 months) at 4°C, 25°C and 45°C were best explained by power law model, but a close relationship were first order, zero order and first order model, respectively.

The release exponent values (n) for power law of all formulations from the release studies after stability test (3 months) at 4°C, 25°C and 45°C using dialysis membrane method is shown in Table 47. The n value obtained from power law

equation of all formula after stability test were in the range from 0.12-0.27. The results indicated that all formula showed drug release by Fickian diffusion mechanism (Table 47). On considering the drug release rate (k) parameter after stability test, the drug release rate of the 35% w/w Eudragit RS-DH (5% w/w) systems after stability test (3 months) at 4°C, 25°C and 45°C were significantly less than that before stability test ($p < 0.05$). The drug release rate of the 35% w/w Eudragit RS-DH (5% w/w) systems containing PEG1500 after stability test (3 months) at 45°C and before stability test did not significantly differ ($p > 0.05$), whereas the drug release rate of the 35% w/w Eudragit RS-DH (5% w/w) systems containing PEG1500 after stability test (3 months) at 4°C and 25°C was significantly higher than that the systems before stability test ($p < 0.05$). The drug release rate of the 35% w/w Eudragit RS-DH (5% w/w) systems containing PO (5% w/w) after stability test (3 months) at 4°C, 25°C and 45°C were significantly less than that before stability test ($p < 0.05$).

The antimicrobial activities of the 35% w/w Eudragit RS-DH (5% w/w), the 35% w/w Eudragit RS- DH (5% w/w) containing PEG1500 (5% w/w) and the 35% w/w Eudragit RS-DH (5% w/w) containing PO (5% w/w) formula after stability test (3 months) at 4°C, 25°C and 45°C against *S. aureus*, *E. coli*, *C. albicans*, *S. mutans* and *P. gingivalis* were investigated by agar diffusion method as shown in Figure 92. The inhibition zone diameter of the 35% w/w Eudragit RS-DH (5% w/w), the 35% w/w Eudragit RS-DH (5% w/w) containing PEG1500 (5% w/w) and the 35% w/w Eudragit RS-DH (5% w/w) containing PO (5% w/w) formula after stability test (3 months) at 4°C, 25°C and 45°C against *S. aureus* was not significantly different from that of before stability test ($p > 0.05$). The inhibition zone diameter of the 35% w/w Eudragit RS-DH (5% w/w), the 35% w/w Eudragit RS-DH (5% w/w) containing PEG1500 (5% w/w) and the 35% w/w Eudragit RS-DH (5% w/w) containing PO (5% w/w) formula after stability test (3 months) at 4°C, 25°C and 45°C against *E. coli* were significantly decreased when compared with before stability test ($p < 0.05$). The inhibition zone diameter of the 35% w/w Eudragit RS-DH (5% w/w), the 35% w/w Eudragit RS-DH (5% w/w) containing PEG1500 (5% w/w) and the 35% w/w Eudragit RS-DH (5% w/w) containing PO (5% w/w) formula after stability test (3 months) at 4°C and 25°C against *C. albicans* was not significantly different from that of before stability test ($p > 0.05$), whereas The

inhibition zone diameter of the 35%w/w Eudragit RS-DH (5% w/w), the 35%w/w Eudragit RS-DH (5% w/w) containing PEG1500 (5% w/w) and the 35%w/w Eudragit RS-DH (5% w/w) containing PO (5%w/w) formula after stability test (3 months) at 45°C against *C. albicans* significantly increased ($p<0.05$). The inhibition zone diameter of the 35%w/w Eudragit RS-DH (5% w/w), the 35%w/w Eudragit RS-DH (5% w/w) containing PEG1500 (5% w/w) and the 35%w/w Eudragit RS-DH (5% w/w) containing PO (5% w/w) formula after stability test (3 months) at 4±1°C, 25±1°C and 45±1°C against *S. mutans* and *P. gingivalis* significantly increased from that of before stability test ($p<0.05$). The results indicated that after stability test, the Eudragit RS-DH systems containing PEG and PO exhibited the antimicrobial activity against *S. aureus*, *E. coli*, *C. albicans*, *S. mutans* and *P. gingivalis* similar to before stability test.

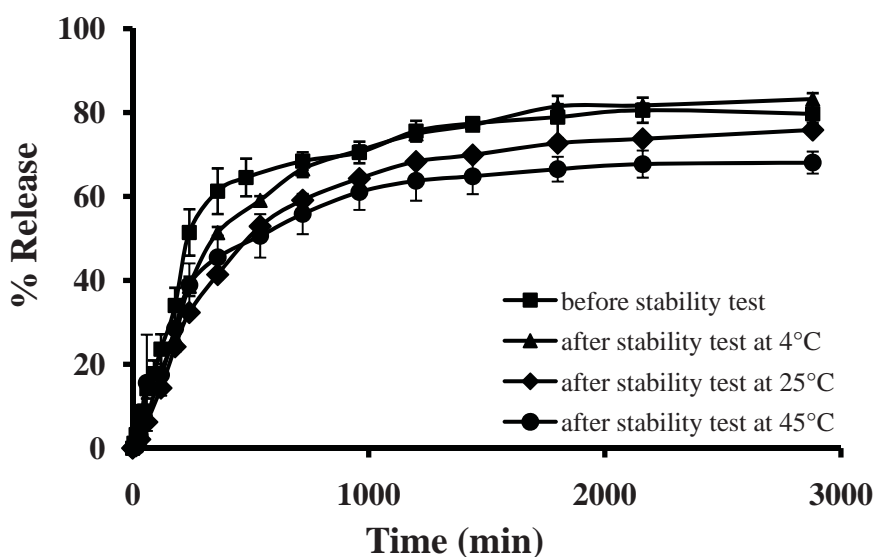


Figure 89 Release profiles of the 35%w/w Eudragit RS-doxycycline hyclate (5%w/w) systems after stability test (3 months) at 4°C, 25°C and 45°C.

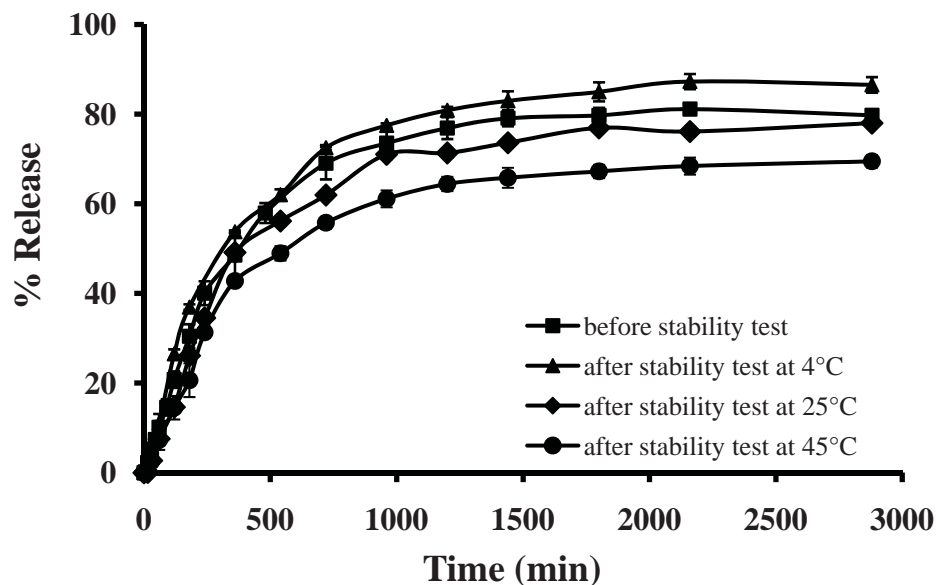


Figure 90 Release profiles of the 35%w/w Eudragit RS-doxycycline hyclate (5%w/w) systems containing polyethylene glycol 1500 (5%w/w) after stability test (3 months) at 4°C, 25°C and 45°C.

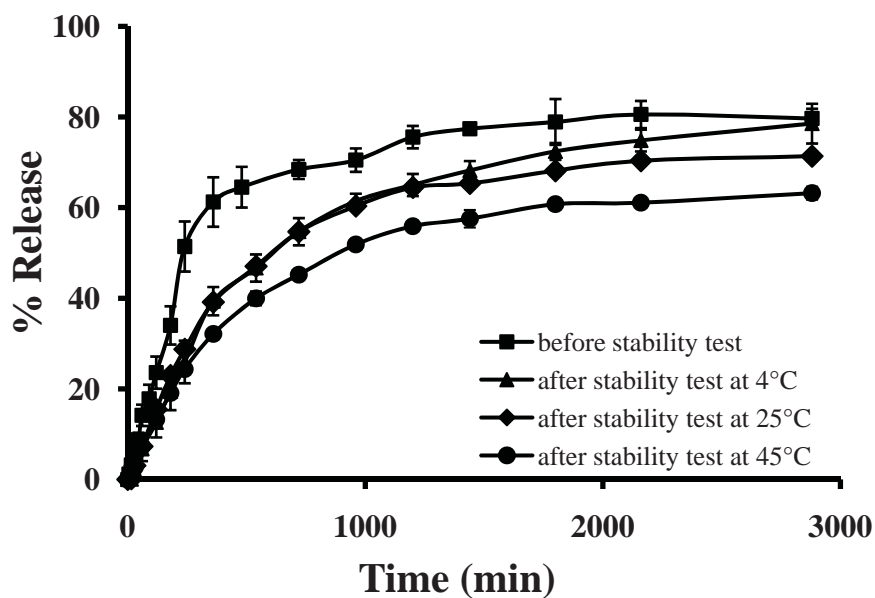


Figure 91 Release profiles of the 35%w/w Eudragit RS-doxycycline hyclate (5%w/w) systems containing peppermint oil (5%w/w) after stability test (3 months) at 4°C, 25°C and 45°C.

Table 46 Comparison of degree of goodness-of-fit from curve fitting of the release profiles of doxycycline hyclate in phosphate buffer pH 6.8 after stability test (3 months) at 4°C, 25°C and 45°C using dialysis membrane method to different release models.

Formula	First order		Higuchi's		Zero order		Power law	
	r ²	msc	r ²	msc	r ²	msc	r ²	msc
ERS 35%, DH 5%								
at 4°C	0.9292	2.25	0.8064	1.31	0.8735	1.50	0.9837	3.57
at 25°C	0.8654	1.67	0.9792	1.44	0.9086	1.82	0.9850	3.14
at 45°C	0.8527	1.47	0.8987	1.95	0.8420	1.40	0.9827	3.51
ERS 35%, PEG1500 5%, DH 5%								
at 4°C	0.9268	2.28	0.7973	1.26	0.8957	1.69	0.9633	2.70
at 25°C	0.8504	1.57	0.7799	1.18	0.8936	1.67	0.9653	2.82
at 45°C	0.8915	1.82	0.9167	1.99	0.8910	1.36	0.9746	3.13
ERS 35%, PO 5%, DH 5%								
at 4°C	0.9306	2.33	0.9059	2.03	0.7826	1.19	0.9869	3.79
at 25°C	0.8482	1.55	0.8536	1.59	0.8825	1.70	0.9740	3.10
at 45°C	0.9379	2.38	0.8842	1.82	0.9362	2.25	0.9768	3.22

Table 47 Estimate parameter from curve fitting of doxycycline hyclate release in phosphate buffer pH 6.8 after stability test (3 months) at 4°C, 25°C and 45°C using dialysis membrane method to different release models.

Formula	$k \pm \text{S.D.}$	$t_l \pm \text{S.D. (min)}$	$n \pm \text{S.D.}$	Release mechanism
ERS 35%, DH 5%				
at 4°C	0.1883 ± 0.0093	174.38 ± 2.66	0.20 ± 0.01	Fickian
at 25°C	0.1431 ± 0.0125	170.20 ± 4.27	0.22 ± 0.01	Fickian
at 45°C	0.2063 ± 0.0519	172.22 ± 2.14	0.14 ± 0.03	Fickian
ERS 35%, PEG1500 5%, DH 5%				
at 4°C	0.3455 ± 0.0022	178.31 ± 0.26	0.12 ± 0.01	Fickian
at 25°C	0.1785 ± 0.0075	173.06 ± 4.44	0.19 ± 0.01	Fickian
at 45°C	0.1557 ± 0.0085	164.48 ± 2.57	0.25 ± 0.01	Fickian
ERS 35%, PO 5%, DH 5%				
at 4°C	0.0945 ± 0.0110	167.86 ± 6.38	0.27 ± 0.01	Fickian
at 25°C	0.1218 ± 0.0026	167.43 ± 1.21	0.23 ± 0.01	Fickian
at 45°C	0.0905 ± 0.0049	172.22 ± 2.14	0.14 ± 0.03	Fickian

k = release rate; t_l = lag time and n = diffusional exponent

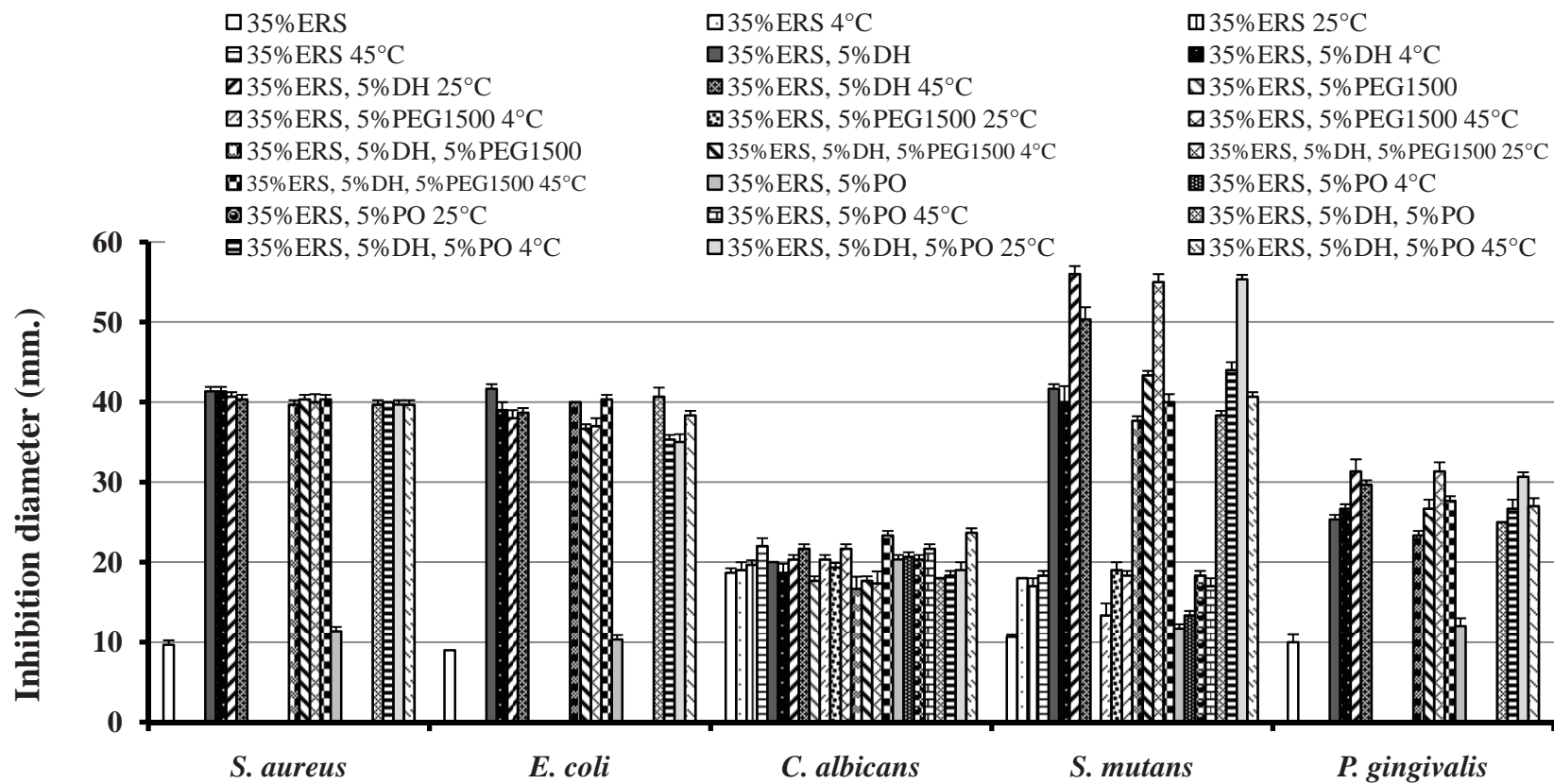


Figure 92 Inhibition zone diameter of the Eudragit RS formula containing polyethylene glycol 1500 and peppermint oil against *S. aureus*, *E. coli*, *C. albicans*, *S. mutans* and *P. gingivalis* after stability test (3 months) at 4°C, 25°C and 45°C.

CHAPTER 5

CONCLUSION

The viscosity and syringeability of *in situ* forming gel was increased when the amount of polymer was increased and the gel appearances were based on type of polymer. All systems (Ethocel, bleached shellac and Eudragit RS) showed the Newtonian behaviors. The Ethocel, bleached shellac and Eudragit RS formula were formed the *in situ* gel in phosphate buffer pH 6.8 when the solvent exchange and polymer precipitation were occurred. The gel formation capacity depended on the polymer amount. The Ethocel and Eudragit RS formula were formed the stiff gels, whereas the bleached shellac formula were formed the elastic gels after injecting into PBS pH 6.8.

In addition, the antimicrobial agents such as doxycycline hyclate, metronidazole and benzoyl peroxide were incorporated into *in situ* forming gel systems. All polymer systems incorporating with doxycycline hyclate, metronidazole and benzoyl peroxide showed the Newtonian flow behavior. The systems comprising each drug could form the *in situ* gel after injecting into PBS pH 6.8. For the drug release studies using dialysis membrane method, the Ethocel (5-10%w/w), bleached shellac (15-30%w/w) and Eudragit RS (15-35%w/w) systems showed the drug release of Fickian diffusion mechanism, which a rate of drug release decreased as a function of time. Whereas the drug release from Ethocel (15-20%w/w) systems were anomalous or non-Fickian diffusion, which indicated that the drug release was controlled by both mechanisms of diffusion and polymeric chain relaxation. In the addition, the drug release studies using membrane-less method, the doxycycline hyclate released from each polymer formula were fitted well with first order model. All formula (Ethocel (5-20%w/w), bleached shellac (15-30%w/w) and Eudragit RS (25-35%w/w) were anomalous (non-Fickian) diffusion controlled release except only Eudragit RS (15%w/w) that showed drug release by Fickian diffusion. It suggested that the doxycycline hyclate release from each polymer formula decreased with increased

polymer amount. On the other hands, the increased Eudragit RS amount did not change the metronidazole release. The SEM structures of all Ethocel formula containing DH (5% w/w) were continuous phase, which the pore sizes of structure were decreased with the increasing Ethocel amount. The SEM structures of all Eudragit RS formula containing DH (5% w/w) were spherical which connected together and formed into the continuous phase. The particle size of Eudragit RS was decreased with increasing of Eudragit RS amount. For antimicrobial activities studies, the inhibition zone diameter against *S. aureus*, *E. coli*, *S. mutans* and *P. gingivalis* of all systems containing DH and MT were significantly higher than that of the gel base ($p < 0.05$). Whereas the inhibition zone diameter against *P. gingivalis* of the Ethocel, bleached shellac and Eudragit RS systems containing BP were significantly higher than that of the gel base ($p < 0.05$). The increased Ethocel amount in the systems containing DH, MT and BP (5% w/w) did not affect the antimicrobial activity against all microbes ($p > 0.05$). When the Eudragit RS and bleached shellac amount were increased, the inhibition zone diameter against all microbes significantly decreased ($p < 0.05$) supported that the increased bleached shellac and Eudragit RS amount could demonstrate the sustain release drugs system.

The increased PEG1500 and PO amounts in the systems increased the viscosity and syringeability of the Eudragit systems. Both dialysis membrane and membrane-less methods, the doxycycline hyclate release from all Eudragit RS systems containing PEG1500 and PO were significantly slower than that of the gel base systems ($p < 0.05$). The increased PEG1500 amounts did not change the drug release profiles, whereas the doxycycline hyclate release was decreased when the PO amount was increased. All systems containing PEG1500 and PO showed the drug release by Fickian diffusion mechanism, which a rate of drug release decreased as a function of time, due to a decrease in the concentration gradient. The SEM structures of all Eudragit RS formula containing PEG1500 were continuous phase and the pore sizes of structure were increased with an increase of PEG1500 amount. The SEM structures of all Eudragit RS formula containing PO were continuous cells and the cell structures were more fused, thickened and the porous was more pronounced as the increasing PO amount. The increased PEG1500 amounts in the Eudragit systems did not affect the

antimicrobial activity against microbes. The increasing of PO amount did not significantly alter the antimicrobial activity against *S. aureus*, *E. coli* and *P. gingivalis* ($p>0.05$), whereas the antimicrobial activity against *C. albicans* and *S. mutans* of the 35%w/w Eudragit RS systems with DH (5%w/w) were significantly decreased with increasing PO amount ($p<0.05$). After stability test for 3 months at 4°C, 25°C and 45°C, the Eudragit RS-DH systems containing PEG and PO exhibited the antimicrobial activity against *S. aureus*, *E. coli*, *C. albicans*, *S. mutans* and *P. gingivalis* similar to that of freshly prepared systems. However, the storage at 4°C was the suitable condition for the *in situ* forming gel systems.

In conclusion, the *in situ* forming gel systems containing doxycycline hyclate, metronidazole and benzoyl peroxide showed the antimicrobial activity against *P. gingivalis* ATCC 33277 and the combination between doxycycline hydrochloride and metronidazole also showed the synergist effect against *P. gingivalis* ATCC 33277, which has revealed certain interesting for periodontal treatment. Only Eudragit RS system did not seem to be promising as periodontal *in situ* gel whereas the incorporation of PO in the systems could form the soft and elastic gels after injected into phosphate buffer pH 6.8. Especially, the burst release that was the disadvantage of injectable *in situ* forming gel systems could be minimized by the incorporation of PO.

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APPENDICES

APPENDIX A

Standard curve for the *in vitro* release study

1. Determination of the amount of doxycycline hyclate released

Standard : Doxycycline hyclate
Method : UV-vis spectrophotometry
Detector : at 379 nm
Concentration ($\mu\text{g/mL}$): 4.8, 10.2, 15.0, 25.2, 30.0, 34.8

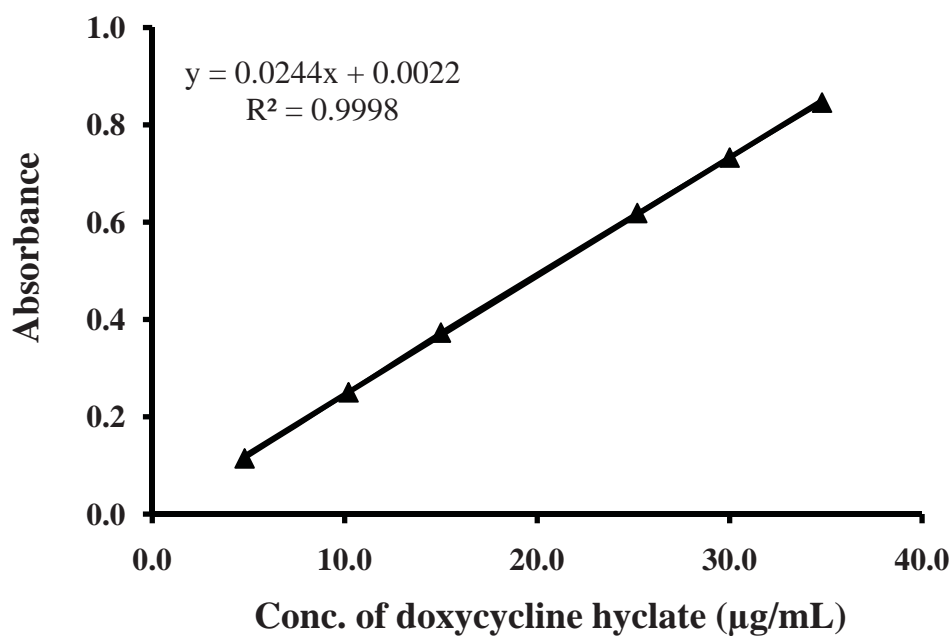


Figure 93 Standard curve of doxycycline hyclate in phosphate buffer pH 6.8 for the *in vitro* release study.

2. Determination of the amount of metronidazole released

Standard : Metronidazole
Method : UV-vis spectrophotometry
Detector : at 320 nm
Concentration ($\mu\text{g/mL}$): 0.3, 0.6, 0.9, 1.5, 1.8

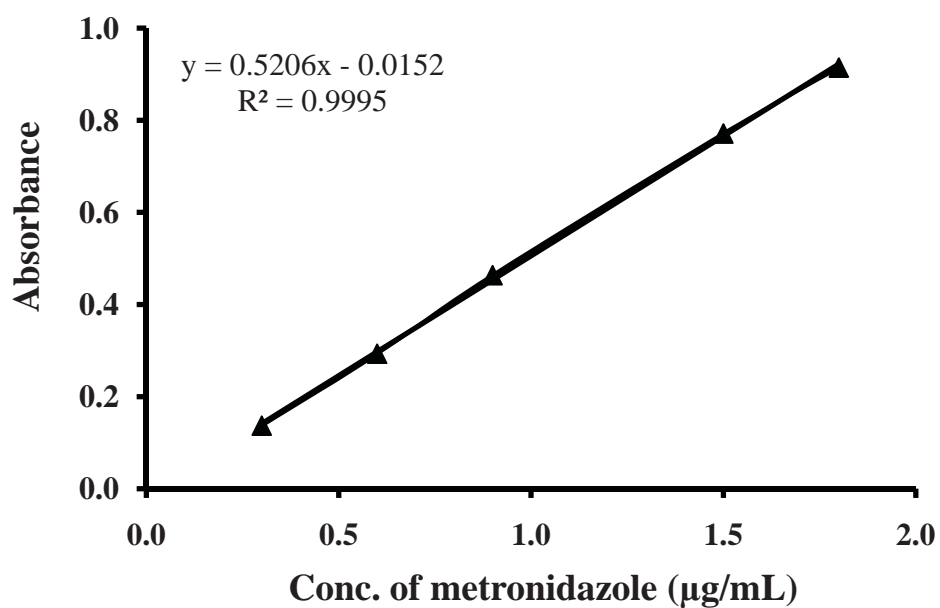


Figure 94 Standard curve of metronidazole in phosphate buffer pH 6.8 for the *in vitro* release study.

3. Determination of the amount of benzoyl peroxide released

Standard : Benzoyl peroxide
Method : UV-vis spectrophotometry
Detector : at 275 nm
Concentration ($\mu\text{g/mL}$): 15.0, 30.0, 60.0, 75.0, 105.0

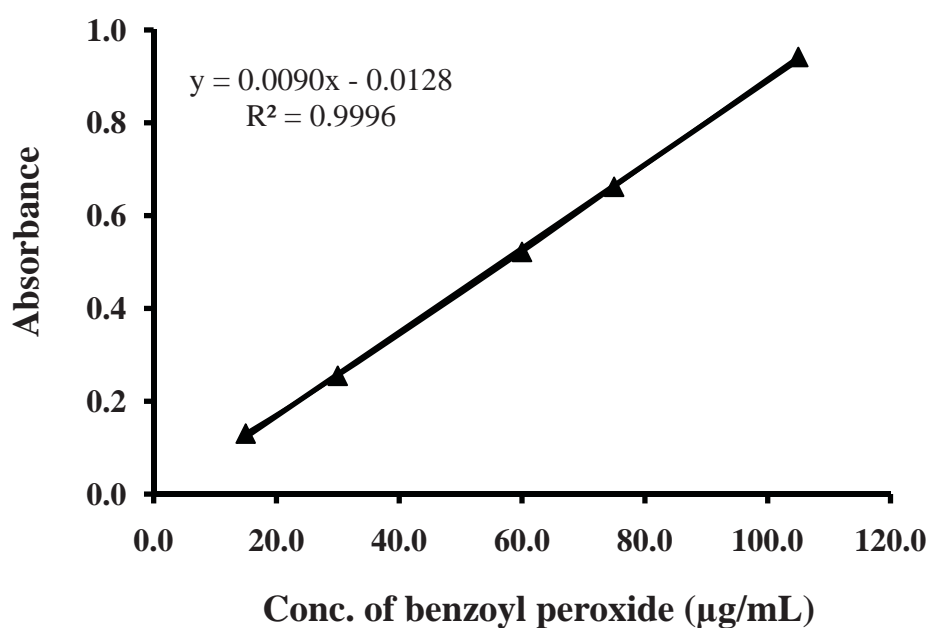


Figure 95 Standard curve of benzoyl peroxide in phosphate buffer pH 6.8 : ethanol (5:2) for the *in vitro* release study.

APPENDIX B

Release measurement

Table 48 Effect of Ethocel amount on the release profile of doxycycline hyclate in phosphate buffer pH 6.8 using dialysis membrane method at 37°C, 50 rpm.

Time (min)	Cumulative amount of doxycycline hyclate release							
	Ethocel formula containing DH (5%w/w)							
	5%w/w EC		10%w/w EC		15%w/w EC		20%w/w EC	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
5	3.0020	0.2432	2.1475	0.1877	1.8511	0.1454	1.7967	0.1783
15	5.7739	0.1753	4.8275	0.2657	3.8395	0.1804	3.6844	0.2308
30	13.7501	3.1712	11.1033	2.1286	9.1196	1.0567	8.9250	1.2510
45	19.6180	1.8868	15.8826	1.3800	15.2634	0.7909	14.8203	0.2377
60	29.7753	2.1554	26.0676	1.1217	22.6266	1.4555	20.0249	0.7827
90	46.3104	1.2501	37.3558	1.5129	30.4391	3.3216	29.7200	2.3631
120	59.3673	2.8862	47.1673	2.6949	42.5234	1.4724	38.8663	1.5929
180	80.1055	3.2169	63.2172	2.3817	56.9816	2.2840	50.2234	2.1194
240	88.2064	1.2541	78.6012	5.0202	68.7462	1.0965	64.1276	1.8035
300	88.5744	2.1071	85.9563	4.7599	80.0971	1.2608	73.9798	0.6290
360	90.2098	0.7001	87.7370	2.5378	84.3414	0.7264	80.9191	0.5058
420	91.1191	0.2906	89.3646	0.4728	85.6879	2.1055	83.4271	0.7253
480	92.4645	2.1000	89.7708	0.8445	88.1617	2.0061	86.2085	0.5404
540	92.1310	1.7212	89.1970	1.4815	87.8299	2.3456	87.0945	1.9376
600	90.9388	1.5713	89.2361	0.6398	88.0164	2.3592	86.9197	0.7990
720	90.6146	2.1069	89.2158	0.3336	87.2942	3.2402	86.6876	0.6214
960	90.4808	2.1241	88.8491	0.2424	86.5589	3.5818	85.3495	2.9688

Table 49 Effect of Ethocel amount on the release profile of doxycycline hyclate in phosphate buffer pH 6.8 using membrane-less method at 37°C, 50 rpm.

Time (min)	Cumulative amount of doxycycline hyclate release							
	Ethocel formula containing DH (5% w/w)							
	5% w/w EC		10% w/w EC		15% w/w EC		20% w/w EC	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
5	3.4637	0.5572	2.9668	0.3104	2.4863	0.3848	2.0750	0.1698
15	6.1767	0.1980	5.0320	0.4807	4.5972	0.1732	2.9167	0.1975
30	14.1886	1.5611	8.9560	0.7800	6.7872	0.3709	4.0817	0.0525
60	18.3528	1.9982	13.2422	1.0255	10.5562	0.9454	6.4801	0.6697
90	21.9749	1.1402	16.9391	0.6949	13.9687	0.7142	8.2438	0.2610
120	25.1082	1.1393	20.2931	0.9347	17.1036	0.2282	10.3889	0.5774
180	28.4676	1.3566	22.3736	0.3581	20.1301	0.4385	12.8419	0.6383
240	36.5609	1.5592	25.5561	0.7578	21.7097	0.7414	16.1045	0.4575
360	45.5512	1.9133	29.3423	2.6638	27.4518	1.2754	20.5641	0.4454
480	52.0115	2.2667	36.6980	1.6926	35.1795	1.2599	25.7105	0.1669
720	62.5575	2.4289	48.0238	1.6489	43.8713	2.7922	33.0041	1.7974
960	74.0544	2.0589	57.0805	2.0487	51.8409	1.2436	40.5948	1.1037
1440	87.1971	2.0772	72.0876	1.4618	63.1559	2.2496	50.7688	3.6653
1800	89.9946	0.6568	79.9525	0.7181	73.3642	0.7174	61.6781	2.4376
2160	90.2820	0.6256	85.6397	1.7883	81.4615	1.2917	73.2543	3.1210
2880	88.3965	1.4007	87.4130	1.1981	86.0349	0.9803	84.3010	4.5320
3240	88.8405	0.8662	87.6801	2.1791	88.2412	1.8696	85.1027	3.1241

Table 50 Effect of bleached shellac amount on the release profile of doxycycline hyclate in phosphate buffer pH 6.8 using dialysis membrane method at 37°C, 50 rpm.

Time (min)	Cumulative amount of doxycycline hyclate release							
	Bleached shellac formula containing DH (5% w/w)							
	15% w/w BS		20% w/w BS		25% w/w BS		30% w/w BS	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
5	0.4046	0.2221	0.3549	0.3287	0.4653	0.3585	0.1179	0.1322
15	3.2940	0.1276	2.9162	0.5531	2.5481	0.2835	1.8703	0.1913
30	5.3843	0.2569	4.0352	0.5916	3.3658	0.1958	2.6068	0.3372
45	2.0600	0.1109	6.5506	0.4316	5.3169	0.1716	4.3954	0.4658
60	14.4251	4.4558	8.2695	0.2182	7.7708	0.8043	5.6854	0.4504
90	24.7697	6.4939	13.9302	0.6368	12.3661	1.6419	9.4223	0.4764
120	31.1909	1.8632	20.5431	0.7170	16.0329	0.6060	14.4404	1.8311
180	43.6822	3.1317	26.6582	1.6317	20.7753	1.9639	18.5233	1.4681
240	52.1226	1.4309	34.0462	3.4948	26.5172	0.5805	23.6800	0.8536
360	63.7700	1.1034	45.5244	0.5848	36.0017	0.4828	31.2630	2.4691
480	70.1173	1.6560	51.6291	1.2159	41.8390	1.3693	36.4438	2.1197
720	74.5424	0.6594	57.5487	2.6693	49.0733	0.5560	43.6301	2.7667
960	74.8401	1.2072	62.9586	0.5925	56.6105	1.9638	49.8873	3.0767
1200	75.1889	1.6012	67.4117	0.9031	60.8892	0.3108	55.5546	4.1490
1440	75.8016	1.2488	69.6358	0.9623	64.0952	0.8531	59.0456	3.8013
1560	75.5289	0.9259	69.7771	0.9966	64.3817	0.1163	59.6803	5.2290
1800	75.6574	1.1002	70.0733	1.5137	65.6773	0.5937	61.2578	3.2654
1920	76.1453	1.2044	70.3783	1.5393	65.9790	0.3011	62.1127	2.9604
2045	75.7100	0.9752	71.2259	1.1592	67.1762	0.4111	63.6072	2.9141
2880	75.8594	1.1555	73.0340	1.3088	69.7902	0.2780	66.6664	2.4918

Table 51 Effect of bleached shellac amount on the release profile of doxycycline hyclate in phosphate buffer pH 6.8 using membrane-less method at 37°C, 50 rpm.

Time (min)	Cumulative amount of doxycycline hyclate release							
	Ethocel formula containing DH (5% w/w)							
	15% w/w BS		20% w/w BS		25% w/w BS		30% w/w BS	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
5	0.8176	0.5830	0.5222	0.1254	0.6025	0.0812	0.4966	0.1384
15	2.2200	0.1703	2.0775	0.1696	1.6835	0.2284	1.5976	0.1917
30	3.2725	0.2544	3.2676	0.9740	2.6552	0.2315	2.5043	0.1949
60	4.2894	0.5319	3.8898	0.6675	3.5112	0.4875	3.1946	0.4051
90	5.6984	0.6514	4.8676	0.6859	4.4027	0.6609	3.7115	0.4176
120	7.2623	0.5478	5.9320	0.6199	5.3448	0.4263	4.8538	0.5121
180	8.8119	0.6217	7.3626	0.9282	6.4799	0.5614	5.7289	0.1830
240	11.8353	0.3945	10.7460	0.0453	9.3086	0.3174	7.2169	0.3551
360	13.8761	0.3555	12.8254	0.7902	11.3770	0.2778	9.0454	0.3603
480	18.6917	1.0417	15.5418	0.2009	14.0399	0.3335	10.3202	0.4676
720	26.2031	3.5910	20.3599	2.2104	18.4070	0.9145	14.3466	3.1114
960	33.4102	3.5189	28.5575	0.7361	22.8936	3.1041	18.1587	0.6451
1440	42.5978	3.6210	37.9822	2.6958	30.9644	1.1477	24.4176	1.2818
1800	48.2267	2.2963	43.6216	1.3819	36.4882	1.5650	29.9811	1.6946
2160	55.2484	1.3497	49.8008	0.5705	42.0200	1.9699	35.5229	1.4301
2880	62.9203	3.4239	56.5608	1.5017	48.9491	2.4355	43.3930	2.4348
3240	68.6743	2.9945	60.6136	1.6764	54.6779	2.5012	47.7229	1.9420

Table 52 Effect of Eudragit RS amount on the release profile of doxycycline hyclate in phosphate buffer pH 6.8 using dialysis membrane method at 37°C, 50 rpm.

Time (min)	Cumulative amount of doxycycline hyclate release							
	Eudragit RS formula containing DH (5% w/w)							
	15% w/w ERS		25% w/w ERS		30% w/w ERS		35% w/w ERS	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
5	2.8441	0.2042	1.7807	0.0477	1.6500	0.0378	1.4780	0.0560
15	5.4835	0.3529	5.2497	0.1554	5.1715	0.1148	4.5878	0.1286
30	9.5229	1.3498	8.6578	0.6256	8.3182	0.3326	8.2094	0.2582
45	14.6725	2.1525	13.8898	1.9908	13.4929	2.3040	11.2722	0.8465
60	20.4942	2.9207	19.0443	2.1644	18.4517	1.6873	16.5903	0.7289
90	37.9600	1.2499	35.7691	0.6757	34.5756	0.4268	30.7137	1.6594
120	52.7774	1.0455	48.7224	0.9994	46.4399	1.3873	39.0014	2.0633
180	72.7205	1.2075	66.7954	0.7400	62.9389	0.4346	53.9541	1.5744
240	81.4803	0.9803	75.2437	0.6825	69.4472	0.2951	64.4644	0.8219
300	85.4312	0.3252	81.0464	2.9816	76.2189	0.4896	72.0342	0.5235
360	87.9685	0.9974	83.9558	2.4991	79.9003	0.5516	76.6838	0.9747
420	91.2938	1.2002	86.0286	0.4206	82.7295	0.3538	80.0244	1.1147
480	92.1832	0.9299	88.7868	1.4907	83.9781	1.1559	81.5249	0.6537
540	91.7930	0.9682	91.0890	1.2668	86.6313	0.5137	83.4378	1.6016
600	91.5225	0.8167	90.8743	0.2965	87.6354	2.2046	84.5776	1.6945
720	91.5729	1.7198	91.0953	0.3248	88.7754	0.9626	85.7503	1.4805
960	90.3173	4.2733	90.6900	0.6629	90.0538	0.6294	88.5472	1.3847

Table 53 Effect of Eudragit RS amount on the release profile of doxycycline hyclate in phosphate buffer pH 6.8 using membrane-less method at 37°C, 50 rpm.

Time (min)	Cumulative amount of doxycycline hyclate release							
	Eudragit RS formula containing DH (5% w/w)							
	15% w/w ERS		25% w/w ERS		30% w/w ERS		35% w/w ERS	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
5	1.2094	1.4320	0.3910	0.2229	0.4819	0.2209	0.5180	0.8706
15	5.5772	1.6212	3.4869	2.3363	2.3220	0.4184	2.0803	0.2499
30	8.6802	1.9931	5.8905	3.1554	3.4434	0.6412	2.9766	0.7172
60	11.5191	1.5139	8.3808	3.9356	5.2494	0.8905	4.3719	0.9055
90	15.1471	2.2720	10.8022	4.0130	7.7377	0.8694	6.7128	1.9857
120	18.0716	2.4864	12.7978	4.2653	8.8629	0.8244	8.3764	1.5290
180	21.1759	4.1943	15.6851	4.2301	11.2488	1.1304	10.8997	1.8370
240	26.5757	2.0785	18.3042	4.5885	13.7188	1.5256	14.8660	3.8649
360	32.7542	2.1629	22.4510	5.0068	16.8411	1.6616	17.1836	2.0642
480	38.0961	1.6569	29.8447	3.8212	21.5491	1.6101	21.6430	2.0660
720	50.8932	2.0434	36.7897	4.0863	29.8235	1.8158	30.1805	2.3104
960	64.5398	2.8590	46.6687	4.1331	36.8955	1.5190	34.3930	3.1153
1440	75.2420	1.0573	57.7750	4.0970	48.6840	2.5949	44.9548	3.6373
1800	77.2222	2.8135	66.7836	2.2965	55.5856	1.9603	50.4879	3.1821
2160	81.7495	1.2853	68.8585	2.3665	63.7291	6.4943	56.3511	2.9250
2880	88.1821	1.1833	75.8629	2.0328	71.0709	5.3614	65.9373	4.1042
3240	91.5069	3.4014	79.5534	2.4453	71.8176	1.2309	68.9819	5.5261

Table 54 Effect of Eudragit RS amount on the release profile of metronidazole in phosphate buffer pH 6.8 using dialysis membrane method at 37°C, 50 rpm.

Time (min)	Cumulative amount of metronidazole release							
	Eudragit RS formula containing MT (5% w/w)							
	15% w/w ERS		25% w/w ERS		30% w/w ERS		35% w/w ERS	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
5	0.4477	0.2779	0.8291	0.4550	1.1038	0.3491	1.1441	0.4646
15	0.7327	0.1065	0.7331	0.1595	0.9003	0.0781	1.0668	0.3094
30	1.6013	0.2451	1.5041	0.3167	1.6693	0.1814	1.7507	0.1222
60	2.9039	0.3342	3.2009	0.9127	3.4427	0.1855	4.1449	0.6004
120	7.0128	0.8601	8.1109	0.8168	6.3985	0.8106	6.4920	0.2463
180	9.2151	0.5642	9.8874	0.6168	8.5744	0.3916	8.7216	0.2854
240	9.6553	0.7095	10.0581	0.2067	8.9823	0.7018	9.4555	0.6551
360	11.4660	0.5255	11.3595	0.9596	10.8246	0.1528	10.5250	0.5764
480	10.2165	0.4811	10.8684	0.5655	11.0003	0.7472	10.0314	0.0993
720	10.5292	0.2131	10.6517	0.6433	10.8030	1.1577	10.3484	0.5796
960	11.1691	0.4897	11.2655	0.0738	10.9232	0.1745	10.9457	0.0350

Table 55 Effect of polyethylene glycol 1500 amount in 35%w/w Eudragit RS-doxycycline hyclate (5%w/w) on the release profile of doxycycline hyclate in phosphate buffer pH 6.8 using dialysis membrane method at 37°C, 50 rpm.

Time (min)	Cumulative amount of doxycycline hyclate release							
	35% w/w Eudragit RS-DH (5% w/w) formula containing PEG1500							
	2.5% w/w PEG1500		5% w/w PEG1500		7.5% w/w PEG1500		10% w/w PEG1500	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
5	0.2608	0.2397	0.1762	0.1319	0.1158	0.1370	0.2201	0.1397
15	1.9266	0.3259	2.2798	0.4881	1.9789	0.3721	1.9172	0.2393
30	4.3101	1.3269	4.5769	0.9250	4.0117	0.7994	4.6706	0.2626
45	6.8281	1.2738	7.4096	0.9428	6.5890	0.5344	6.9098	0.7767
60	9.3477	1.8707	10.1215	1.4123	9.3874	1.1279	8.7716	0.5551
90	13.6755	1.9693	14.5735	1.8069	13.1061	1.1988	12.7057	1.0084
120	19.6153	2.0214	20.7843	1.8343	18.1922	0.7970	18.2190	1.7570
180	29.0449	2.5497	30.4431	2.6957	26.3209	1.7990	26.0324	2.3529
240	40.3136	0.9361	40.0666	2.6602	36.4818	1.4764	37.7772	2.2521
360	50.7954	1.1169	48.5475	5.0090	44.8017	1.3341	46.8417	3.8679
480	58.4192	1.4175	57.9469	2.2169	55.3654	2.5879	56.5789	3.1439
720	66.2629	0.7264	69.0567	3.5910	69.4223	4.3645	63.1842	2.4904
960	69.6890	0.7541	73.5324	3.0198	72.5391	1.3188	70.1696	4.7170
1200	73.0995	0.5638	76.8959	2.4502	78.1571	3.7803	76.4961	3.1388
1440	74.7920	0.8161	79.1052	1.7844	77.4830	2.6446	76.6744	3.3925
1800	75.9632	1.3514	79.7297	1.6901	78.6484	3.1108	78.8367	2.8294
2160	78.0202	1.9725	81.1295	0.9022	80.1063	2.3001	78.7494	2.4951
2880	77.7691	0.7179	79.7489	1.1628	78.6080	2.1512	77.5590	2.8363
3240	77.1804	0.8624	79.3982	0.6643	78.8704	1.2868	77.4049	2.7802
3600	77.5684	1.2529	80.2693	1.6372	79.2878	1.1278	78.5064	2.7519
4320	78.5775	1.6276	80.1761	1.2713	79.4857	1.4197	78.3938	2.7312

Table 56 Effect of polyethylene glycol 1500 amount in 35%w/w Eudragit RS-doxycycline hyclate (5%w/w) on the release profile of doxycycline hyclate in phosphate buffer pH 6.8 using membrane-less method at 37°C, 50 rpm.

Time (min)	Cumulative amount of doxycycline hyclate release							
	35% w/w Eudragit RS-DH (5% w/w) formula containing PEG1500							
	2.5% w/w PEG1500		5% w/w PEG1500		7.5% w/w PEG1500		10% w/w PEG1500	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
5	0.0329	0.0176	0.0337	0.0221	0.0483	0.0420	0.0453	0.0062
15	0.9184	0.4849	0.5143	0.2463	0.6054	0.0536	0.7112	0.2239
30	2.1212	0.5223	1.6346	0.5322	1.8678	0.1928	1.8926	0.4873
45	3.1099	0.8154	3.3929	1.3487	3.7221	0.2848	3.5635	0.6761
60	4.1373	1.0947	4.4551	1.3511	5.1787	0.3132	4.9302	0.8393
90	6.0508	1.6381	6.4404	1.6728	7.2249	0.5000	7.0438	1.1770
120	8.1950	1.6744	8.3548	1.8120	9.0623	0.5017	8.6041	1.1932
180	10.9611	2.0714	10.8214	1.4161	11.6362	0.5585	11.1392	0.6076
240	14.2349	2.0289	14.2295	1.3506	14.7470	0.3839	14.3627	0.5492
360	18.0348	1.8976	18.1658	1.4161	17.7947	0.4463	17.2944	1.0136
480	21.9202	2.2305	22.9668	2.7530	21.8658	0.3594	21.6398	0.5715
720	28.9096	1.7131	28.0152	1.5355	26.1703	0.4505	25.3493	0.8842
960	34.0440	1.1059	35.6043	0.8170	32.5946	0.7841	31.4348	0.7959
1200	40.1459	1.3502	39.8039	0.5256	38.3164	0.4071	35.4172	0.2256
1440	45.2310	2.1016	44.9655	0.3711	42.0447	0.7360	40.8044	0.5921
1800	48.5184	2.3031	48.4818	0.4958	46.9520	1.4728	45.3561	1.0452
2160	52.3957	2.8204	51.5714	0.4566	50.6670	1.9658	50.0625	0.7445
2880	58.9817	0.7466	59.7832	1.5305	59.2439	1.9669	56.7703	0.8342
3240	61.3878	1.5580	61.5180	1.1431	60.5947	1.8579	59.3599	0.0513
3600	62.6397	0.6273	61.9603	1.5146	62.8180	2.0705	61.1781	1.5857
4320	65.4213	1.8740	65.2862	1.0574	65.4718	2.0910	64.7818	1.6367
5760	67.9034	1.4149	68.1570	1.9843	69.0279	3.7535	69.7539	1.7092
8640	70.7035	2.2501	71.4052	2.6536	72.1841	3.1635	72.5044	3.0106
11520	72.4568	3.1082	72.4262	3.4419	72.7101	4.9832	73.1422	3.8250

Table 57 Effect of peppermint oil amount in 35%w/w Eudragit RS-doxycycline hyclate (5%w/w) on the release profile of doxycycline hyclate in phosphate buffer pH 6.8 using dialysis membrane method at 37°C, 50 rpm.

Time (min)	Cumulative amount of doxycycline hyclate release							
	35%w/w Eudragit RS-DH (5%w/w) formula containing PO							
	2.5%w/w PO		5% w/w PO		7.5%w/w PO		10%w/w PO	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
5	0.0152	0.0005	1.1844	1.0469	0.3430	0.4145	0.5955	0.3619
15	2.1185	0.0872	3.2301	1.1098	4.5645	1.4043	4.7984	1.3161
30	5.6153	1.1638	5.7370	0.3179	5.6978	0.8497	5.7411	0.2523
45	8.1430	0.4218	8.7871	1.5452	11.4311	0.9576	9.2881	1.3263
60	14.7450	0.6447	14.1786	2.3567	13.2390	0.1000	12.8523	1.0689
90	18.7960	0.7092	17.7846	3.1396	17.3606	0.9663	16.3181	0.6658
120	24.5790	0.4279	23.6022	3.5514	22.6826	2.6177	20.7346	0.6938
180	40.7055	4.3353	34.0113	4.2228	33.2520	3.6524	29.1913	0.5518
240	58.3384	2.0801	51.4173	5.5254	44.8499	5.9100	38.5816	0.7101
360	67.2543	2.9163	61.2512	5.4550	54.0710	1.4427	45.5641	0.7843
480	70.2119	1.0569	64.5225	4.4895	59.1266	2.1022	48.3816	1.1112
720	73.5375	1.7897	68.4288	2.0947	63.5834	0.7983	51.7481	0.8264
960	76.3374	1.7152	70.4921	2.5843	65.3587	3.7529	56.3846	0.6608
1200	79.5004	1.4192	75.5693	2.4774	70.4883	0.9025	61.5577	0.1006
1440	81.2674	1.1457	77.4033	1.2422	72.2705	2.3553	63.3446	2.6411
1800	82.3174	0.8506	78.9146	5.0498	73.7029	0.8959	66.0841	1.2668
2160	83.6439	0.6282	80.5588	2.9961	75.8789	0.9365	70.0623	2.1008
2880	82.5324	1.0300	79.6912	2.1172	75.9657	1.3290	69.8848	2.2645
3600	81.9660	1.8378	78.1476	1.5949	75.5469	0.3691	70.1663	2.2575

Table 58 Effect of peppermint oil amount in 35%w/w Eudragit RS-doxycycline hyclate (5%w/w) on the release profile of doxycycline hyclate in phosphate buffer pH 6.8 using membrane-less method at 37°C, 50 rpm.

Time (min)	Cumulative amount of doxycycline hyclate release							
	35%w/w Eudragit RS-DH (5%w/w) formula containing PO							
	2.5%w/w PO		5% w/w PO		7.5%w/w PO		10%w/w PO	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
5	0.2740	0.1000	0.0317	0.0331	0.1558	0.0490	0.5777	0.8599
15	1.2915	0.2230	1.1979	0.1617	1.0538	0.2188	1.2306	0.1507
30	2.2847	0.2573	2.0651	0.1395	1.7499	0.1848	1.9345	0.1411
45	2.9148	0.2966	3.0355	0.3708	2.3332	0.2006	2.6622	0.1077
60	4.0777	0.4383	4.2763	0.5817	3.1927	0.2898	3.5581	0.2165
90	5.2228	0.6983	5.0988	0.4581	4.0910	0.4212	4.2216	0.3771
120	6.5248	0.8139	6.2430	0.5521	5.0764	0.4070	5.4297	0.2449
180	9.4375	1.1122	10.1377	0.8307	8.0965	1.2444	8.0626	0.7985
240	13.0485	1.0642	13.8524	2.0359	11.7290	1.9842	10.8689	0.8111
360	18.2024	0.7558	18.4622	1.8475	15.2731	1.7664	12.3227	0.8114
480	23.4438	1.7005	22.6420	0.9511	19.4130	2.6404	15.4426	1.1214
720	32.2489	0.8834	31.8555	3.1226	26.6099	3.0013	17.5516	0.3093
960	38.1932	1.4091	37.4908	4.4167	31.9639	3.3191	21.1682	0.2032
1200	51.7778	0.9411	47.0810	3.2894	41.3323	0.8624	26.2880	1.4465
1440	61.2731	0.3542	55.3363	3.0744	48.1439	2.2482	31.8651	1.7595
1800	71.1965	1.5224	65.4631	0.9585	55.5324	3.3131	38.0835	0.3653
2160	80.9317	0.8775	74.2299	1.5021	63.9149	0.9741	48.4177	1.1898
2880	84.6005	0.1110	79.6053	0.6475	68.4211	0.9959	55.7679	1.0052
3600	85.5302	0.9908	80.3385	1.7425	71.2061	1.1079	59.1791	1.4753
4320	84.5748	0.5326	81.3796	3.0384	73.2186	0.9685	62.4918	2.0368
5760	82.6408	1.9828	79.4668	1.8930	72.8163	2.6977	63.5657	2.5210

Table 59 Effect of polyethylene glycol 1500 amount in 35% w/w Eudragit RS-benzoyl peroxide (5% w/w) on the release profile of benzoyl peroxide in phosphate buffer pH 6.8 using dialysis membrane method at 37°C, 50 rpm.

Time (min)	Cumulative amount of benzoyl peroxide release							
	35% w/w Eudragit RS-BP (5% w/w) formula containing PEG1500							
	2.5% w/w PEG1500		5% w/w PEG1500		7.5% w/w PEG1500		10% w/w PEG1500	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
5	0.6592	0.0068	0.6728	0.0202	0.6740	0.0150	0.6703	0.0154
15	2.4119	1.3668	1.0745	0.1982	0.9936	0.2270	0.9705	0.0189
30	3.1655	0.2440	2.8113	0.2496	2.2712	0.2226	2.0231	0.1253
45	5.1055	0.7758	4.6692	0.1065	3.7967	0.1594	3.6475	0.2211
60	6.2537	0.0722	6.3195	0.4925	4.9713	0.1394	5.3365	0.8964
90	7.6831	0.2293	7.7930	0.8947	6.8356	0.5591	6.1885	0.0497
120	10.9230	0.7652	10.2060	0.9821	8.8950	0.5482	8.5220	0.3755
180	13.3256	0.5205	12.7238	1.2833	11.1667	0.9766	11.9007	1.1236
240	16.4227	0.3016	16.2210	1.0669	13.6772	0.2024	13.9547	0.2163
360	21.1495	1.1482	22.1801	0.7162	17.7221	0.4901	18.1479	0.2829
480	23.8572	0.8296	23.7439	1.8362	21.3311	0.8569	21.4100	0.7475
720	27.8028	0.1211	28.4856	0.8929	25.8891	0.7373	25.8708	0.5370
960	29.1973	0.1871	29.4912	0.4886	27.3617	0.1582	27.8945	0.7491
1200	29.8453	0.1513	30.6937	0.9243	28.7347	0.1392	29.4411	0.4304
1440	30.9948	0.1050	31.4514	1.5135	29.4686	0.0552	30.2440	0.1786
1680	30.6424	0.2110	31.0265	0.3634	29.8130	0.1051	30.2551	0.0627
1920	31.2364	0.6000	31.0794	0.2768	30.0495	0.3128	30.4659	0.0874
2160	30.9018	0.3082	31.3579	0.2771	30.1672	0.1796	30.4615	0.1002
2880	31.4112	0.4817	31.4825	0.3483	30.7142	0.3401	30.9913	0.2615
5760	31.3854	0.3410	31.6043	0.2027	31.0220	0.4414	31.5258	0.4356
7200	31.3412	0.3883	31.8141	0.1276	31.1448	0.6047	31.5525	0.4068

Table 60 Stability test (3 months) of the 35% Eudragit RS-DH (5% w/w) formula at 4°C, 25°C and 45°C on the release profile of doxycycline hyclate in phosphate buffer pH 6.8 using dialysis membrane method at 37°C, 50 rpm.

Time (min)	Cumulative amount of doxycycline hyclate release					
	35% w/w Eudragit RS-DH (5% w/w) formula					
	after stability test (3 months) at					
	4°C		25°C		45°C	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
15	0.7803	1.2806	0.5729	0.8998	0.3315	0.2217
30	2.8707	0.7765	2.0914	0.5936	3.0656	0.2012
60	7.1752	0.5839	6.2178	2.1289	15.5885	11.4746
120	18.1550	4.6706	14.3179	2.6243	17.4669	4.9570
180	26.5341	1.7651	24.1638	1.4669	28.5097	5.6007
240	38.5791	2.2979	32.3328	0.3899	38.9125	5.1342
360	51.3644	1.3699	41.3743	2.1954	45.4950	5.1668
540	59.0674	1.0134	52.8900	2.2561	50.6002	5.1798
720	66.5568	1.9331	59.1098	1.3262	55.8263	4.8217
960	71.0034	1.1426	64.2963	0.9270	61.0620	4.2742
1200	74.9090	1.0856	68.3170	1.4129	63.7202	4.7357
1440	77.0253	1.3463	69.8941	0.8664	64.8192	4.2645
1800	81.4620	0.5554	72.7051	0.5927	66.5013	2.9594
2160	81.7023	0.4020	73.7448	1.1930	67.7194	3.2347
2880	83.2145	1.4118	75.8510	0.9391	68.0761	2.6168

Table 61 Stability test (3 months) of the 35% Eudragit RS-DH (5%w/w) formula containing PEG1500 (5%w/w) at 4°C, 25°C and 45°C on the release profile of doxycycline hyclate in phosphate buffer pH 6.8 using dialysis membrane method at 37°C, 50 rpm.

Time (min)	Cumulative amount of doxycycline hyclate release					
	35%w/w Eudragit RS-DH (5%w/w) formula containing PEG1500 (5%w/w)					
	after stability test (3 months) at					
	4°C		25°C		45°C	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
15	3.0237	3.6608	0.0964	0.0194	0.4458	0.5281
30	5.7233	0.8577	2.6981	0.7200	2.6347	1.3771
60	11.1066	2.0098	7.5547	1.5192	7.3154	2.2199
120	26.5464	0.9749	14.6538	1.4416	14.5193	2.6219
180	36.9598	0.6287	26.1092	4.5649	20.6501	3.7597
240	42.1021	0.8145	34.5395	1.7054	31.3154	1.2427
360	53.7680	0.2930	49.1292	0.2311	42.7722	0.9646
540	62.0328	1.2108	56.1551	1.3020	48.9678	1.6165
720	72.5146	0.5730	61.9533	1.6660	55.7640	1.2668
960	77.4811	0.4669	71.0368	1.4490	61.1133	1.8910
1200	80.8605	0.7728	71.3389	2.1483	64.4320	1.6012
1440	82.9711	2.1455	73.6141	1.6341	65.8180	2.2283
1800	84.9906	2.1294	76.9271	0.9467	67.2285	1.4896
2160	87.2886	1.6865	76.1086	1.5781	68.4243	1.8905
2880	86.5395	1.7334	77.9969	2.3148	69.4759	1.3938

Table 62 Stability test (3 months) of the 35% Eudragit RS-DH (5%w/w) formula containing PO (5%w/w) at 4°C, 25°C and 45°C on the release profile of doxycycline hyclate in phosphate buffer pH 6.8 using dialysis membrane method at 37°C, 50 rpm.

Time (min)	Cumulative amount of doxycycline hyclate release					
	35%w/w Eudragit RS-DH (5%w/w) formula containing PO (5%w/w)					
	after stability test (3 months) at					
	4°C		25°C		45°C	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
15	0.0555	0.0075	0.1554	0.0864	1.6175	1.3546
30	1.9281	0.6293	3.0532	0.3351	2.7417	0.6711
60	6.7462	2.6852	7.2843	0.2739	7.3350	0.2471
120	12.5933	3.2981	15.4619	1.2158	13.2737	1.2576
180	19.4348	4.1193	22.9392	0.6105	19.0837	0.5473
240	25.9473	4.6962	28.7280	1.5691	24.3716	1.1253
360	39.3587	3.1263	39.1390	0.4948	32.1266	0.1551
540	46.6805	3.0028	47.0405	1.4091	39.9865	1.5738
720	54.6971	2.9972	54.6679	2.9874	45.2008	0.2368
960	61.4287	1.6523	60.3394	3.7831	51.8774	0.0954
1200	65.0029	2.4446	64.4384	3.3572	55.9126	0.9517
1440	68.1930	2.0977	65.3325	2.6242	57.5509	1.9374
1800	72.3940	1.8540	68.1182	2.2394	60.7719	1.1303
2160	74.8040	2.4039	70.3330	2.5149	61.0972	0.6092
2880	78.5411	4.3925	71.3393	2.9927	63.2085	1.4502

APPENDIX C

Calibration curve of microbes

1. Calibration curve of *Staphylococcus aureus* ATCC 6853P

Microbe : *Staphylococcus aureus* ATCC 6853P
Method : UV-vis spectrophotometry
Detector : at 530 nm

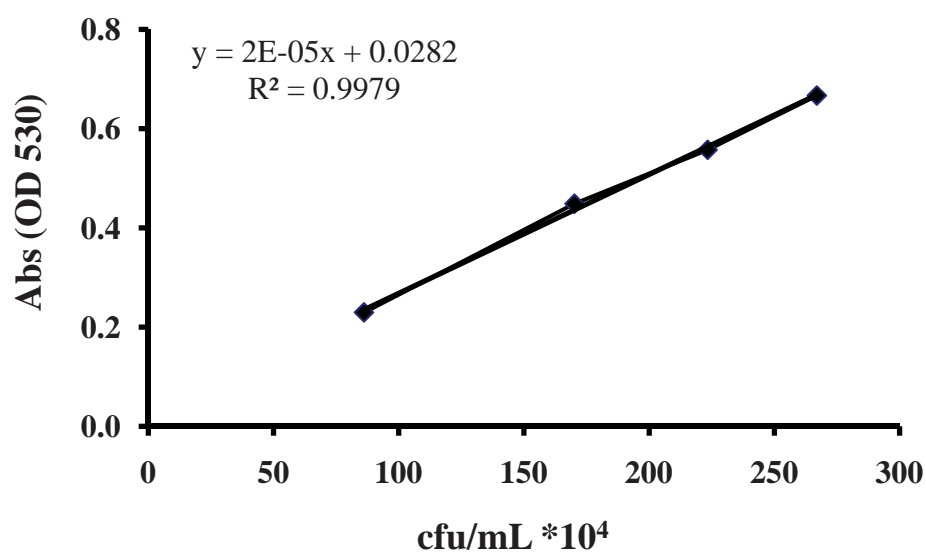


Figure 96 Calibration curve of *Staphylococcus aureus* ATCC 6853P.

2. Calibration curve of *Escherichia coli* ATCC 25922

Microbe : *Escherichia coli* ATCC 25922

Method : UV-vis spectrophotometry

Detector : at 530 nm

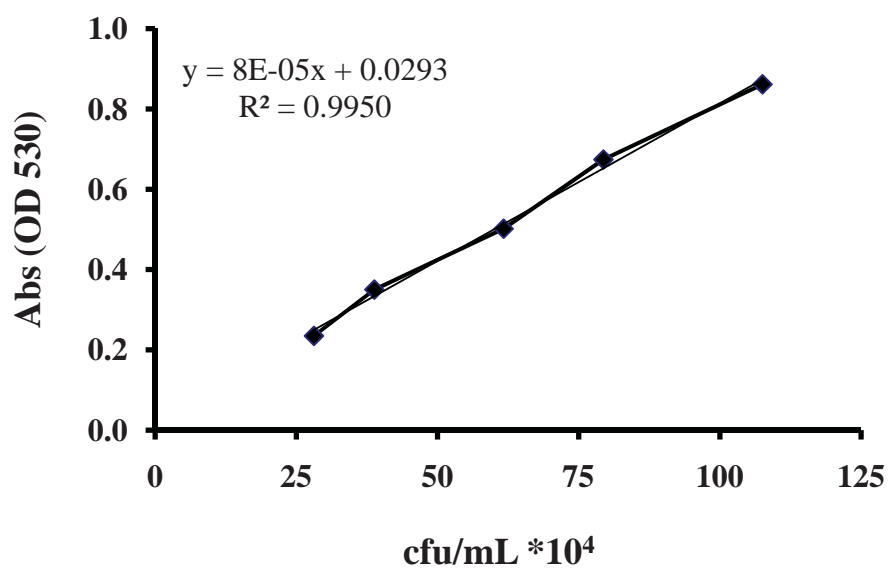


Figure 97 Calibration curve of *Escherichia coli* ATCC 25922

3. Calibration curve of *Candida albicans* ATCC 17110

Microbe : *Candida albicans* ATCC 17110
Method : UV-vis spectrophotometry
Detector : at 530 nm

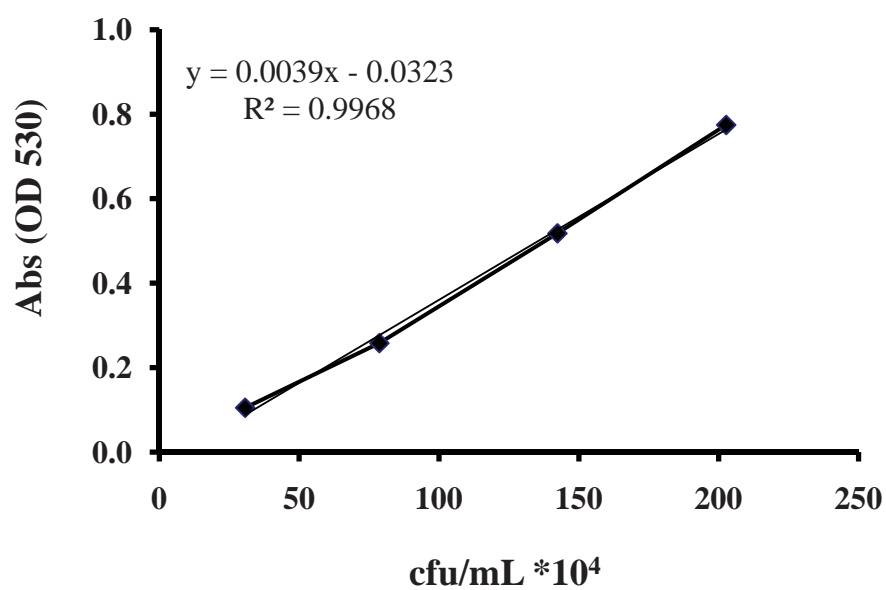


Figure 98 Calibration curve of *Candida albicans* ATCC 17110

4. Calibration curve of *Streptococcus mutans* ATCC 27175

Microbe : *Streptococcus mutans* ATCC 27175
Method : UV-vis spectrophotometry
Detector : at 540 nm

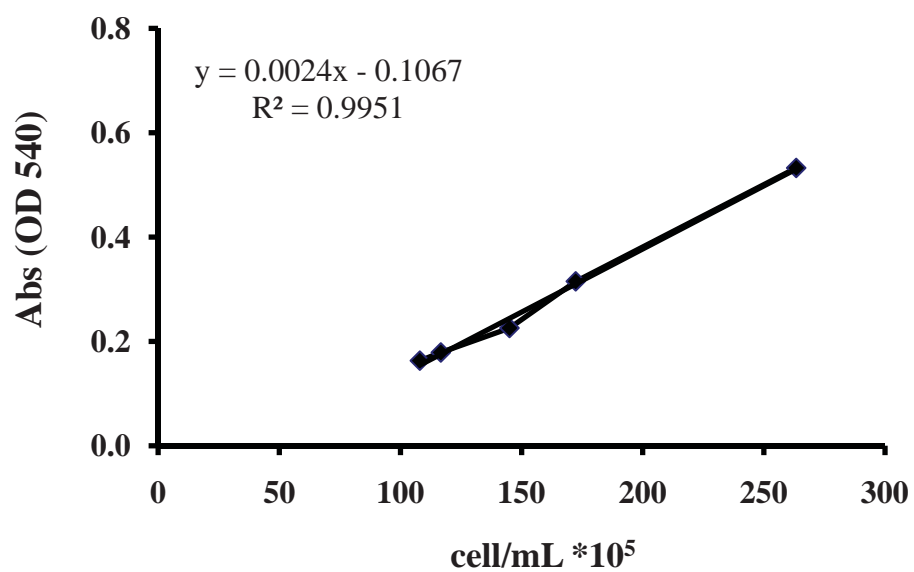


Figure 99 Calibration curve of *Streptococcus mutans* ATCC 27175

5. Calibration curve of *Porphyromonas gingivalis* ATCC 33277

Microbe : *Porphyromonas gingivalis* ATCC 33277
Method : UV-vis spectrophotometry
Detector : at 540 nm

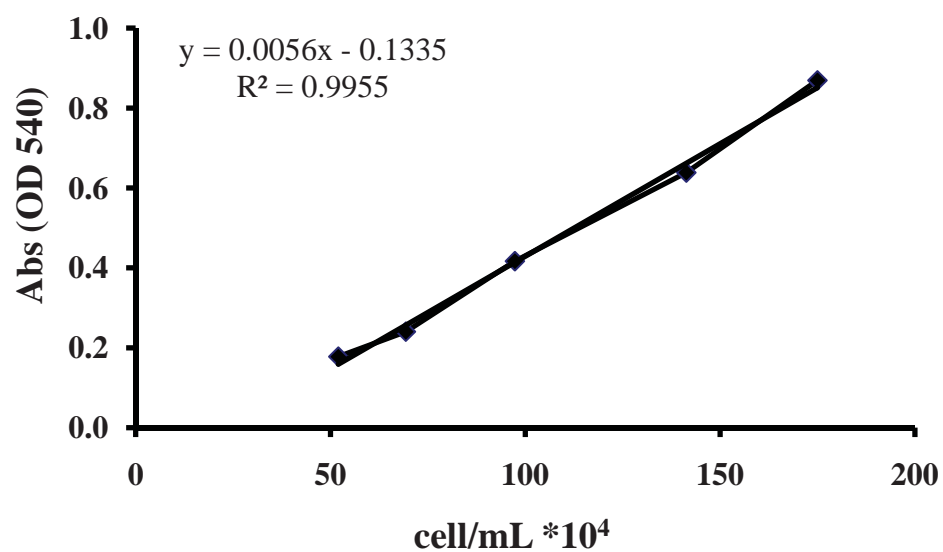


Figure 100 Calibration curve of *Porphyromonas gingivalis* ATCC 33277

BIOGRAPHY

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Education

2008 (June) - 2012 (May)	Doctor of Philosophy, Ph.D. in Pharmaceutical Technology Silpakorn University, Thailand
2007 (June) - 2008 (Jan)	Master of Pharmacy Silpakorn University, Thailand
2000 (June) - 2004 (Jan)	Bachelor of Pharmacy Silpakorn University, Thailand

Award

2008	Honorable mention (Poster presentation) in the 2 nd Silpakorn University International Conference on Academic Research, Thailand. (Characterization of Typical and Nano Zinc Oxide)
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Presentation

Poster

1. Jongjan Mahadlek, Thawatchai Phaechamud, Supab Choopun and Juree Charoenteeraboon. (2008). Characterization of Typical and Nano Zinc Oxide. The 2nd Silpakorn University International Conference on Academic Research at Silpakorn University, Bangkok, Thailand. (December 18-19, 2008)

2. Jongjan Mahadlek, Juree Charoenteeraboon J, Choopun S, Phaechamud T. (2009). Role of Zinc Oxide on Rheology of Thermosensitive Gel Developed for Periodontitis Treatment. International conference on Functionalized and Sensing Materials at Chulabhorn Convention Center, Bangkok, Thailand. (December 7-9, 2009)
3. Jongjan Mahadlek, Juree Charoenteeraboon and Thawatchai Phaechamud. (2010). Zinc Oxide Gels for Periodontitis Treatment. The 6th International Conference on Materials Science and Technology at Miracle Grand Convention Hotel, Bangkok, Thailand. (August 26-27, 2010)
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3. Jongjan Mahadlek, Juree Charoenteeraboon, Supab Choopun and Thawatchai Phaechamud. (2010). Role of Zinc Oxide on Rheology of Thermosensitive Gel Developed for Periodontitis Treatment. Adv Mat Res. 93-94: 479-484.
4. Jongjan Mahadlek, Juree Charoenteeraboon and Thawatchai Phaechamud. (2010). Zinc Oxide Gels for Periodontitis Treatment. J Met Mater Miner. 20: 159-163.

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