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นางสาวธารทิพย์ วชิรศักดิ์วงศ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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ปีการศึกษา 2555

DISSOLUTION ENHANCEMENT AND STABILITY EVALUATION OF *PASSIFLORA FOETIDA* EXTRACT IN SELF EMULSIFYING SYSTEM

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	DISSOLUTION ENHANCEMENT AND STABILITY EVALUATION OF *PASSIFLORA FOETIDA* EXTRACT IN SELF EMULSIFYING SYSTEM
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การศึกษานี้มีวัตถุประสงค์ที่จะพัฒนาระบบเกิดอิมัลชันเองเพื่อเพิ่มการละลายของสารสกัดกะทกรกและประเมินความคงตัว สูตรตำรับที่ศึกษาประกอบด้วย น้ำมัน ได้แก่ แคปเทกซ์ 200 แคปเทกซ์ 300 หรือ เอธิลโอลีเอท สารลดแรงตึงผิว ได้แก่ ทวีน 80 และ/หรือครีโมฟอร์ อีแอล ตัวทำละลายร่วมได้แก่ โพรพิลีนกลัยคอล หรือพอลิเอธิลีนกลัยคอล 400 ศึกษาการละลายในน้ำ กรดไฮโดรคลอริคความเข้มข้น 0.1 นอร์มัล และฟอตเฟตบัฟเฟอร์ พีเอช 6.8 และศึกษาความคงตัวของสารสกัดในระบบเกิดอิมัลชันเองที่สภาวะของการเก็บต่าง ๆ โดยติดตามปริมาณไวเท็กซิน สารฟลาโวนอยด์ และสารฟีนอลิก ผลการศึกษาพบว่าระบบเกิดอิมัลชันเองสามารถเตรียมจากความเข้มข้นของสารลดแรงตึงผิวร้อยละ 50-88.9 โดยน้ำหนัก โดยมีลักษณะใสหลังจากกระจายตัวในน้ำ สูตรตำรับที่มีเอธิลโอลีเอทและมีตัวทำละลายร่วมหรือสารลดแรงตึงผิวร่วมมีความสามารถการบรรจุสารสกัดในปริมาณมาก การละลายและความคงตัวของสารสกัดกะทกรกสามารถอธิบายได้ดีที่สุดเมื่อใช้ไวเท็กซินเป็นตัวชี้วัด โดยพบว่าไวเท็กซินละลายได้จากของเหลวเกิดอิมัลชันเองมากที่สุด น้อยลงมาตามลำดับ คือการละลายจากสารสกัด แกรนูลเกิดอิมัลชันเอง และแกรนูลของสารสกัด การเตรียมแกรนูลเกิดอิมัลชันเองโดยการดูดซับของเหลวเกิดอิมัลชันเองด้วยไมโครคลิสตัลลีนเซลลูโลสทำให้ไวเท็กซินมีการละลายของลดลงแต่มีความคงตัวเพิ่มขึ้น ในการศึกษานี้ระบบเกิดอิมัลชันเองที่มีศักยภาพในการเพิ่มการละลายประกอบด้วยเอธิลโอลีเอท ร้อยละ 25/ ทวีน 80 ร้อยละ 56.3/โพรพิลีนกลัยคอล ร้อยละ 18.8 แกรนูลของระบบเกิดอิมัลชันนี้มีความคงตัว โดยมีไวเท็กซินเหลืออยู่ถึงร้อยละ 95 หลังจากที่เก็บในตู้เย็นและที่อุณหภูมิ 25 องศาเซลเซียส เป็นเวลา 4 เดือน

ภาควิชา วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม ลายมือชื่อนิสิต

สาขาวิชา เภสัชอุตสาหกรรม ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม

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The aim of this study was to develop self-emulsifying system to improve the dissolution of *Passiflora foetida* extract and evaluate its stability. The formulations studied were composed of Captex 200, Captex 300 or ethyl oleate as oils, Tween 80 and/or Cremophor EL as surfactants, propylene glycol or polyethylene glycol 400 as co-solvents. Dissolution in water, 0.1 N HCl and pH 6.8 phosphate buffer, and stability of the extract in the self-emulsifying systems at varied storage conditions, were investigated by monitoring the amount of vitexin, total flavonoids and total phenolic compounds. The results showed that the self-emulsifying systems could be obtained with surfactant concentrations between 50-88.9 %w/w, where they provided clear appearance after dispersing in water. A high extract loading capacity was achieved by formulation composed of ethyl oleate in the presence of co-solvent or co-surfactant. The dissolution and stability of *Passiflora foetida* extract were shown to be best described by using vitexin as marker. It was shown that vitexin could be dissolved from the self-emulsifying liquids > extract > self-emulsifying granules > extract granules. Preparation of self-emulsifying granules by adsorption of self-emulsifying liquid on microcrystalline cellulose led to decreased vitexin dissolution but improved stability. In this study, the potential self-emulsifying liquid providing enhanced vitexin dissolution to the greatest extent was composed of 25% ethyl oleate/ 56.3 % Tween 80/ 18.8% propylene glycol. Its granules were also stable, i.e. vitexin remaining up to 95% after storage in refrigerated and at 25 °C conditions for 4 months.

Department: Pharmaceutics and Industrial Pharmacy Student's Signature .....
Field of Study: Industrial Pharmacy Advisor's Signature .....
Academic Year: 2012 Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

%	=	Percentage
µg	=	Microgram (s)
°C	=	Degree celcius (centrigrade)
Et al.	=	et alii, ‘and other’
g	=	Gram (s)
h	=	Hour(s)
HCl	=	hydrocholicde
HPLC	=	High Performance Liquid Chromatography
MCC	=	Microcrystalline cellulose
mg	=	Milligram(s)
min	=	Minute(s)
ml	=	Milliliter(s)
RSD	=	Relative standard deviation
SD	=	Standard deviation
SEM	=	Scanning electron microscopy
SE	=	Self-emulsifying
SES	=	Self-emulsifying system
TEM	=	Transmission electron microscopy

CHAPTER I
INTRODUCTION

Approximately 40% of new drugs have poor water solubility (Kumar et al., 2010). Oral administration of these drugs often results in poor bioavailability mainly due to limited dissolution rate. Good formulation can offer improved their water solubility and/or membrane permeability (Zvonar et al., 2010). Among several approaches to improve dissolution and/or bioavailability, self-emulsifying drug delivery systems have attracted growing interest for the delivery of poor orally absorbed drugs.

Self-emulsifying drug delivery system is isotropic mixture of oil, surfactant, co-surfactant and drug. Upon mild agitation in aqueous media, such as gastrointestinal fluids, the system can form fine oil droplets in water. Emulsions formed are thermodynamically stable, transparent dispersions stabilized by an interfacial film of surfactant. Self-emulsifying drug delivery systems have many advantages, such as bioavailability enhancement of poorly water soluble drugs (Reddy et al., 2011), reduction of food effect (Perlman et al., 2008)), ability to deliver peptides that are prone to enzymatic hydrolysis in gastrointestinal tract, and ease of manufacturing and scale up (Kumar et al., 2010).

Although self-emulsifying drug delivery systems could increase the dissolution rate and bioavailability of poorly water soluble drugs, stability problem may exist (Kumar et al., 2010). Solid self-emulsifying systems may overcome this problem. They can be produced by loading liquid self-emulsifying systems on solid carriers using different technologies.

Passiflora foetida or "ka-tok-rok" in Thailand is native species which can be found as climbing weed all over the country. It is also widely grown in India. This plant has been extensively used in the folklore medicine (Dhawan et al., 2004). The leaves have been applied for giddiness and headache and the leaf infusion has been

used for treatment of hysteria and insomnia. *In vivo* antioxidant activity was obtained from ethanolic extract prepared from callus of this plant (Rasool et al., 2011). The major constituents of *Passiflora foetida* leaves are C-glycosyl flavonoids such as vitexin (Chivapat et al., 2011) which provides various pharmacological activities, including neuroprotective (Abbasi et al., 2012) and anti-depressant-like (Can et al., 2013) effects. Vitexin is slightly soluble in water leading to poor bioavailability (Zu et al., 2012). In the present study, attempt was made to improve dissolution of the constituents of *Passiflora foetida* leaf extract by formulating in self-emulsifying systems. Their stability was also evaluated.

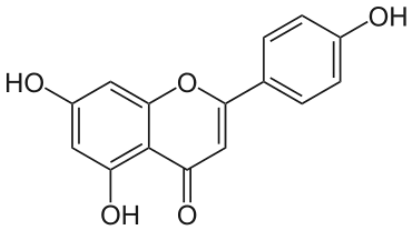
Objectives of the study

1. To improve dissolution of *Passiflora foetida* extract by formulation in self-emulsifying system.
2. To study the dissolution of major phyto-constituents in *Passiflora foetida* extract from self- emulsifying systems.
3. To evaluate stability of *Passiflora foetida* extract self-emulsifying systems.

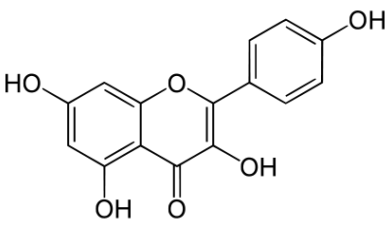
CHAPTER II
LITERATURE REVIEW

1. *Passiflorra foetida* extract

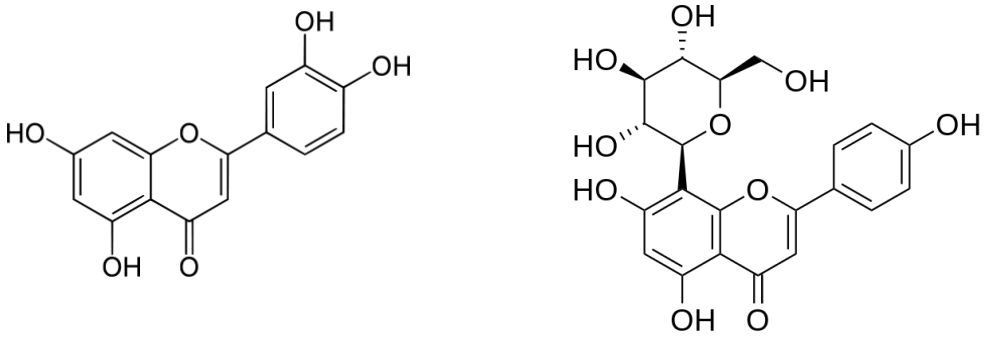
The *Passiflora* comprising about 500 species is the largest genus in the Passion flower family, Passifloraceae (Dhawan et al., 2004). The species of *Passiflora* are found in the warm temperature and tropical area, particularly in Asia, Australia and tropical Africa. *Passiflora foetida* or "ka-tok-rok" in Thailand is native species which can be found as climbing weed all over the country. *Passiflora foetida* is also widely grown in India. This plant has been extensively used in the folklore medicine (Dhawan et al., 2004). The leaves have been applied for giddiness and headache and the leaf infusion has been used for treatment of hysteria and insomnia. *In vivo* antioxidant activity was obtained from ethanolic extract prepared from callus of this plant (Rasool et al., 2011). Phyto-constituents in *Passiflora foetida* has been reviewed by Dhawan et al. (2004). These constituents includes flavonoids pachypodol, 7,4′dimethoxyapigenin, ermanin, 4′,7-*O*-dimethyl-naringenin, 3,5-dihydroxy-4,7-dimethoxy flavanone, C-glycosyl flavonoids chrysoeriol, apigenin, isovitexin, vitexin, 2′′-xylosylvitexin, luteolin-7-β-D-glucoside, kaempferol, cyanohydrin glycosides tetraphyllin A, tetraphyllin B, tetraphyllin B sulphate, deidaclin , volkenin, fatty acids (linoleic acid and linolenic acid), alpha-pyrones named passifloricins. The major constituents of *Passiflora foetida* leaves are C-glycosyl flavonoids such as apigenin, luteolin, kaempferol, isovitexin and vitexin (Chivapat et al., 2011).



(a) Apigenin



(b) Kaempferol



(c) Leuteolin (d) Vitexin

Figure 1 Chemical structures of some C-glycosyl flavonoids found in *Passiflora foetida*

Vitexin is a C-glycosyl flavonoid (5, 7, 4-trihydroxyflavone-8-glucoside). Vitexin is insoluble in water (7.6172 μg/ml) leading to a poor bioavailability (Zu et al., 2012). It has various pharmacological activities such as hypotensive and anti-inflammatory action (Prabhakar et al., 1981). Vitexin protected against myocardial ischemia/reperfusion injury in rat hearts (Dong et al, 2011). Recently, vitexin was found to provide neuroprotective (Abbasi et al., 2012) and anti-depressant-like (Can et al., 2013) effects. Chivapat et al. (2011) found that 40% ethanolic extract from *Passiflora foetida* leaves which contained 0.84% vitexin did not cause toxicity in mice and its LD50 should be more than 40 g/kg.

2 Determination of vitexin

Zeraik and Yariwake (2010) determined isoorientin and total flavonoids in *Passiflora edulis* fruit pulp by high performance liquid chromatography (HPLC) with photo-diode array detection. The method development was carried out with linear gradient of 0.2 % formic acid in water (solvent A) and 0.2% formic acid in acetonitrile (solvent B) using rutin as external standard The chromatogram was monitored at 330 nm. Pongpun et al. (2007) determined vitexin and analyse *Passiflora foetida* leaf extract by reversed phase HPLC with photo-diode array detection. The mobile phase was isocratic isopropanol: tetrahydrofuran: water (5: 15: 80, v/v/v) and 0.3% formic acid**.** The major peaks, including a separated peak of

vitexin could be resolved within 18 min. Yang et al. (2011) employed HPLC with UV detector at 360 nm and gradient of 0.2% acetic acid in water (solvent A) and acetonitrile (solvent B) as mobile phase to determine vitexin and isovitexin in mung bean.

3 **Self-emulsifying system** (SES)

Self-emulsifying system is an isotropic mixture of oil, surfactant with or without co-surfactant or co-solvent. The mixture emulsifies to form fine emulsion or oil droplets in aqueous medium under gentle agitation or gastric mobility. The droplet size ranges from 100-300 nm to less than 50 nm. The system of which the size is less than 50 nm, producing transparent microemulsion, often classified as self-microemulsifying system (Gursoy and Benita, 2004). Mechanism of self-emulsification has been discussed. In conventional emulsion, the free energy is a function of the energy required to create a new surface between oil and water. The oil and water tend to separate with time to reduce interfacial area and hence the free energy of the system. The surfactant in the emulsion formulation forms a layer surrounding oil droplets to reduce interfacial energy. For SES, the free energy required to form emulsion is either very low and positive or negative, so emulsification occurs spontaneously (Constantinides, 1995). Sef-emulsification may be also involved with formation of liquid crystalline phase between the oil/surfactant and water phases (Craig et al., 1995). The ease of self-emulsification relies on the ease of water penetration into liquid crystal or gel phases formed at the oil-water interface (Rang and Miller, 1999).

SES is one of the strategies employed to improve oral bioavailability of the hydrophobic crystalline drugs where dissolution is often rate limiting step of absorption. The SES can improve the rate and extent of oral absorption and provides more consistent absorption behavior. Poorly soluble drugs can be dissolved in the SES and encapsulated to be administered orally. When the formulation is introduced into gastrointestinal fluid, under digestive mobility, it forms fine oil-in-water emulsions or microemulsion and the drug remains in dissolved state. The SES must be formulated to ensure that the drug remained dissolved throughout the transit in

gastrointestinal tract (Pouton, 2000). Therefore, bioavailability can be improved as the dissolution step is avoided. The small oil droplets provide large interfacial area for partitioning of the drug between the oil and aqueous gastrointestinal fluid (Pouton, 1997). The drug dissolved in oil may also be absorbed into the lymphatic system resulting in reduction of first-pass metabolism (Narang et al., 2007).

The SES is also beneficial in preparing oral dosage form for water insoluble drug without food effect. Hong et al. (2006) reported that the SES composed of Transcutol®, Pluronic® L64 and tocopherol acetate improved bioavailability of itraconazole in rat and the formulation could be taken without food effect. The SES formulation containing medium chain triglyceride/Triacetin/Polysorbate 80/Capmul CMC (20/30/20/30) also reduced food effect on absorption of torcetrapib in dogs (Perlman et al., 2008).

The marketed pharmaceutical products which are formulated as SES are such as Sandimmune® (cyclospoin A), Neoral® (cyclosporine A), Norvir® (ritonavir) and Fortase® (saquinavir).

4 Excipient in SES

It was proposed by Pouton (1985) that specific proportion of oil and surfactants was important in formulation of self-emulsification system. The amount of surfactant in the formulation of SES is usually more than 25%. The high content of surfactant may cause problem of utilizing the SES due to toxicological issue. Non-ionic surfactants are also preferred to ionic surfactants as they are considerably less toxic and less affected by pH and ionic strength.

Basically, prior to prepare SES, screening studies were carried out for selecting oils and non-ionic surfactants which have ability to form SES. Bachynsky et al. (1997) suggested that HLB of 10-15 were suitable for formation of fine emulsion droplets.

In addition, solvent capacity of selected oils should be determined. The SES formulation should have adequate solvent capacity for incorporating the drug of a unit dose. Triglycerides show high solvent capacity for the hydrophobic drug having log P >4 but low solvent capacity for less hydrophobic drug. In the latter case, the solvent capacity could be improved by adding other oily excipient such as mixture of mono- and di-glycerides (Pouton, 2000). In addition, Pouton (2000) suggested that the use of hydrophilic surfactant (HLB > 12) or water soluble co-solvent i.e. propylene glycol and polyethylene glycol could improve the solvent capacity of the SES formulation for the drug with 2<log P<4.

A high proportion of oil in the formulation may be required in order to incorporate more drug, but this can provide adverse effect on emulsion stability (Pouton, 1985). The oil in the formulation is also important factor on digestability and/or solubilization by mixed micelles of bile salts and phospholipids, forming colloidal dispersion. The formulation with reduced oil proportion and addition of surfactant and co-solvent is less susceptible to digestion and self-emulsification is dependent on initial emulsification process (Pouton, 2000). Apart from facilitating self-emulsification and solubilizing the hydrophobic drug, the oil in the formulation improves the transport of hydrophobic drug through the lymphatic pathway, depending on nature of triglycerides (Gershanik and Benita, 2000).

Self-emulsification efficiency is dependent on a specific pair of oil/surfactant, oil to surfactant ratio and the temperature (Gursoy and Benita, 2004). In early work, Pouton (1997) reported that SES can be formulated from medium chain triglyceride oils and ethoxylated non-ionic surfactants.

Addition of co-solvent such as ethanol, propylene glycol and polyethylene glycol may improve the drug solubility in the oil and/or miscibility. Tang et al. (2008) showed that ethyl oleate as oil formulated with Tween 80/propylene glycol or Tween 80-Cremophor EL (1:1)/propylene glycol provided greater self-emulsification region, comparing with Miglyol 812 (medium chain triglycerides). Addition of co-

surfactant could reduce emulsion droplet size as it reduces angle of curvature during droplet formation.

The effect of increased amount of surfactant was also shown by Perlman et al. (2008) who reported that there was an increase in absorption of torcetrapib in dogs when Cremophor RH40 content in the SES formulation of Miglyol 812/Triacetin/Cremophor RH40/Capmul MCM was increased. They explained that Cremophor RH40 could inhibit lipolysis and assist in solubilization of the drug.

The drug loading capacity of the SES system is dependent on the properties of the drug and the system which should be compatible (Gursoy and Benita, 2004). The drug loading often interferes self-emulsification process. Change in self-emulsification may result in varied size distribution of emulsion droplet as a function of drug concentration (Charman et al., 1992). The complex formulation, providing smaller emulsion droplet are likely to be affected by adding the drug (Constantinides, 1995).

CHAPTER III
MATERIALS AND METHODS

1. MATERIALS

1.1 Drugs

-Vitexin reference standard (Lot BCBF7415V, 96.1% purity, Fluka, Buchs, Switzerland)

-Gallic acid (Lot No.080M0001V, 102.5% purity, 4% LOD, Sigma, USA)

-Quercetin (Lot No.020M1566, 98% purity, Sigma,USA)

-*Passiflora foetida* leaf extract (in ethanol)

1.2. Oils

-Caprylic/capric triglycerides (Captex 300, Lot No. 100209UT12, Abitec Corporation, Janesville, USA)

-Propylene glycol dicaprylate/dicaprate (Captex 200, Lot No. 070709-7, Abitec Corporation, Janesville, USA)

-Ethyl oleate (Lot No. BCBD8137V, Aldrich, Buchs, Switzerland)

1.3 Emulsifiers

-Polyoxyethylene 20 sorbitan monooleate (Tween 80, Lot No. 809861 Nof Corporation,Tokyo, Japan)

-Polyoxyl 35 castor oil (Cremophor EL, Lot No. 29209724U0, BASF, Ludwigshafen, Deutschiand, Germany)

-Propylene glycol (Lot No. 91512234I0, Ludwigshafen, Deutschiand, Germany)

-Propylene ethylene glycol 400 (Lot No. E132080208, Srichand United Dispensary, Bangkok, Thailand)

1.4 Adsorbents

-Microcrystalline cellulose (Avicel PH 102, Lot No. 2155, Asahi Kasei Corporation, Japan)

-Hydrophilic fumed silica (Areosil 200, Lot No. ZB55869, Germany)

-Veegum (Lot No. 2009131837, Srichand United Dispensary, Bangkok, Thailand)

1.5 Chemicals

-Methanol HPLC grade (Lot No. L7CG1H, Burdick&Jackson, Korea)

-Acetonitrile HPLC grade (Batch No. 11060370, RCI Labscan, Bangkok, Thailand)

-Sulfuric acid (Mallinkrodt, Phillipsburg, USA)

2. EQUIPMENT

-High performance liquid chromatography system consisted of Waters Model Alliance 2695 column heater and photodiode array detector Model 2998 (Water Corporation, Micromass UK Ltd., Manchester, UK).

-Column:ACE®C18, 5 µm particle size, 4.6 mm diameter x 250 mm length, Advanced Chromatography Technologies, Aberdeen, Scotland

-The liquid chromatography mass spectrometry (LC–MS) system consisted of an Agilent 1100 series, equipped with a G1311A binary pump and a G13114C diode array detector, connected to an Agilent G1968A LC/MS

-Dissolution Apparatus (Model VK7000, Vankel, NJ, USA)

-Zetasizer Nano Malvern ZS (Malvern Instruments Ltd., Worcestershire, UK)

-Scanning Electron Microscope (Model JSM-5800LV, Jeol Ltd., Tokyo, Japan)

-Transmission Electron Microscope (JEM-2100 (200KV), Jeol Ltd., Tokyo, Japan)

-Shaking incubator (LSI-3016A, Daihan Labtech Co., Ltd., Korea)

3. METHODS

1 Preparation of *Passiflora foetida* leaf extract

To prepare the *Passiflora foetida* leaf extract, 50 kg of leaves were macerated with 0.92 kg ethanol (3 times). The extract was concentrated by rotary evaporator. The yield of *Passiflora foetida* extract was 15.86%.

2 Development and validation of stability-indicating analytical method for vitexin

2.1 Column chromatography

The *Passiflora foetida* extract was separated by using HPLC column and conditions as described in section 3. Four main fractions were collected from the HPLC column at different retention time beginning at 5, 7, 9, 10 min, respectively. Each fraction was re-injected into the HPLC column to comfirm peak purity and analysed by LC-MS (see section 2.2). The fraction of interest was collected during the retentime 10-11 min which was around that of vitexin standard (10.4 min).

2.2 Liquid chromatography mass spectrometry (LC-MS)

The LC-MS was used to determine molecular weight of compound in the extract fraction obtained from section 2.1. Each fraction was separated by a Hypersil C-18 (10 μm particle size, 5 mm diameter × 150 mm length) using the flow rate at 1 mL/min. The mobile phase consisted of 0.06% sulfuric acid, methanol and acetonitrile as shown in Table 2. Electrospray ionization mass spectrometry was performed with drying gas temperature at 360 °C.

3 High Performance Liquid Chromatography (HPLC)

Instruments and analytical column

HPLC system consisted of Waters model Alliance 2695 equipped with a photodiode array detector model 2998. The chromatographic method was developed using ACE® C-18 columns (5 μm particle size, 4.6 mm diameter x 250 mm length), operating at 45°C.

Chromatographic conditions

The mobile phase composed of 0.06% sulfuric acid, methanol and acetonitrile are shown in Table 2. A mobile phase flow rate of 1 mL/min and an injection volume of 20 μL were applied. A photodiode array detector was used with the monitoring wavelength set at 350 nm.

Table 2 Mobile phase compositions

Time (min)	0.06% Sulfuric acid in water	Methanol	Acetonitrile
0	75	13.5	11.5
16	70	17	13
31	60	18	22
41	75	13	12
60	75	13	12

4 Degradation study

The method was modified from the method described by Walash et al. (2011).

4.1 Acid-induced degradation

Vitexin reference standard of 10 mg was accurately weighed into a 10 mL volumetric flask. Then, 1N HCl solution was added to dissolve vitexin and adjusted to volume. After that the solution was heated at 80 ^{o}C in water bath for 7 days. One milliliter of the heated solution was then pipetted into a 10 mL volumetric flask and diluted to volume with methanol. The final vitexin concentration was 0.1 mg/mL.

4.2 Base-induced degradation

Vitexin reference standard of 10 mg was accurately weighed into a 10 ml volumetric flask. Then, 1N NaOH solution was added to dissolve vitexin and adjusted to volume. After that the solution was heated at 80 ^{o}C in water bath for 5 h. One milliliter of the heated solution was pipetted into a 10 ml volumetric flask and diluted to volume with methanol. The final vitexin concentration was 0.1 mg/ml.

4.3 Hydrolysis degradation

Vitexin reference standard of 10 mg was accurately weighed into a 10 ml volumetric flask. Then, vitexin was dissolved with for 7 days. One milliliter of the heated solution was then pipetted into a 10 ml volumetric flask and diluted to volume with methanol. The final vitexin concentration was 0.1 mg/ml.

4.4 Oxidative degradation

Vitexin reference standard of 10 mg was accurately weighed into a 10 ml volumetric flask. Then, vitexin was dissolved with 30% H_2O_2 solution and adjusted to volume. After that the solution was heated at 80 ^oC in water bath for 5 h. One milliliter of the heated solution was then pipetted into a 10 ml volumetric flask and diluted to volume with methanol. The final vitexin concentration was 0.1 mg/ml.

4.5 Photo degradation

Vitexin reference standard of 10 mg was accurately weighed into a 10 ml volumetric flask and kept in a photostability chamber for 7 days at 30 ^oC. Then, vitexin was dissolved with methanol and adjusted to volume. One milliliter of the solution was then pipetted into a 10 mL volumetric flask and diluted to volume with methanol. The final vitexin concentration was 0.1 mg/mL.

5 Preparation of vitexin standard solution

Vitexin reference standard of 5 mg was accurately weighed into a 5 ml volumetric flask. An amount of methanol was added into the flask. The solution was sonicated for 5 min and adjusted to volume with methanol (1 mg/ml). Then, 1 ml of resulted solution was pipetted into a 10 ml volumetric flask and diluted with methanol to volume. The solution was filtered with 0.45 μm nylon membrane before injection using chromatographic conditions above.

6 Preparation of sample solution

Passiflora foetida extract of 50 mg was accurately weighed into a 50 ml volumetric flask. An amount of methanol was added into the flask. The solution was sonicated for 5 min and adjusted to volume with methanol (1 mg/ml). Then, 1 ml of resulted solution was pipetted into 10 ml volumetric flask and diluted with methanol to volume. The solution was filtered with 0.45 μm nylon membrane before injection using chromatographic conditions above.

7 Validation of stability-indicating analytical method

7.1 Accuracy

The accuracy of analytical method was determined by adding known concentration of vitexin reference standard to the *Passiflora foetida* extract solution. The actual and measured concentration were compared at three different concentration levels i.e. 0.05, 0.5 and 1 mg/ml of known vitexin concentration. For each concentration, the solution was injected in triplicate. The mean percentage of recovery of vitexin ranging in 80-120% with relative standard deviation (%RSD) of peaks less than 2.0% indicates accuracy of the analytical method

7.2 Precision

Repeatability and intermediate precision was evaluated. The solution of vitexin reference standard was prepared and diluted with methanol to obtain the final concentration of 0.1 mg/ml. Six determinations of the vitexin reference standard solution were carried out on the same day and on different days. The relative standard deviation (%RSD) of peaks of vitexin for both within and between runs of less than 2.0% indicates precision of the analytical method.

7.3 Linearity

The linearity of an analytical method refers to the linearity of the relationship of known drug concentration and assay measurement. Vitexin reference standard of 10 mg was accurately weighed into a 10 ml volumetric flask. An amount of methanol was added to dissolve vitexin. The solution was sonicated for 5 min and adjusted to volume with methanol. Then, 0.5, 1.25, 2.5 and 7.5 ml of the resulted solution was pipetted into 10 ml volumetric flasks and diluted with methanol to volume. The solution was injected. The peak area was plotted against the concentration and correlation coefficient of the regression line was calculated.

7.4 Specificity

Specificity is the ability to measure the analyte in the presence of components that may be expected to be present. The excipients in the formulation should not

interfere with the peak of active ingredient i.e. vitexin. The excipients used were Captex 200, Captex 300, ethyl oleate, Cremophor EL, Tween80, propylene ethylene glycol 400 and propylene glycol. Vitexin reference standard of 10 mg was accurately weighed into a 10 ml volumetric flask. An amount of methanol was added to dissolve vitexin. Then, one milliliter of vitexin solution was pipetted into the flask containing the excipient of 1 g which was then diluted with methanol to volume.

8 Analytical method of total flavonoids

The total flavonoids was analysed using UV spectrometry method modified from the method published by Pourmorad et al. (2006)

8.1 Construction of standard curve

Quercetin of 15 mg was accurately weighed into a 100 ml volumetric flask and dissolved with methanol (0.15 mg/ml). Then, 10 ml of resulted solution was pipetted into 100 ml volumetric flask and diluted with methanol to volume, which was used as stock solution (0.015 mg/ml). After that 2, 3, 4, 5, 6 and 6.5 ml of the stock solution was pipetted into separate 10 ml volumetric flask and added with 0.2 ml of 10% aluminium chloride, 0.2 ml of 1 M potassium acetate and 3 ml of methanol. The solutions were diluted to volume with distilled water and stored in ambient temperature for 30 min. Absorbance of each solution was determined by UV spectrometry at a maximum absorbance of 411 nm using 0.2 ml of 10% aluminium chloride, 0.2 ml of 1 M potassium acetate and 3 ml of methanol which was diluted with 6 ml methanol and sufficient water to volume of 10 ml as blank solution.

8.2 Sample preparation

An accurately weighed amount of 20 mg extract were added into 100 ml volumetric flask and dissolved with methanol. The solution of 6 ml was pipetted into 10 ml volumetric flask and then 0.2 ml of 10% aluminium chloride, 0.2 ml of 1 M potassium acetate and 3 ml of methanol was added into the flask. The resulted solution was diluted to volume with distilled water and stored in ambient temperature for 30 min. Absorbance of each solution was determined by UV spectrometry at a maximum absorbance of 411 nm using 0.2 ml of 10% aluminium chloride, 0.2 ml of 1

M potassium acetate and 3 ml of methanol which was mixed and diluted with 6 ml methanol and sufficient water to volume of 10 ml as blank solution.

9 Analytical method of total phenolic compounds

The total phenolic compounds were analysed using UV spectrometry method modified from the method published by Pourmorad et al. (2006).

9.1 Construction of standard curve

An amount of gallic acid of 15 mg was accurately weighed into a 100 ml volumetric flask and dissolved in methanol (0.1 mg/ml). Then, 10 ml of resulted solution was pipetted into a 100 ml volumetric flask and diluted with methanol to volume, which was used as a stock solution (0.01 mg/ml). After that 2, 3, 4, 5, 6 and 6.5 ml of the stock solution was pipetted into separate 10 ml volumetric flasks and added with 3 ml of 2% Na_2CO_3 and 1 ml of 50% of Folin-ciocalteu reagent. The solutions were diluted to volume with distilled water and stored in ambient temperature for 30 min. Absorbance of each solution was determined by UV spectrometry at a maximum absorbance of 669 nm using 3 ml of 2% Na_2CO_3 and 1 ml of 50% of Folin-ciocalteu which was mixed and diluted with water to volume of 10 ml as blank solution.

9.2 Sample preparation

An accurately weighed amount of 20 mg extract were added into a 100 ml volumetric flask, dissolved and adjusted to volume with methanol. The solution of 6 ml of was pipetted into a 10 ml volumetric flask and then 3 ml of 2% Na_2CO_3 and 1 ml of 50% of Folin-ciocalteu reagent was added into the flask. The resulted solution was diluted to volume with distilled water and stored in ambient temperature for 30 min. Absorbance of each solution was determined by UV spectrophotometry at a maximum absorbance of 669 nm using 3 ml of 2% Na_2CO_3 and 1 ml of 50% of Folin-ciocalteu which was diluted with 6 ml methanol and sufficient water to volume of 10 ml as blank solution.

10 Solubility study of *Passiflora foetida* extract

The solubility of *Passiflora foetida* extract in oils, surfactant and co-surfactant was determined. An excess *Passiflora foetida* extract was added into 50 g of oils, surfactants and co-surfactants and mixed by vortex mixer at 90 Hertz for 3 min. The dispersion was shaken at a speed of 100 rpm and incubated at 25 °C. The supernatant was taken at 1, 3, 8, 24, 48, 72 h, respectively. The sample was filtered through 0.45 μm membrane before quantification of vitexin by HPLC. Total flavonoids and total phenolic compounds were quantified by UV spectrometry.

11 Screening for self-emulsifying mixture

11.1 Construction of pseudoternary phase diagrams

The pseudoternary phase diagrams were constructed by three component mixtures of oil, surfactant with or without co-surfactant or co-solvent and water at ambient temperature. The mixture was made to 3 g of total weight with varied ratios of surfactant to co-surfactant or co-solvent, if any, at 1:1, 2:1 or 3:1 and mixed by vortex mixer at 90 Hertz for 3 min. After that the mixture was visually observed at 0 and 3 days. The ratios of oil, surfactant and/or co surfactant mixture which appeared as clear dispersion would be selected for further study.

11.2 Self-emulsification assessment

One gram of the mixtures of surfactant, with or without co-surfactant/co-solvent providing clear dispersion selected from pseudoternary phase diagram were prepared and diluted with 1 fold distilled water using vortex at 90 Hertz for 5 min. The solution was visually observed at 0 and 24 h. The dispersions which were clear were selected for further dilution study.

11.3 Dilution study

One gram of the mixtures of surfactant, with or without co-surfactant/co-solvent providing clear dispersion upon 1 fold dilution were prepared and diluted with 200 fold distilled water using a dissolution apparatus II with a speed of 100 rpm at 37 °C for 15 min. The solution was visually observed at 0 and 24 h. The dispersions

which was clear were selected for further studies i.e. droplet size and zeta potential determinations as described below.

The sample

11.4 Determination of *Passiflora foetida* extract loading capacity of self-emulsifying mixtures

Three grams of self-emulsifying mixture containing more than 20% oil, surfactant, with or without co-surfactant/co-solvent providing clear dispersion after 200 fold dilution (section 11.3), were prepared. One hundred and fifty (150) milligrams of *Passiflora foetida* extract was added into the self-emulsifying mixture which was blended by vortex mixer at 90 Hertz for 5 min. After that the resulting mixture was visually observed at 0 and 24 h. If there was no precipitation occurring after 24 h, 50 mg of the extract was subsequently added until precipitation was observed.

12. Self-emulsifying mixture containing *Passiflora foetida* extract

12.1 Preparation of self-emulsifying mixture containing of *Passiflora foetida* extract

Self-emulsifying mixtures containing surfactant less than 75% and having loading capacity more than 100 mg per 1 g of self-emulsifying mixture were chosen for this study.

Three hundred milligrams (300 mg) of *Passiflora foetida* extract was dissolved in 3 g of the chosen self-emulsifying mixtures. The final mixture was diluted with 200 fold distilled water (600 g) in a dissolution apparatus II with a speed of 100 rpm at 37 ^{o}C for 15 min.

12.2 Characterization of self-emulsifying mixture containing *Passiflora foetida* extract

12.2.1 Visual observation

The dispersion was visually observed after preparation and storage for 24 h at ambient temperature to ensure that there was no sedimentation occurred.

12.2.2 Transmission electron microscopy

The morphology of oil droplets was observed by transmission electron microscopy. The self-emulsifying mixture was diluted with 50 and 100 fold distilled water using magnetic stirrer or a dissolution apparatus II with a speed of 100 rpm at 37 $^{\circ}$C, respectively, for 15 min. Then, a drop of sample obtained after dilution was placed on copper grids. Excess liquid was drawn off by filter paper. The sample was stained with 1% phosphotungstic acid solution before observation.

12.2.3 Determination of droplet size, size distribution and zeta potential

One gram of sample was diluted with 200 fold distilled water. Z-Average size and polydispersity index (PdI) were measured using photon correlation spectroscopy. Zeta potential of diluted sample was also determined. The results were compared with blank self-emulsifying mixtures.

12.2.4 *In vitro* dissolution

Dissolution profiles of self emulsifying mixture were determined by using USP dissolution apparatus II at 37±5 ^{o}C and a rotation speed of 100 rpm in 500 ml distilled water. Each vessel contained 1 g of self-emulsifying mixture containing *Passiflora foetida* extract, prepared as described above. During the study, 20 ml aliquots were withdrawn at 15, 30, 60, 120 and 180 min from the dissolution medium and 20 ml of fresh medium was replaced. The amounts of total flavonoids and phenolic compounds released in the solution medium were determined by UV spectrophotometry at a maximum absorbance of 411 nm and 669 nm, respectively. The solution was also filtered with 0.45 μm nylon membrane and vitexin released was determined by HPLC method using chromatographic conditions mentioned above. The self-emulsifying mixture which showed more dissolution of phyto-contituents in water was selected to be adsorbed on solid carrier.

13 Preparation and characterization of self-emulsifying granules of *Passiflora foetida* extract

13.1 Selection of solid carrier

The solid carrier, i.e. microcrystalline cellulose (Avicel PH102), colloidal silicon dioxide (Aerosil 200) or veegum was added to adsorb self-emulsifying mixture containing *Passiflora foetida* extract, prepared with 300 mg extract incorporated in 3 g of self-emulsifying mixture, i.e. total weight 3.3 mg. Only ET3PG2 (22.2% ethyl oleate/58.4 Tween 80/19.5% propylene glycol) was used in this screening study. The mixture was mixed in mortar and pestle. The amount of solid carrier of 500 mg was added consecutively until the mixture was sufficiently dry.

The constant amount (4.8 g) of each solid carrier was also added to adsorb 3.3 g self-emulsifying mixture (ET3PG2) of *Passiflora foetida* extract, i.e. SE mixture: solid carrier, 1:1.5. The resultant mixture of 2 g was then subject to dissolution study in water.

The solid carrier which provided dry granules at a small proportion and gave more release of vitexin, total flavonoids and phenolic compounds was selected for preparing self-emulsifying granules.

13.2 Preparation of self-emulsifying granules of *Passiflora foetida* extract

Self-emulsifying granules of *Passiflora foetida* extract were prepared with chosen self-emulsifying mixture and solid carrier.

Passiflora foetida extract of 300 mg was weighed into self-emulsifying mixture of 3 g. The mixture was mixed by vortex mixer at 90 Hertz for 5 min. An amount, 3.3 g of selected solid carrier was added into the mixture and further mixed with mortar and pestle. The granules were kept in a desiccator before characterization.

13.2.1 Morphology

Scanning electron microscope (SEM) was employed to investigate morphology of self emulsifying granules. The sample was prepared by gold sputtering technique prior to examination.

13.2.3 Transmission electron microscopy

The morphology of oil droplets was observed by transmission electron microscopy. Self-emulsifying granules (2 g) were dispersed in 400 g of distilled water using a dissolution apparatus II with a speed of 100 rpm at 37 ^{o}C for 15 min. Then, a drop of filtered sample obtained after dispersing was placed on copper grids. Excess liquid was drawn off by filter paper. The sample was stained with 1% phosphotungstic acid solution before observation

13.2.2 Determination of droplet size, size distribution and zeta potential

Two grams of self-emulsifying granules were dispersed in 600 g of distilled water, as described in section 13.2.3. Z-Average and polydispersity index were measured using photon correlation spectroscopy. The dispersion was left until the solid content precipitated and the supernatant was used for measurement**.**

13.2.4 Assay

One gram (1 g) of self-emulsifying liquids or 2 g of self-emulsifying granules containing *Passiflora foetida* extract prepared as described above was weighed into a 100 ml volumetric flask. Then, an amount of methanol was added into the flask. The solution was sonicated for 5 min and adjusted to volume with methanol. The amounts of total flavonoids, total phenolic compounds and vitexin release were analyzed as previously described.

13.2.5 In vitro dissolution

Dissolution profiles of 1 g self-emulsifying liquids and 2 g granules of *Passiflora foetida* extract were determined by using USP Dissolution apparatus II at 37±5 ^{o} C and a rotation speed of 100 rpm in 500 ml distilled water, 0.1 N HCl and phosphate buffer pH 6.8. During the study, 20 ml aliquots were withdrawn at 15, 30, 60, 120 and 180 min from the dissolution medium and 20 ml of fresh medium was replaced. The amount of total flavonoids and total phenolic compounds released in the solution medium was determined by UV spectrophotometry at a maximum absorbance of 411 nm and 669 nm, respectively. The solution was also filtered with

0.45 um nylon membrane and vitexin released was determined by HPLC method using chromatographic conditions mentioned above.

13.2.6 Stability study

The *Passiflora foetida* extract**,** self emulsifying liquids and self emulsifying granules were kept into a light brown glass bottles and subjected to stability study under conditions shown in Table 3 The samples were analyzed for the content (0, 30, 60, 90, 120 days) and dissolution (0, 120 days) of vitexin, total flavonoids and phenolic compounds.

Table 3 Lists of samples and conditions of stability study

Tested samples	Stability test conditions
1. *Passiflora foetida* extract 2. Self emulsifying liquids 3. Self emulsifying granules	(a) Refrigerated (5± 3°C)
	(b) air conditioned room (approximately 25 °C)
	(c) 30 °C± 2, 75%RH
	(d) 30 °C± 2/UV light, 75%RH (30 days)
	(e) 40 °C± 2, 75%RH

CHAPTER IV
RESULTS AND DISCUSSION

The chemical marker for quality control the *Passiflora foetida* extract was vitexin. In this study, the amounts of total flavonoids, equivalent to the amount of quercetin, and total phenolic compounds, equivalent to gallic acid, were also determined. The HPLC method for determination of vitexin in the *Passiflora foetida* extract was developed based on the work carried out by Zeraik and Yariwake (2010). The method was intended to quantify isoorientin and total flavonoids in *Passiflora edulis* fruit pulp using a linear gradient of 0.2% formic acid in water (solvent A) and 0.2% formic acid in acetonitrile (solvent B) as mobile phase. However, although the gradient was varied, this method was not possible to characterize vitexin in the *Passiflorra foetida* extract. Modification of mobile phase was made by substituting acetic acid or sulfuric acid for formic acid and adjusting the amount of acid to achieve pH about 2.0-2.5. Initially, it was found that using of 1% acetic acid (solvent A) and methanol (solvent B) could not resolve the constitute patterns of the extract. The improvement of resolving vitexin chromatogram was achieved by replacing 1% acetic acid with 0.06% sulfuric acid. Addition of acetonitrile to the mobile phase was required as an organic modifier to shorten the analysis time. Hence, the vitexin peak in the *Passiflora* foetida leaf extract chromatogram was separated successfully (Figure 5) by using mobile phase comprising 0.06% sulfuric acid in water, methanol and acetonitrile as described in Table 2, Chapter III. By comparing the leaf extract chromatogram to the vitexin standard chromatogram, the apparent retention time of vitexin was 10.4 min. The vitexin peak was confirmed by UV spectrum match plot and the results from LC-MS as the followings.

1. Liquid chromatography mass spectrometry (LC-MS)

The chromatogram of *Passiflora foetida* extract is shown in Figure 5. The fraction which was separated and collected from the HPLC column at the retention time around 10-11 min was re-injected into the HPLC column. The chromatogram of this fraction appeared as single peak at the retention time of 10.4 min which

corresponded to the retention time of vitexin (Figure 6-7). Peak purity of the extract fraction was also evaluated by using UV spectrum of vitexin standard as a reference spectrum and the result is shown in Figure 8.

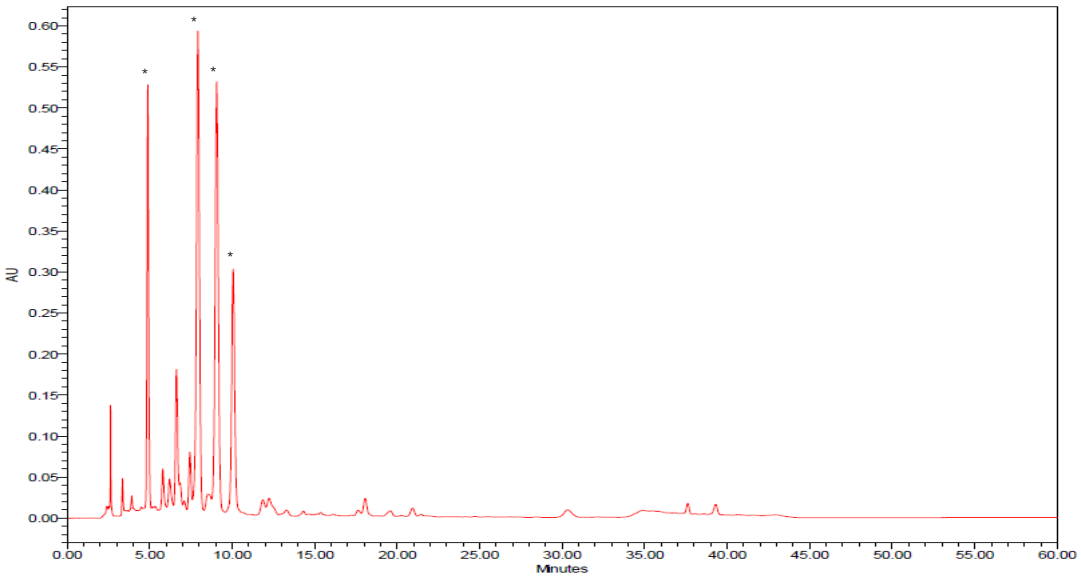


Figure 5 Chromatogram of *Passiflora foetida* extract; asterisk (*) peak was separated and collected for re-injection in the HPLC column

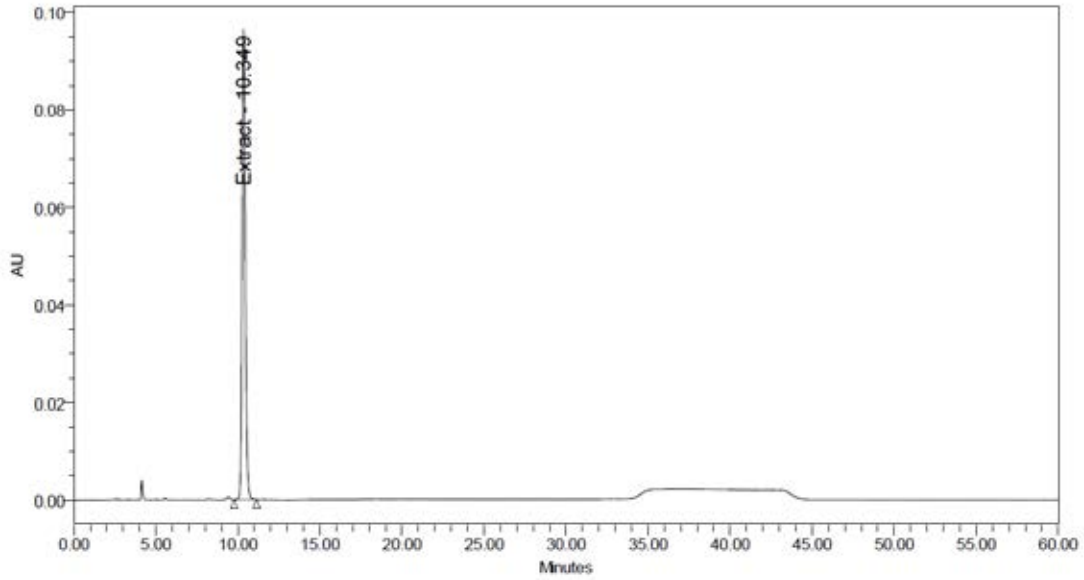


Figure 6 Chromatogram of the extract fraction separated at the retention time of 10-11 min

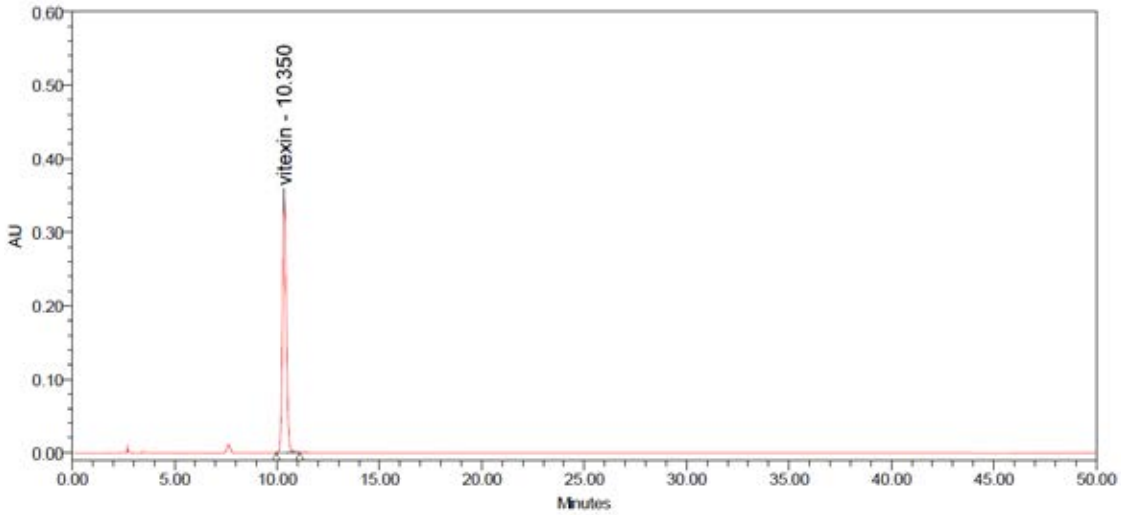


Figure 7 Chromatogram of the vitexin standard

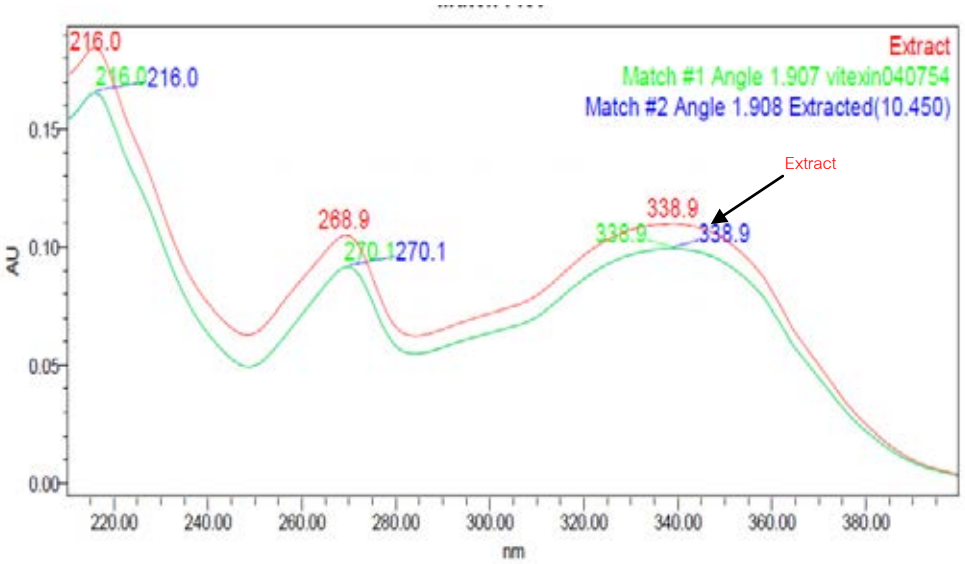


Figure 8 Match plot between UV spectrum of the extract fraction separated at the retention time of 10-11 min and that of vitexin standard.

In addition, the product ion mass spectra transitions were *m*/*z* 433 which was matched to that of vitexin standard.

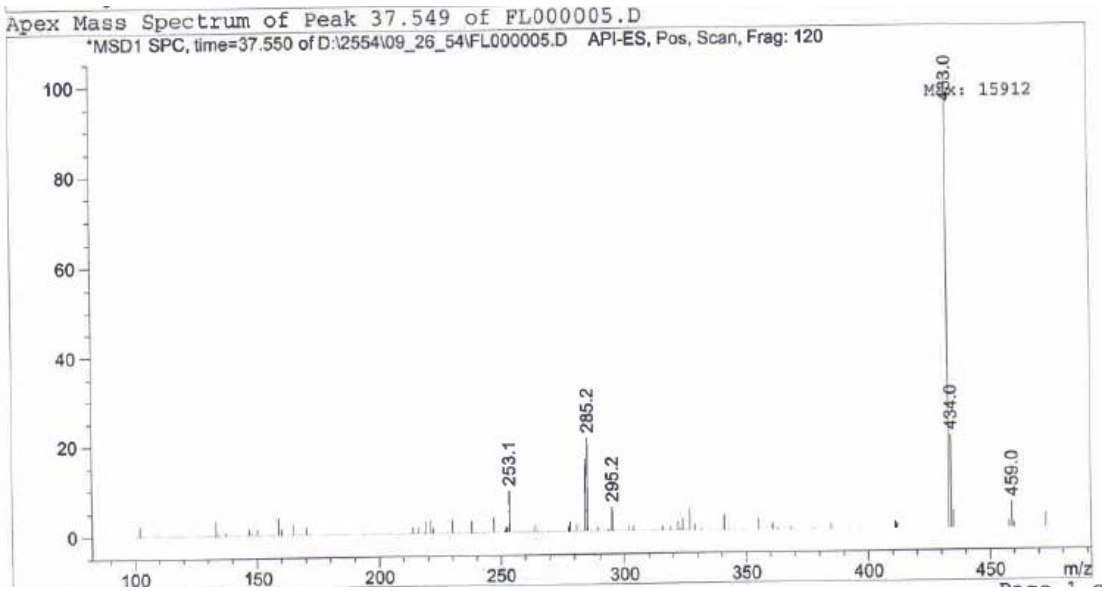


Figure 9 The product ion mass spectra of the extract fraction separated at the retention time of 10-11 min.

Therefore, overall results indicated that the peak at the retention time of 10.4 min in the extract chromatogram was of vitexin.

2. Validation of stability-indicating analytical method

The developed HPLC was validated and the summarized data are tabulated (Table 2) and the details are shown in the Appendix. The method development and validation were also reported by Wachirasakwong et al. (2012).

Table 4 Validation data of the developed HPLC method

Parameters	Results
Range	0.05-0.75 mg/mL
Linearity	$R^2 = 0.999$
Recovery (%, n = 3)	
- Concentration at 0.05 mg/mL	98.3
- Concentration at 0.5 mg/mL	99.2
- Concentration at 0.1 mg/mL	89.4
Precision (n=6, 0.5 mg/mL)	
-Repeatability	RSD= 0.13%
-Intermediate precision	RSD= 0.42%
Robustness :	
Column No.1(Batch V10-2985)	
-Retention time:	10.10
-Tailing factor:	1.02
Column No.2 (Batch V10-2768)	
-Retention time:	10.12
-Tailing factor:	1.10

Vitexin was not markedly degraded after heat exposure at 80 °C for 7 days in either 1 N HCl or aqueous solution. It was also stable after exposure to UV light for 7 days. However, degradation was detected upon exposure to 1 N NaOH and to 30% H_2O_2 at 80 °C for 5 h.

3. Solubility study of constituents in *Passiflora foetida* extract

Table 5 Solubility of vitexin, total flavonoids and phenolic compounds in water, oils, surfactants and co-solvents studied, (n = 3)

Type	Vitexin (μg/ml)	Total flavonoids (μg/ml)	Total phenolic compounds (μg/ml)	*Passiflora foetida* extract (USP 34)
Water	11.1	13.5	13.7	1: 189
Captex 200	11.5	32.2	29.4	1: 982
Captex 300	11.1	29.7	27.1	1: 1459
Ethyl oleate	114.2	34.9	29.9	1: 412
Tween 80 (HLB 15)	56.6	34.0	26.9	1: 44
Cremophor EL (HLB 13)	20.7	31.7	21.1	1: 38
Propylene glycol	57.9	34.8	26.6	1: 1455
Polyethylene glycol 400	68.8	33.8	27.9	1: 1219

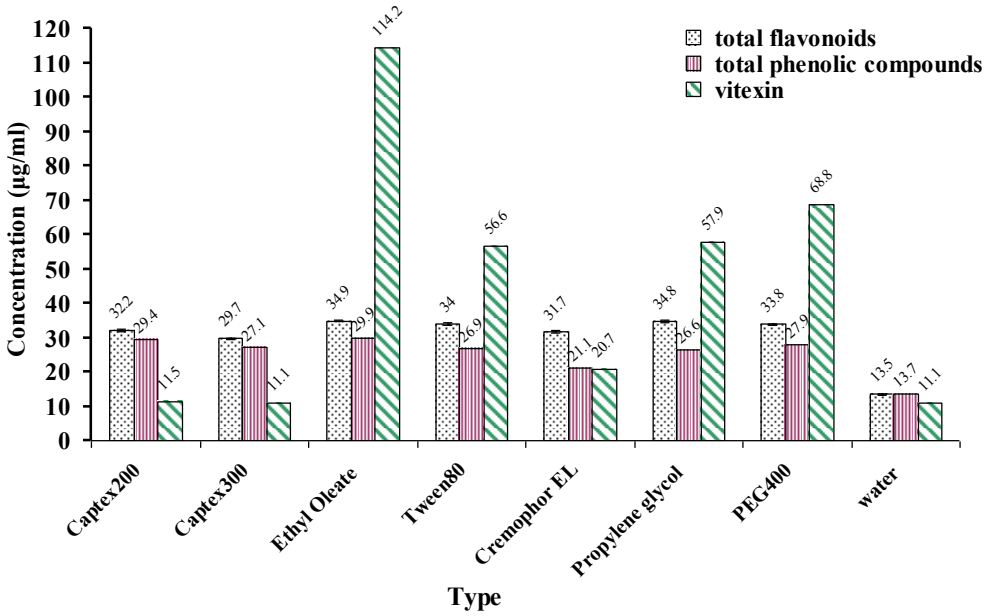


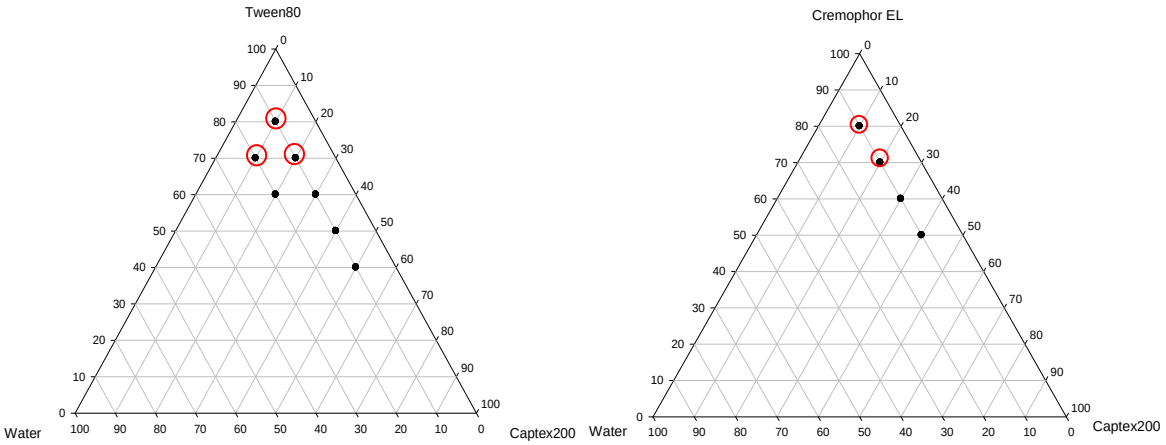
Figure 10 Solubility comparison between vitexin, total flavonoids and phenolic compounds in water, oils, surfactants and co-solvents studied, (n =3)

Passiflora foetida ethanolic extract appeared as black viscous semi-solid. The solubility of *Passiflora foetida* ethanolic extract in water was 1: 189, classified as slightly soluble (USP 34). The solubility of constituents, i.e., vitexin, total flavonoids equivalent to quercetin and phenolic compounds equivalent to gallic acid in *Passiflora foetida* extract in water, oils, surfactants and co-solvents was determined. The results are shown in Table 5 and Figure 10. This study was aimed to determine solvent or solubilizing capacity of oils, surfactants and co-solvents for the constituents in *Passiflora foetida* extract. The results showed that ethyl oleate could dissolve vitexin better than Captex 200 and Captex 300. This may be caused by relatively long chain (C18) of ethyl oleate which contributes to be more hydrophobic, comparing with Captex 300 and Captex 200 which are C6-C12 medium chain triglycerides.

The solubility of flavonoids and phenolic compounds in ethyl oleate and Captex 200 was rather similar and slightly greater than that in Captex 300. The screening results also showed that propylene glycol and polyethylene glycol 400 (PEG 400) could dissolve vitexin better than Captex 200 and Captex 300. However, the co-solvents did not improve solubility of flavonoids and phenolic compounds, comparing with the oils studied.

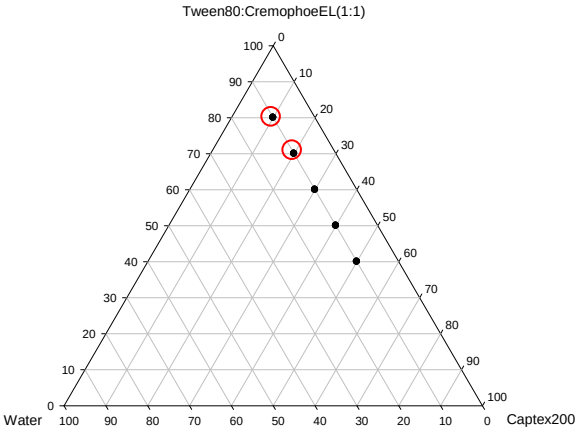
4 Construction of pseudoternary phase diagrams

4.1 Self-emulsification assessment and dilution study

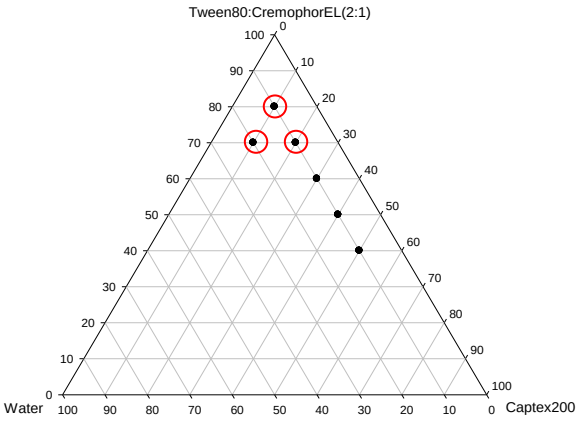


(a)

(b)

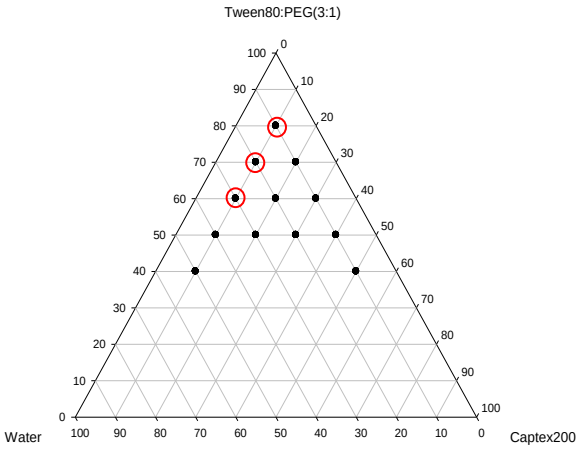


(c)

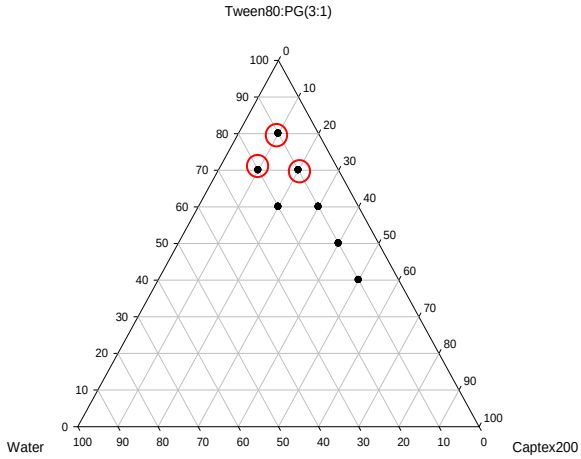


(d)

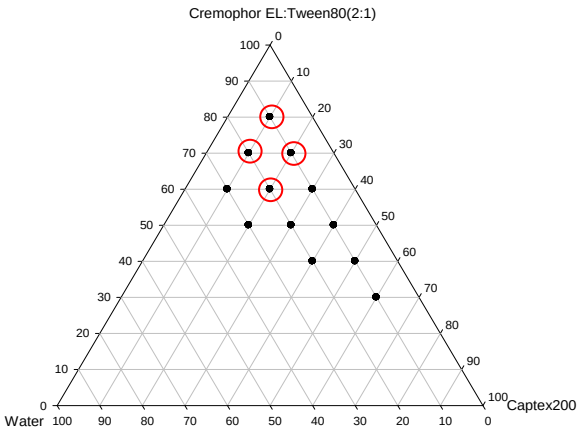
Figure 11 Pseudoternary phase diagrams of water, Captex 200 and surfactant, with or without co-surfactant: (a) Tween 80, (b) Cremophor EL, (c) Tween 80:Cremophor EL (1:1), (d) Tween 80:Cremophor EL (2:1); ● Clear emulsion; ○ Clear stable dispersion after 200 fold dilution



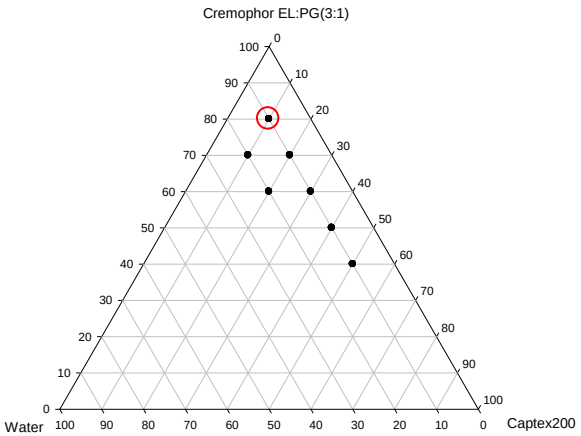
(e)



(f)

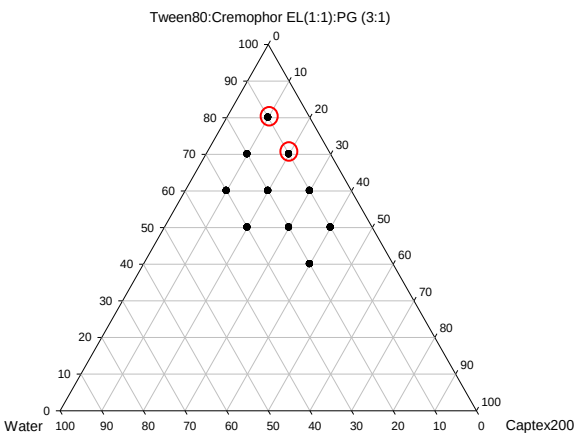


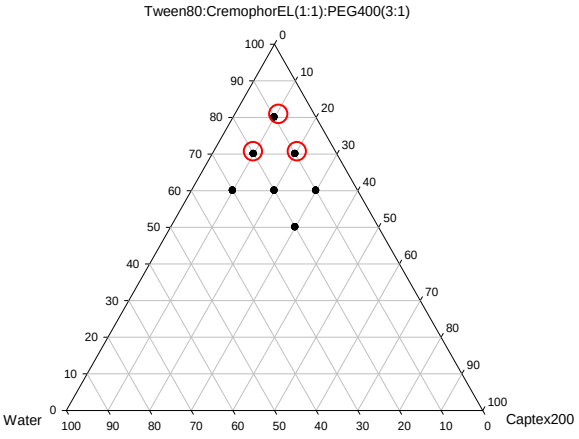
(g)



(h)

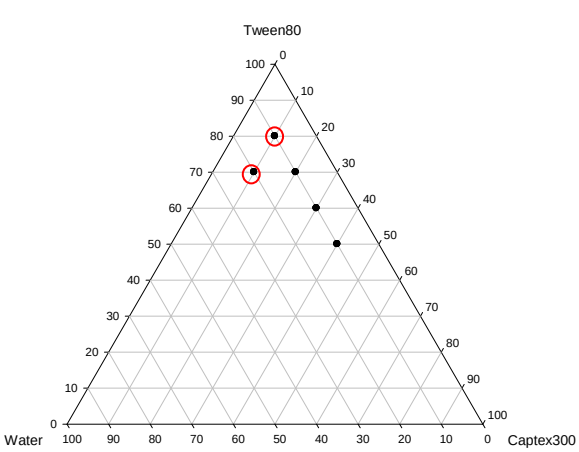
Figure 11 (continue) Pseudoternary phase diagrams of water, Captex 200 and surfactant, with co-surfactant or co-solvent: (e) Cremophor EL:Tween 80 (2:1), (f) Tween 80:propylene glycol (3:1), (g) Tween80:polyethylene glycol 400 (3:1), (h) Cremophor EL:propylene glycol (3:1); • Clear emulsion; ○ Clear stable dispersion after 200 fold dilution



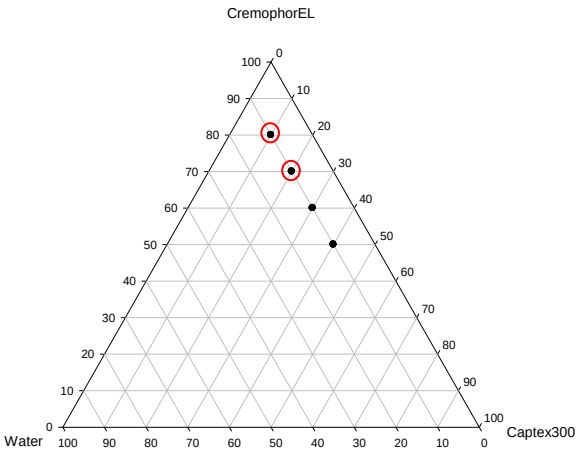


(i) (j)

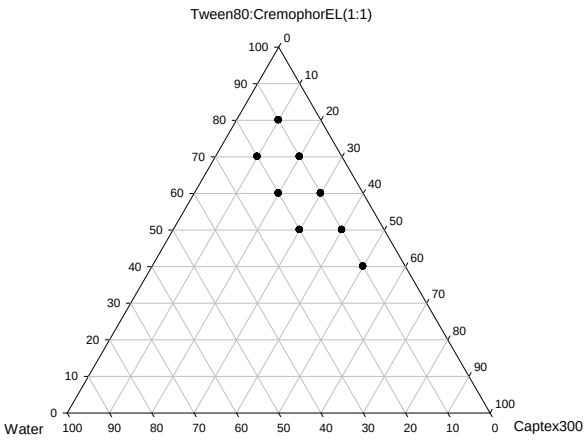
Figure 11 (continue) Pseudoternary phase diagrams of water, Captex 200 and surfactant with co-surfactant and co-solvent: (i) [Tween 80:Cremophor EL (1:1)]:propylene glycol (3:1), (j) [Tween 80:Cremophor EL (1:1)]:polyethylene glycol 400 (3:1); • Clear emulsion; ○ Clear stable dispersion after 200 fold dilution



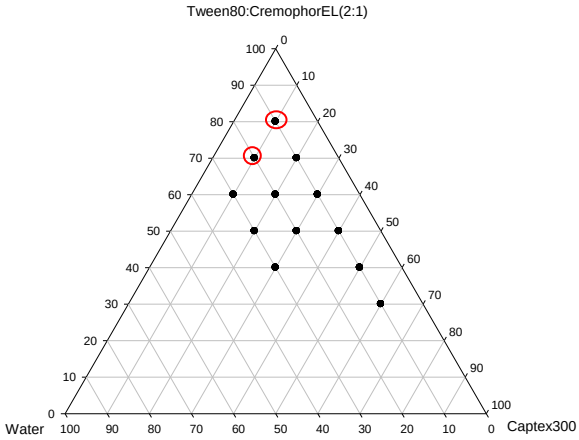
(a)



(b)

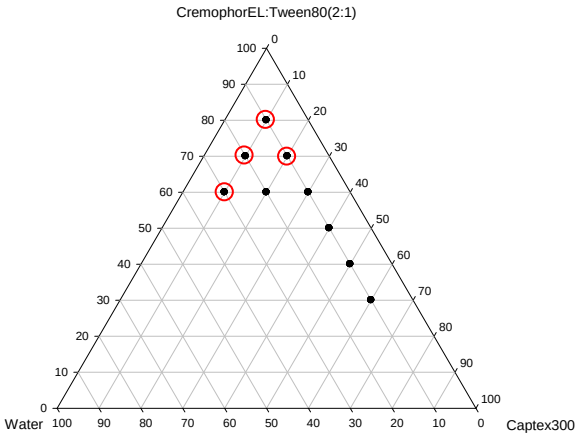


(c)

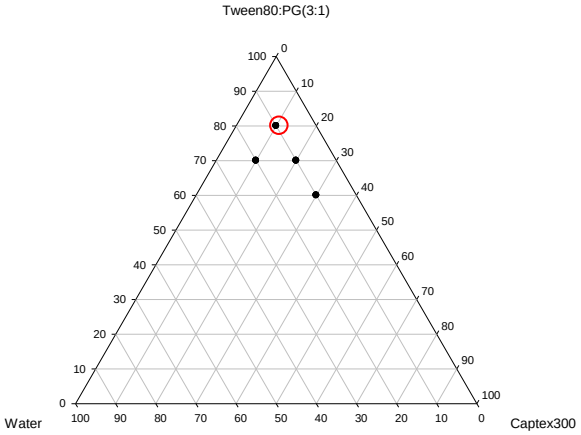


(d)

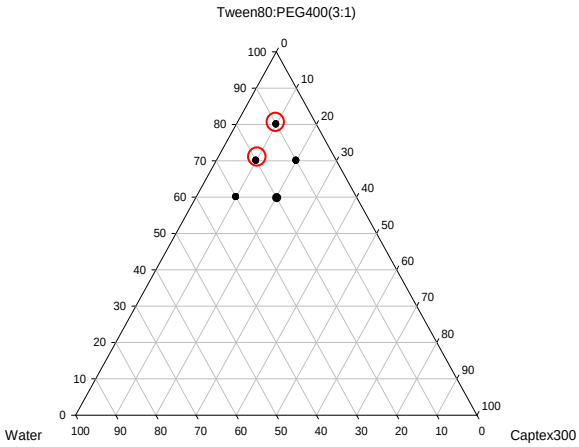
Figure 12 Pseudoternary phase diagrams of water, Captex 300 and surfactant with or without co-surfactant: (a) Tween 80, (b) Cremophor EL, (c) Tween 80:Cremophor EL (1:1), (d) Tween 80:Cremophor EL (2:1); ● Clear emulsion; ○ Clear stable dispersion after 200 fold dilution



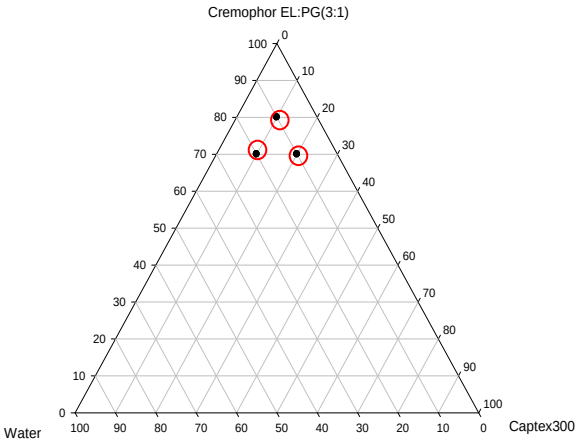
(e)



(f)

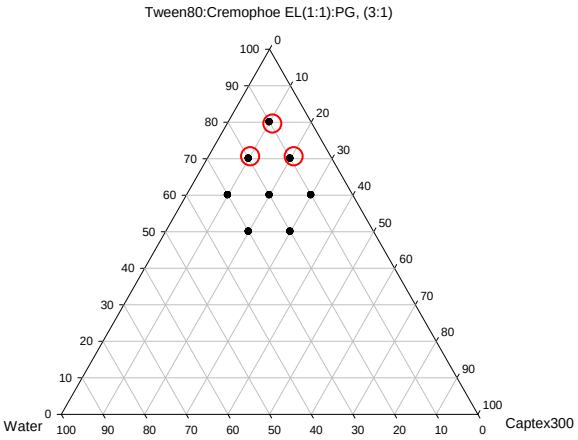


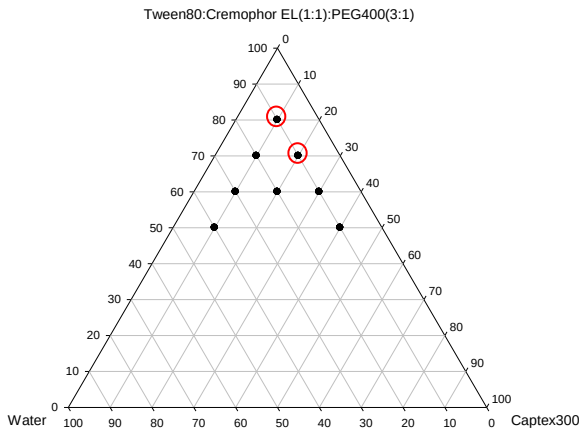
(g)



(h)

Figure 12 (continue) Pseudoternary phase diagrams of water, Captex 300 and surfactant with co-surfactant or co-solvent: (e) Cremophor EL:Tween 80 (2:1), (f) Tween 80:propylene glycol (3:1), (g) Tween 80:propylene glycol (3:1), (h) Cremophor EL: propylene glycol (3:1); ● Clear emulsion; ○ Clear stable dispersion after 200 fold dilution





(i) (j)

Figure 12 (continue) Pseudoternary phase diagrams of water, Captex 300 and surfactant with co-surfactant and co-solvent: (i) [Tween 80: Cremophor EL (1:1)]:propylene glycol (3:1), (j) [Tween80:Cremophpr EL (1:1)]:polyethylene glycol 400 (3:1); ● Clear emulsion; ○ Clear stable dispersion after 200 fold dilution.

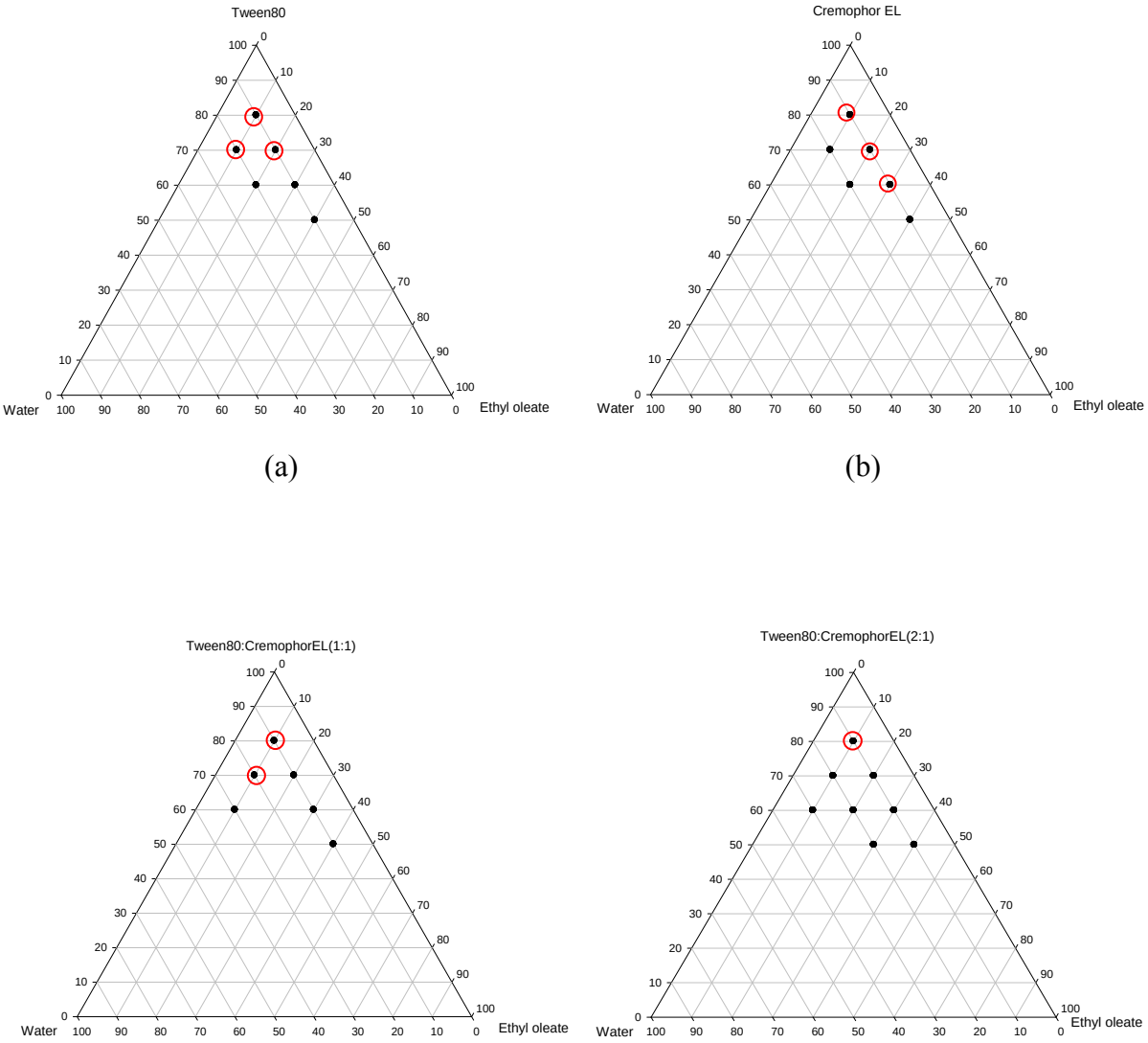
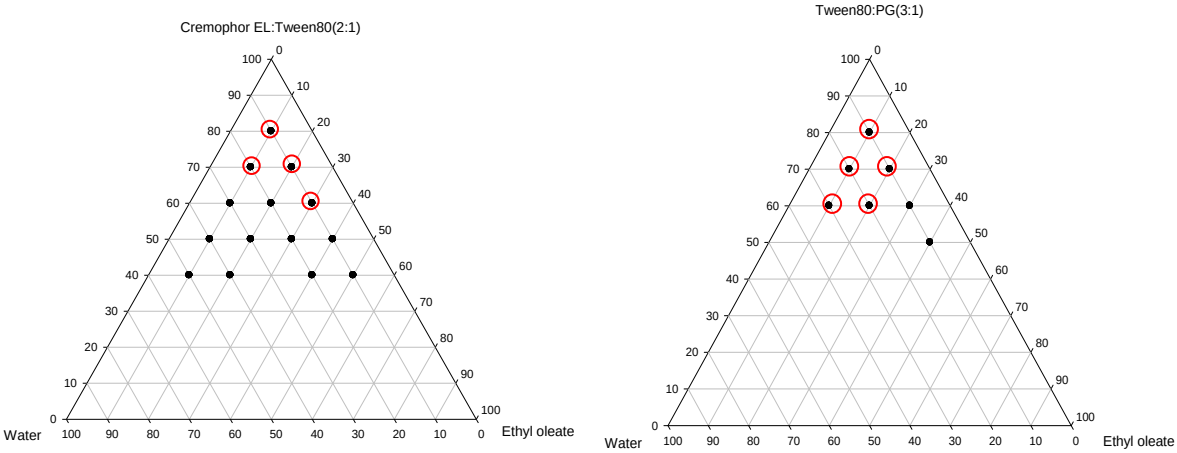
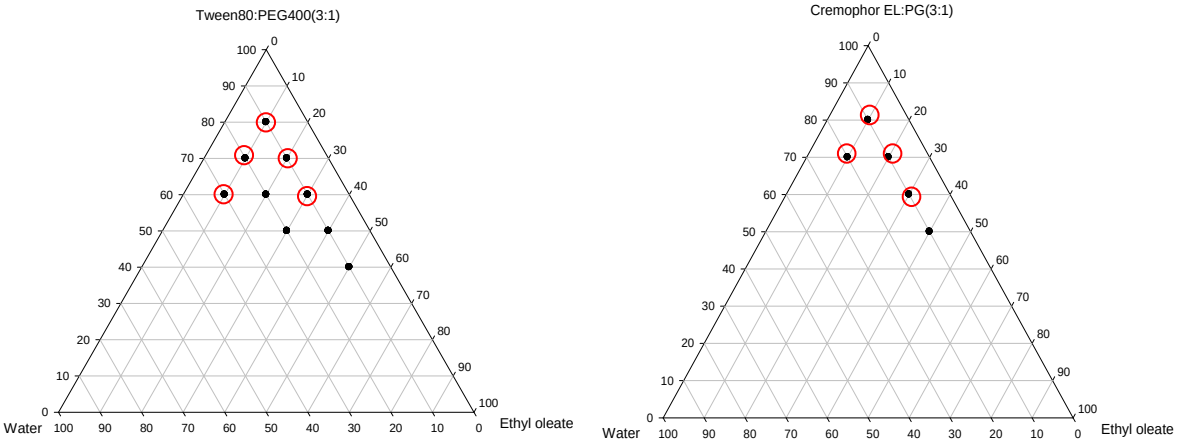


Figure 13 Pseudoternary phase diagrams of water, ethyl oleate and surfactant with or without co-surfactant: (a) Tween 80, (b) Cremophor EL, (c) Tween 80:Cremophor EL (1:1), (d) Tween 80:Cremophor EL (2:1), • Clear emulsion; ○ Clear stable dispersion after 200 fold dilution

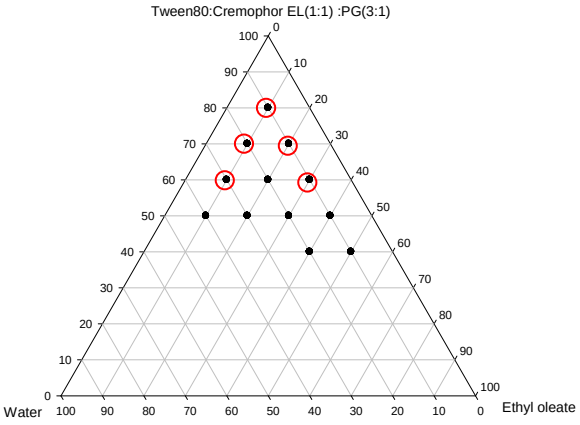


(e) (f)

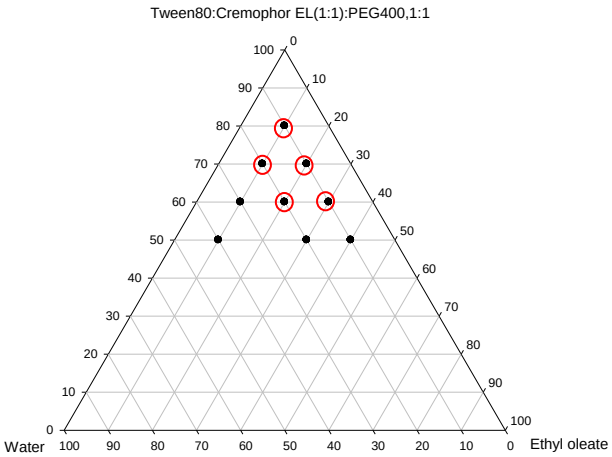


(g) (h)

Figure 13 (continue) Pseudoternary phase diagrams of water, ethyl oleate and surfactant with co-surfactant or co-solvent: (e) Cremophor EL:Tween 80 (2:1), (f) Tween 80:propylene glycol (3:1), (g) Tween 80:polyethylene glycol 400 (3:1), (h) Cremophor EL:propylene glycol (3:1); ● Clear emulsion; ○ Clear stable dispersion after 200 fold dilution



(i)



(j)

Figure 13 (continue) Pseudoternary phase diagrams of water, ethyl oleate and surfactant with co-surfactant and co-solvent: (i) [Tween 80:Cremophor EL (1:1)]:propylene glycol (3:1), (j) [Tween 80:Cremophor EL (1:1)]:polyethylene glycol 400 (3:1); ● Clear emulsion; ○ Clear stable dispersion after 200 fold dilution

From pseudoternary phase diagrams (Figure 11-13), the systems of oil, surfactant with or without co-surfactant/co-solvent, and water which gave clear emulsion after storage of 3 days (• in the phase diagrams) at ambient temperature were of interest. These systems may be microemulsion.

To utilize the formulation of microemulsion as self-emulsifying system (SES), the formulation must be diluted without change in phase behavior. In this study, the mixture of oil, surfactant(s) and co-solvent, without water, which emulsified in 1 part of water and was not precipitated out upon 200 fold dilution, giving transparent dispersion after storage for 24 hours, circled (○) in the pseudoternary phase diagrams, was chosen to prepare self-emulsifying (SE) mixtures (Figure11-13). Among the oils studied, ethyl oleate predominantly provided the SES. The unstable systems were excluded from the study.

The SES is usually formulated with nonionic surfactant with high HLB values. The surfactants having high hydrophilicity could provide rapid self-emulsification. The surfactant concentration providing stable SES was reported as 30-60% w/w of the formulation (Gursoy and Benita, 2004). In this study, self-emulsification could be obtained with Tween 80 or Cremophor EL or mixture of Tween 80: Cremophor EL where the HLB values were in the range of 13-15. The surfactant concentration in the SE mixture was 50-88.9 %w/w. All the SE mixtures could emulsify immediately under gentle agitation in dissolution apparatus.

The mixed surfactants could expand the microemulsion region in the phase diagram, but it did not show marked influence for self-emulsification region (Figure11-13). The co-solvent was also added to aid in dissolving the drug. The amount of co-solvents such as propylene glycol and PEG 400 should be limited because the co-solvent may have deteriorated effect to the capsules shells if the formulation is encapsulated. Therefore, the co-solvent used in this study was limited at about 20%.

When the proportion of oil in the formulation is high, it is possible to incorporate more hydrophobic constituents. While, the formulation with reduced oil proportion and addition of surfactant and co-solvent is less susceptible to digestion and self-emulsification is dependent on initial emulsification process (Pouton, 2000).

In this study, the SE mixtures composed of more than 20 % w/w oil were selected for determining loading capacity.

4.2 Determination of *Passiflora foetida* extract loading capacity of self-emulsifying systems

The *Passiflora foetida* extract was incorporated into the SE mixtures containing more than 20 %w/w oils. The maximum loading capacity of each mixture is showed Table 6. The loading amount is dependent on the extract solubility in the formulation and is specific to the physicochemical compatibility of the extract/system (Gursoy and Benita, 2004). Estimated solubility of the extract in the SES was based on the loading extract, ranging from 1: 20 to 1:4, soluble to freely soluble, which was greater than the solubility in water. It was found that the ET3PG2 formulation had greatest solvent capacity, 250 mg per 1 g SE mixture or 1:4. However, the loading capacity of the SE mixtures was not directly related to solvent capacity ethyl oleate; not all the formulations composed of ethyl oleate having high loading capacity.

The addition of more polar cosolvent or co-surfactant may improve solubility of the extract, depending on the formulation. The extract loaded in ET2 formulation composed of 22.2 % ethyl oleate/ 77.8 % Tween 80 was improved, from 50 mg to 250 mg per 1 g SE mixture, by substituting 19.5 % propylene glycol for the surfactant in the formulation ET3PG2. The extract solubility in C2Cr2 formulation composed of 22.2 % Captex 200/ 77.8 % surfactant (Cremophor EL) was improved, from < 50 mg to 133.3 mg per 1 g SE mixture, using combined surfactants (51.9 % Cremophor EL and 25.9 % Tween 80).

The loading capacity is important. It must be high enough so that the volume of the formulation to be administered for the therapeutic dose is minimized. In other words, the formulation selected should have great extent of solvent capacity. As a large amount of surfactant may cause GI irritation (Gursoy and Benita, 2004), the SE mixtures with loading capacity more than 100 mg/1 g SE mixture and were composed of less than 75% surfactant were selected for further study.

Table 6 Selected self-emulsifying mixtures which contained more than 20 % oil and loading capacity

System	% Oils			% Surfactant or co-solvent				Extract loading (mg) in 1g of SE mixture
	Captex 200	Captex 300	Ethyl oleate	Tween 80	Cremophor EL	Propylene glycol	Polyethylene Glycol 400	
C2T2	22.2	-	-	77.8	-	-	-	66.7
C2Cr2	22.2	-	-		77.8	-	-	< 50
C2TCr2	22.2	-	-	38.9	38.9	-	-	150
C2T2Cr2	22.2	-	-	51.9	25.9	-	-	< 50
C2Cr2T2	22.2	-	-	25.9	51.9	-	-	133.3
C2Cr2T10*	25	-	-	25	50	-	-	233.3
C2T3PG2*	22.2	-	-	58.4	-	19.5	-	133.3
C2(TCr)3PG2	22.2	-	-	29.2	29.2	19.5	-	50
C2(TCr)3PEG2	22.2	-	-	29.2	29.2		19.5	50
C3Cr2	-	22.2	-	-	77.8	-	-	133.3
C3Cr2T2	-	22.2	-	25.9	51.9	-	-	50
C3Cr3PG2*	-	22.2	-	-	58.4	19.5		150
C3(TCr)3PG2	-	22.2	-	29.2	29.2	19.5	-	66.7
C3(TCr)3PEG2	-	22.2	-	29.2	29.2	-	19.5	50
ET2	-	-	22.2	77.8	-	-	-	50
ECr2	-	-	22.2	-	77.8	-	-	150
ECr3*	-	-	33.3	-	66.7	-	-	200

Table 6 (continue) Selected self-emulsifying mixtures which contained more than 20% oil and loading capacity

System	%Oils			% Surfactant or co-solvent				Extract loading (mg) in 1g of SE mixture
	Captex200	Captex300	Ethyl oleate	Tween80	Cremophor EL	Propylene glycol	Polyethylene Glycol 400	
ECr2T2	-	-	22.2	25.9	51.8	-	-	50
ECr2T3	-	-	33.3	22.2	44.5	-	-	50
ET3PG2*	-	-	22.2	58.4	-	19.5	-	250
ET3PG10*	-	-	25	56.3	-	18.8	-	133.3
ET3PEG2*	-	-	22.2	58.4	-	-	19.5	200
ET3PEG3	-	-	33.3	50	-	-	16.7	< 50
E(TCr)3PG2	-	-	22.2	29.2	29.2	19.5	-	50
E(TCr)3PG3	-	-	33.3	25	25	16.7	-	50
E(TCr)3PEG2*	-	-	22.2	29.2	29.2	-	19.5	116.7
E(TCr)3PEG3*	-	-	33.3	25	25	-	16.7	116.7
E(TCr)3PEG10	-	-	25	28.1	28.1	-	18.8	66.7

***** The SE formulation containing less than 75 %w/w surfactant with loading capacity more than 100 mg/1 g SE mixture selected for further study

5. Preparation and characterization of self-emulsifying mixtures containing *Passiflora foetida* extract

It was often found that incorporating the drug into the SES interfering self-emulsification process (Gursoy and Benita, 2004). To investigate efficiency of self-emulsification after incorporating the extract, 3 g of each selected SE mixture was loaded with a constant amount, 300 mg, of the extract. The final mixture was diluted with 200 fold water and characterized for physical properties.

5.1 Visual observation

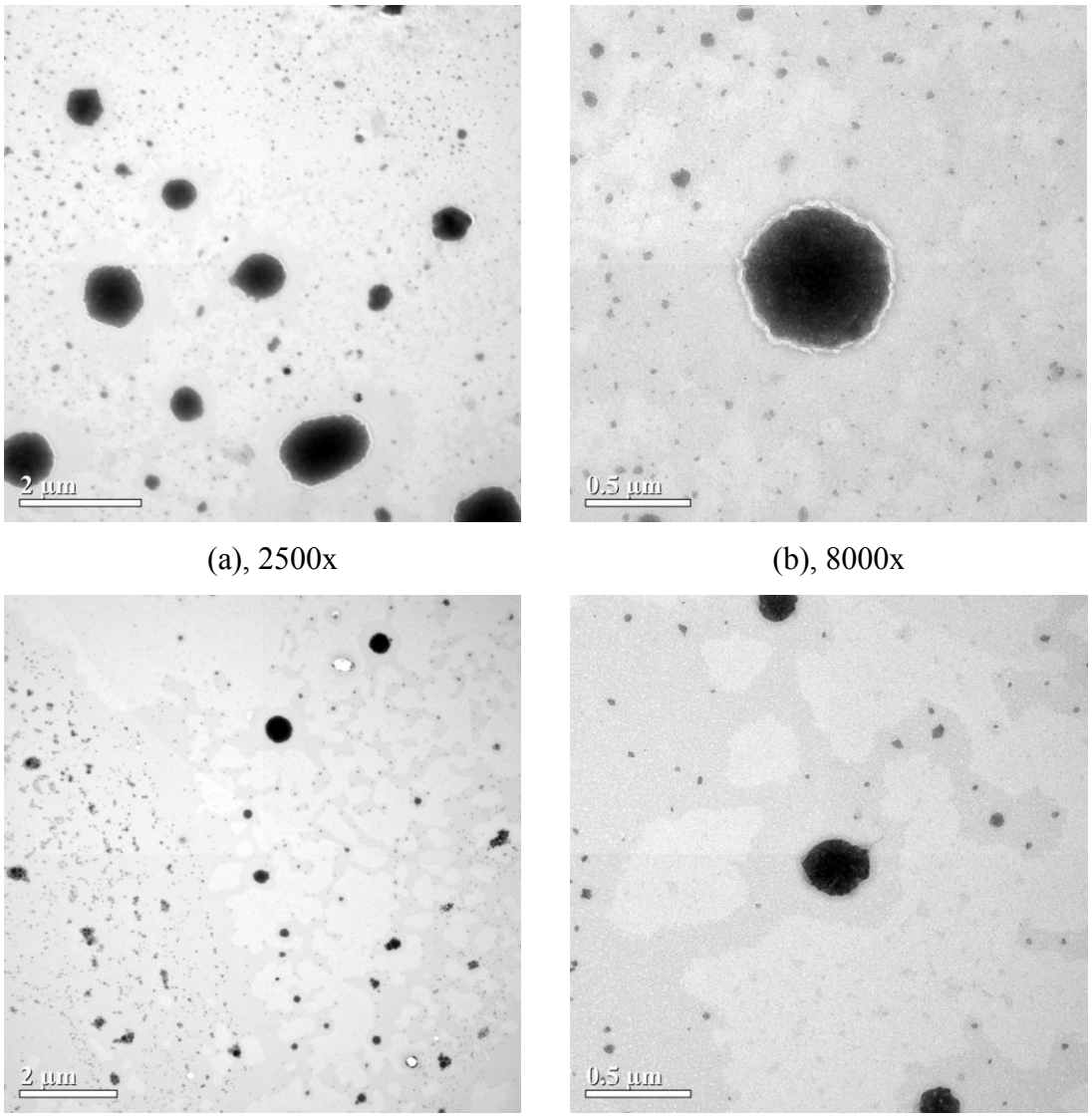
(A) (B)

Figure 14 Dispersion obtained by dilution of ET3PG2 formulation containing *Passiflora foetida extract* with distilled water: (A) 1 fold dilution, (B) 200 fold dilution, after 24 h

A basic consideration was that the constituents in the extract must not precipitate out upon dilution. It was found that all the SES formulation had enough solvent capacity, so that the extract was not precipitated. This may be due to relatively high surfactant concentration (50-75 %w/w) in the formulation. An example of dispersion obtained by dilution of ET3PG2 self-emulsifying mixture is shown in Figure 14. The morphology of droplet emulsion was viewed under TEM, showing round shaped droplets.

However, the incorporation of *Passiflora foetida extract* changed phase behavior of the formulation E(TCr)3PEG2. The formulation was composed of 22.2 % ethyl oleate/ 29.2 % Tween 80/ 29.2 % Cremophor EL/ 19.5 % PEG400 failed to maintain the integrity of the emulsion droplet, indicated by significant increase in the droplet size with board distribution (PdI =1), after incorporating the extract (Table 7). This indicated instability of the system.

5.2 Transmission electron microscopy



(a), 2500x (b), 8000x

(c), 2000x (d), 8000x

Figure 15 TEM photomicrographs of emulsion droplets formed by self-emulsification of ET3PG2 containing *Passiflora foetida* extract in water: (a-b) 50 fold dilution; (c-d) 100 fold dilution

5.3 Determination of droplet size, size distribution and zeta potential

The emulsion droplet size and size distribution is important as they are related to drug release and physical stability. In this study, the SE mixture containing considerably high amount of surfactant, leading to formation of very fine emulsion droplets, mainly less than 100 nm (Table 7). The polydispersity index (PdI) was greater than 0.3, indicating rather board size distribution (Kallakunta, 2013).

When the extract was incorporated, the emulsion droplet size was changed depending on the SE formulation (Table 7). An increase in droplet size after incorporating the extract was found for the complex formulations containing ethyl oleate as oil, Tween 80 as surfactant and co-solvent (ET3PG2, ET3PG10, ET3PEG2). While size reduction was observed for the formulation with Captex 200 and Captex 300, also for the simple formulation of ethyl oleate and Cremophor EL (ECr3). Ethyl oleate was more favorable to the extract, rather than other oils. Further, addition of co-solvent could improve the extract's solubility as discussed earlier. Therefore, the extract may be located in the droplet interior as well as accumulated in the oil-interfacial regions of the emulsion, resulting in increased emulsion droplet size when the extract was incorporated.

The extract contained mixture of hydrophobic and hydrophilic constituents distributing in aqueous-interfacial-oil regions**.** Distribution between three regions i.e., oil, interfacial and aqueous depends on their solubility in each region and on emulsifier concentration. Losada-Barreiro et al. (2012) reported that the hydrophilic phenolic compounds such as gallic acid which is soluble in water, 10 μg/ml (Lu and Lu, 2007) but practically insoluble in oil was mainly located in aqueous-interfacial region of the emulsion, rather than in the oil droplet. For more hydrophobic phenolic compounds, they were located in oil-interfacial region.

The zeta potential of blank SES is negative due to the presence of free fatty acids (Gursoy and Benita, 2004). The incorporation of the extract affected the surface charge of emulsion droplets as indicated by reduction in zeta-potential. Significantly reduced charge was observed for the formulation composed of ethyl oleate as oil.

Zeta potential may be an indicator for the physical stability of the emulsion being formed. High absolute zeta potential values (above30mV) should be achieved in order to ensure the creation of a high-energy barrier against coalescene of the dispersed droplet (Yang and Benita, 2000).

Table 7 Emulsion droplet size (Z-Average), size distribution (polydispersity index, PdI) and zeta potential after diluting self-emulsifying mixtures with 200 fold of deionized water at 37 °C (n= 3)

System	0 h (without extract)			0 h (with extract)			24 h (with extract)		
	Emulsion droplet size (nm), (SD)	PdI	Zeta potential (mV), (SD)	Emulsion droplet size (nm), (SD)	PdI	Zeta potential (mV),(SD)	Emulsion droplet size (nm), (SD)	PdI	Zeta potential (mV), (SD)
C2Cr2T10	37.20 (1.21)	0.423	-13.85 (0.79)	27.49 (1.08)	0.303	-7.32 (0.36)	38.11 (0.58)	0.141	-6.42 (0.29)
C2T3PG2	80.78 (0.80)	0.442	-15.24 (0.81)	53.77 (0.41)	0.328	-4.36 (0.32)	56.28 (0.73)	0.372	-4.47 (0.12)
C3Cr3PG2	107.85 (0.69)	0.691	-6.72 (0.25)	67.87 (0.68)	0.181	-5.22 (0.10)	65.24 (0.77)	0.160	-5.36 (0.20)
ECr3	93.47 (0.82)	0.266	-9.13 (0.41)	34.73 (0.77)	0.209	-4.71 (0.66)	35.80 (0.77)	0.268	-4.43 (0.20)
ET3PG2	38.50 (1.14)	0.474	-24.52 (0.11)	42.23 (0.78)	0.272	-5.31 (0.35)	44.90 (0.58)	0.299	-6.42 (0.20)
ET3PG10	54.78 (1.17)	0.459	-24.85 (0.75)	67.83 (0.30)	0.261	-3.57 (0.25)	69.58 (0.19)	0.269	-8.45 (0.19)
ET3PEG2	21.45 (1.15)	0.156	-15.76 (0.42)	36.89 (0.76)	0.226	-7.30 (0.45)	56.28 (0.51)	0.679	-7.44 (0.29)
E(TCr)3PEG2*	25.84 (1.18)	0.294	-13.67 (0.18)	$8.57x10^4$ (4.16)	1.000	-7.70 (0.23)	$5.02 x10^4$ ($3.73x10^4$)	1.000	-7.63 (0.29)
E(TCr)3PEG3	33.48 (0.54)	0.164	-12.29 (0.92)	32.73 (1.84)	0.342	-7.16 (0.26)	47.20 (0.30)	0.236	-7.31 (0.20)

5.4 *In vitro* dissolution of self-emulsifying mixtures containing *Passiflora foetida*

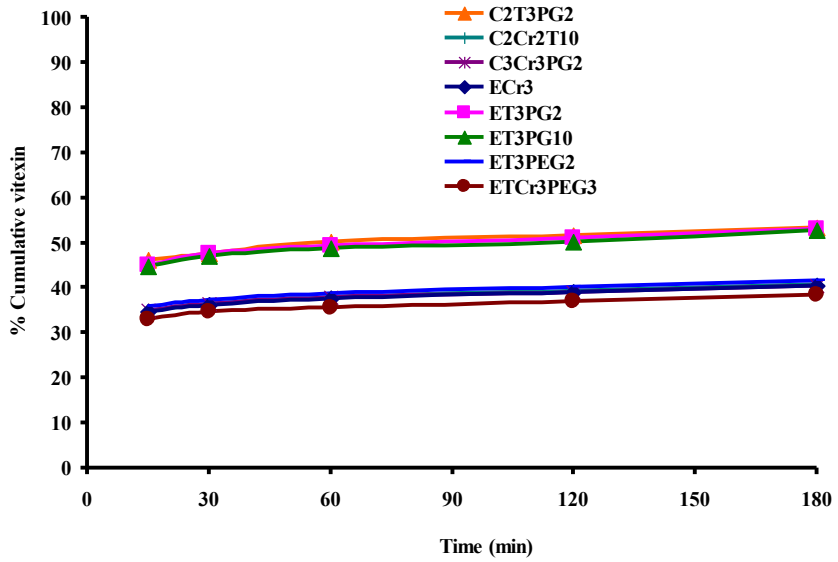


Figure 16.1 Dissolution profiles of **vitexin** from self-emulsifying mixtures **in water**

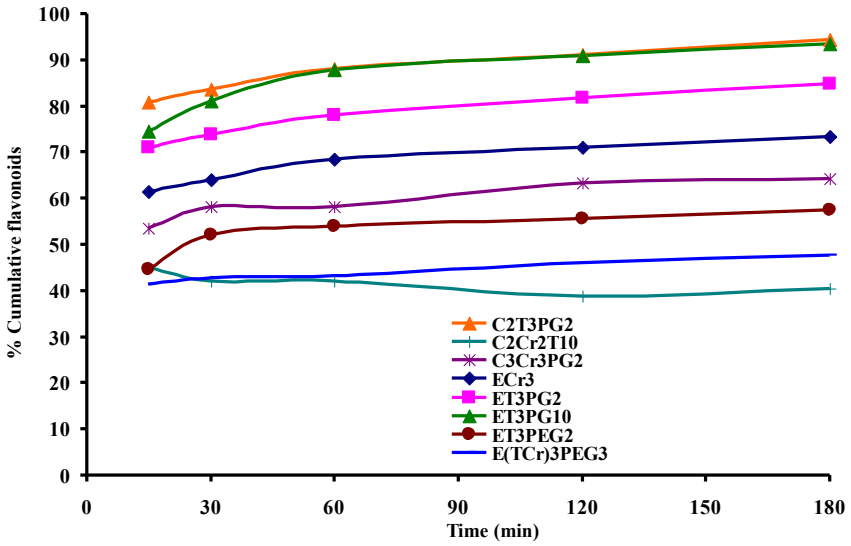


Figure 16.2 Dissolution profiles of **total flavonoids** from self-emulsifying mixtures **in water**

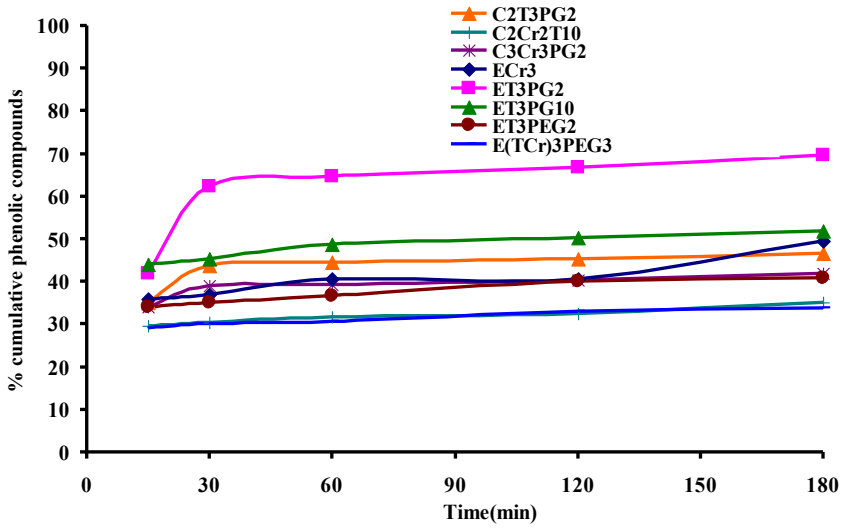


Figure 16.3 Dissolution profiles of **total phenolic compounds** from self-emulsifying mixtures **in water**

The percentage dissolved vitexin, flavonoids and phenolic compounds in water was used for selecting the potential SE formulations to prepare self-emulsifying solids. The results showed that three SE formulations, C2T3PG2, ET3PG2 and ET3PG10, which contained different oils (Captex 200 or ethyl oleate), Tween 80 as surfactant and propylene glycol as co-solvent providing relatively more dissolved vitexin, flavonoids and phenolic compounds were chosen to prepare self-emulsifying solid.

6 Preparation and characterization of self-emulsifying granules containing *Passiflora foetida* extract

6.1 Selection of solid carrier

Table 8 The amount of solid carrier required to adsorb 3.3 g ET3PG2 self emulsifying (SE) mixture containing of Passiflora foetida extract (100 mg extract per 1 g SE mixture)

Type	**Solid carrier used (g)**	**Ratio of SE mixture containing the extract: solid carrier**
Avicel PH102	3.3	1:1
Aerosil 200	2.4	1:0.7
Veegum	4.8	1:1.5

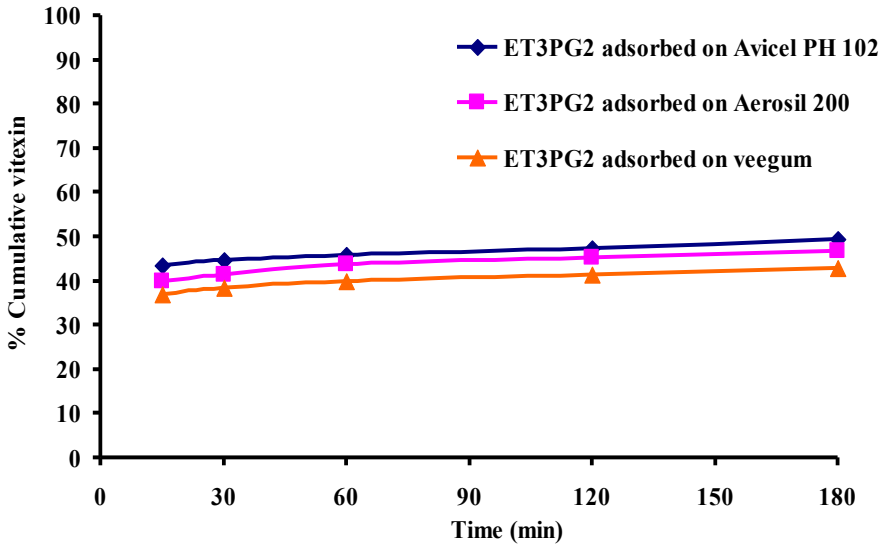


Figure 17.1 Dissolution profiles of **vitexin** from self-emulsifying granules **in water**

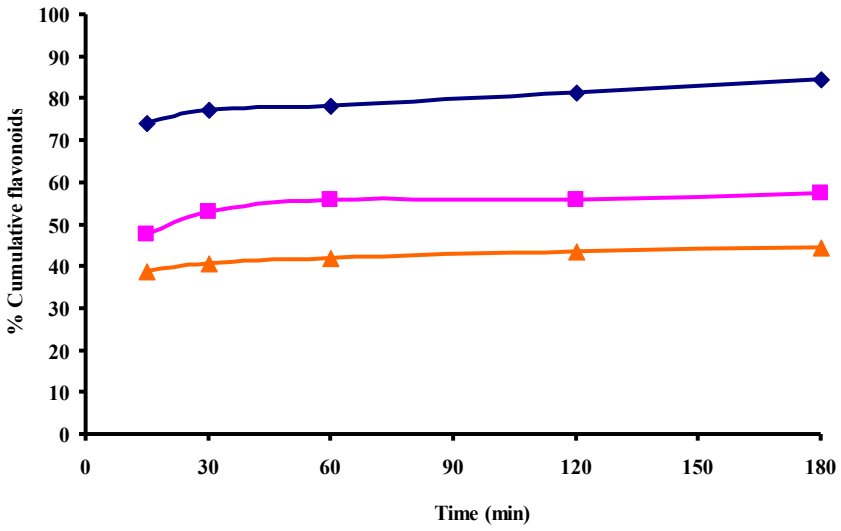


Figure 17.2 Dissolution profiles of **total flavonoids** from self-emulsifying granules in water

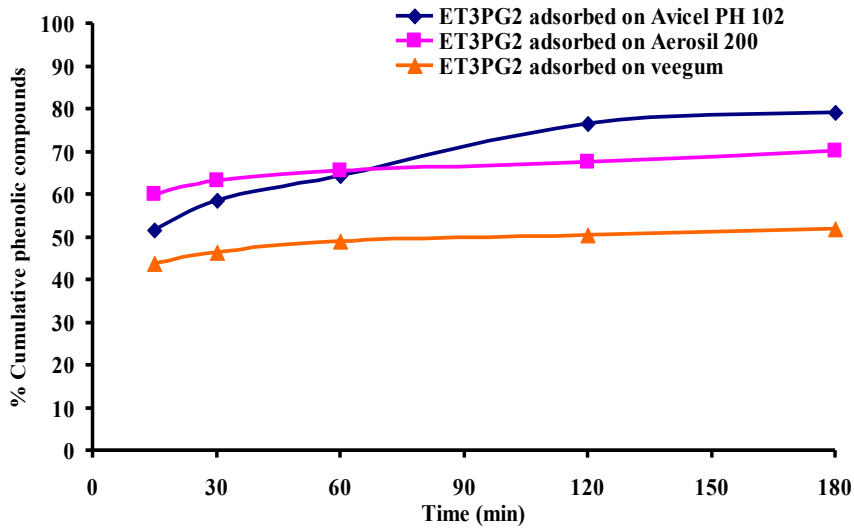
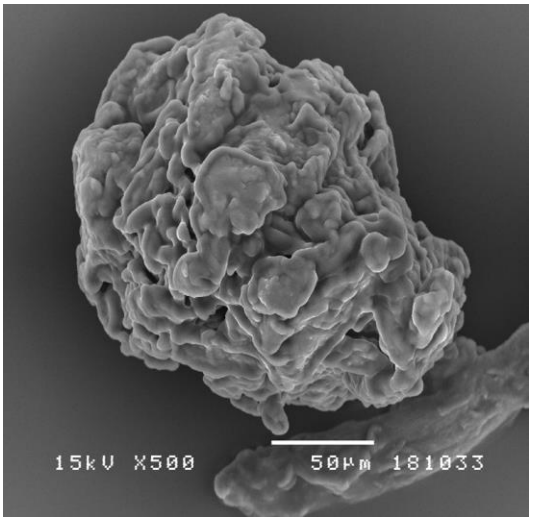


Figure 17.3 Dissolution profiles of **total phenolic compounds** from self-emulsifying granules **in water**

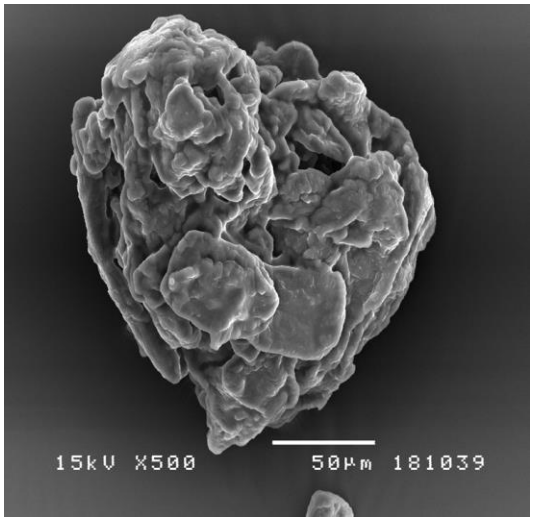
In preliminary study, selection of solid carrier was carried out using formulation of ET3PG2 self -emulsifying liquid containing *Passiflora foetida* extract. The adsorbent used were microcrystalline cellulose (Avicel PH 102), silicon dioxide (Aerosil 200) and veegum. The results showed that Aerosil 200 could adsorb the self-emulsifying liquid better than Avicel PH102 and veegum because the amount of Aerosil 200 required to adsorb the liquid and provided apparently dry mixture was less than Avicel PH102 and veegum. However, the constituents of the extracts may have some interaction with Aerosil 200, resulting in less percentage dissolved amounts of the extract constituents from the granules, as clearly seen in Figure 17.1-17.3. Therefore, Avicel PH 102 was chosen to be solid carrier and used in the 1:1 ratio to SE liquid.

6.2 Morphology of self-emulsifying granules

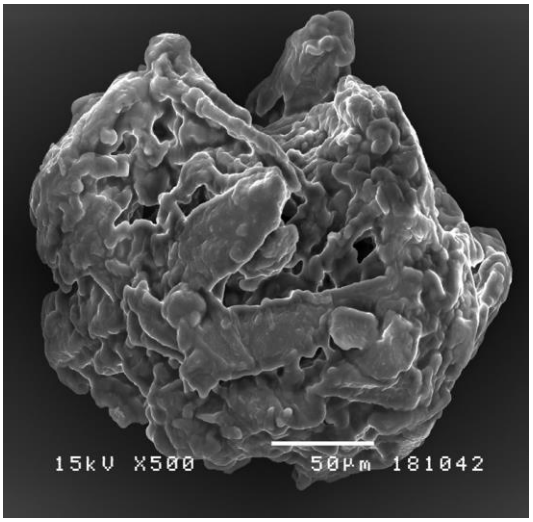
Morphology of self-emulsifying granules was viewed under scanning electron microscopy (SEM). They appeared as irregular shaped agglomerates (Figure 18).



(a)



(b)

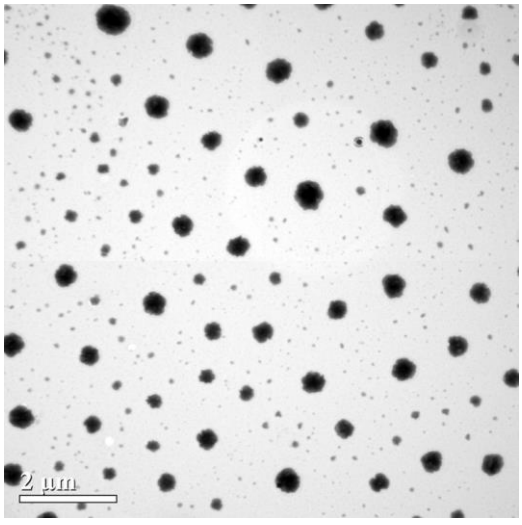


(c)

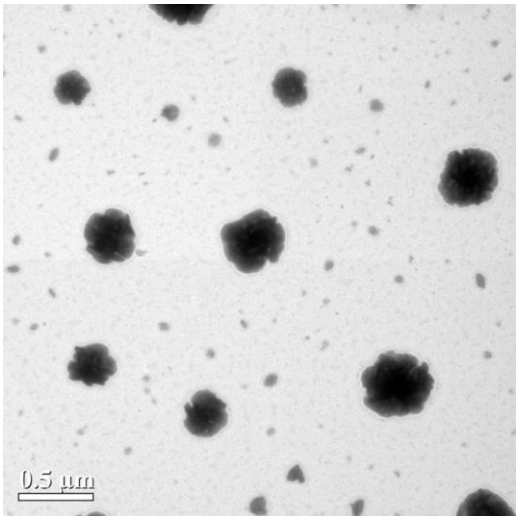
Figure 18 SEM photomicrographs of self-emulsifying granules of (a) C2T3PG2, (b) ET3PG2 and (c) ET3PG10; x 500

6.3 Transmission electron microscopy

Morphology of emulsion droplets formed by reconstitution of self-emulsifying granules viewed under transmission electron microscopy (TEM) was round and varied in size as shown in Figure 19



(a), 2000x



(b), 6000x

Figure 19 TEM photomicrographs of emulsion droplets formed by self-emulsification granules of ET3PG2 in water (200 fold dilution)

Table 9 Emulsion droplet size (Z-Average), size distribution (polydispersity index, PdI) and zeta potential after reconstitution of self-emulsifying granules with 200 fold of deionized water at 37 °C, n = 3

System	Emulsion droplet size (nm), (SD)	PdI	Zeta potential (mV), (SD)
C2T3PG2	81.77 (14.81)	0.241	-10.57 (3.44)
ET3PG2	947.37 (73.86)	0.854	-12.90 (1.18)
ET3PG10	87.65 (8.58)	0.209	-11.15 (2.42)

An increase in droplet size and zeta-potential of the emulsion obtained by reconstitution of self-emulsifying granules was observed. A significant increase in droplet size of reconstituted emulsion and wide size distribution (PdI = 0.854) was observed for the ET3PG2 granules. The reason was unclear. This formulation was not suitable to be designed as self-emulsifying solid.

6.4 Assay

The *Passiflora foetida* extract of 100 mg contained vitexin of 14.1 mg, total flavonoids of 10.6 mg and phenolic compounds of 9.8 mg. The assayed amounts of vitexin, total flavonoids and total phenolic compounds in self-emulsifying liquids and self-emulsifying granules are shown in Table 10-11.

Table 10 Amounts of vitexin, total flavonoids and phenolic compounds in 1 g self-emulsifying (SE) liquids equivalent to 90.9 mg extract

System	vitexin (mg)	Total flavonoids (mg)	Total phenolic compounds (mg)
C2T3PG2	13.0	8.9	6.7
ET3PG2	12.0	8.7	6.9
ET3PG10	14.3	8.8	7.1

Table 11 Amounts of vitexin, total flavonoids and phenolic compounds in 2 g of self-emulsifying (SE) granules equivalent to 90.9 mg extract

System	vitexin (mg)	Total flavonoids (mg)	Total phenolic compounds (mg)
C2T3PG2 granules	12.2	5.1	6.1
ET3PG2 granules	11.3	5.2	6.0
ET3PG10 granules	13.3	5.8	5.6

6.5 *In vitro* dissolution study

The in vitro dissolution study of vitexin, total flavonoids and total phenolic compounds was carried out using USP dissolution apparatus II in 500 ml of distilled water, 0.1 N HCl, pH 6.8 phosphate buffer. Overall results showed that the extract, in particular vitexin, could be dissolved from the SE liquids > extract > SE granules > extract granules. After 180 min, the dissolved vitexin from liquid formulation was ordered as ET3PG10 (60.4%) ~ ET3PG2 (59.9%) ~ C2T3PG2 (58.8%), followed by the extract (53.0%), the solid formulations of ET3PG10 (54.3%) > C2T3PG2 (51.2%) > ET3PG2 (50.4%) and the extract granules (42.6%) (Figure 21). The results indicated that SE liquids could improve dissolution of vitexin. Adsorption of the extract or SE liquids on the solid carrier led to decreased extent of dissolution. Wang et al. (2010) also reported that the release extent of nitrendipine from SE pellets was slightly lower than SE liquids. They explained that there might be strong interaction between the solid carrier and adsorbed SES.

The dissolution of total flavonoids and phenolic compounds in water had similar pattern to that of vitexin but cumulative dissolved amounts were higher and the extent of difference between the formulations was small. Overall results the SE liquid and solid formulation which gave greater dissolved vitexin and flavonoids was ET3PG10. It was noted that the dissolution test could not achieve 100 % release. This might be due to that

the extract constituents were partially adsorbed onto the filter during sample preparation for analysis.

The medium pH should not affect emulsion formed by non-ionic surfactant. However, the dissolution of vitexin, total flavonoids and phenolic compounds was influenced by medium pH. The dissolution in pH 6.8 phosphate buffer was less than that in water and 0.1 N HCl, in particular when the phenolic compounds were monitored. This may be explained that the compounds were likely to be unstable in alkaline. Vitexin was found to degrade under forced exposure to alkaline solution. It was also reported that quercetin was more stable in weak acid than in weak base (Hu et al., 2012).

6.5 Stability study

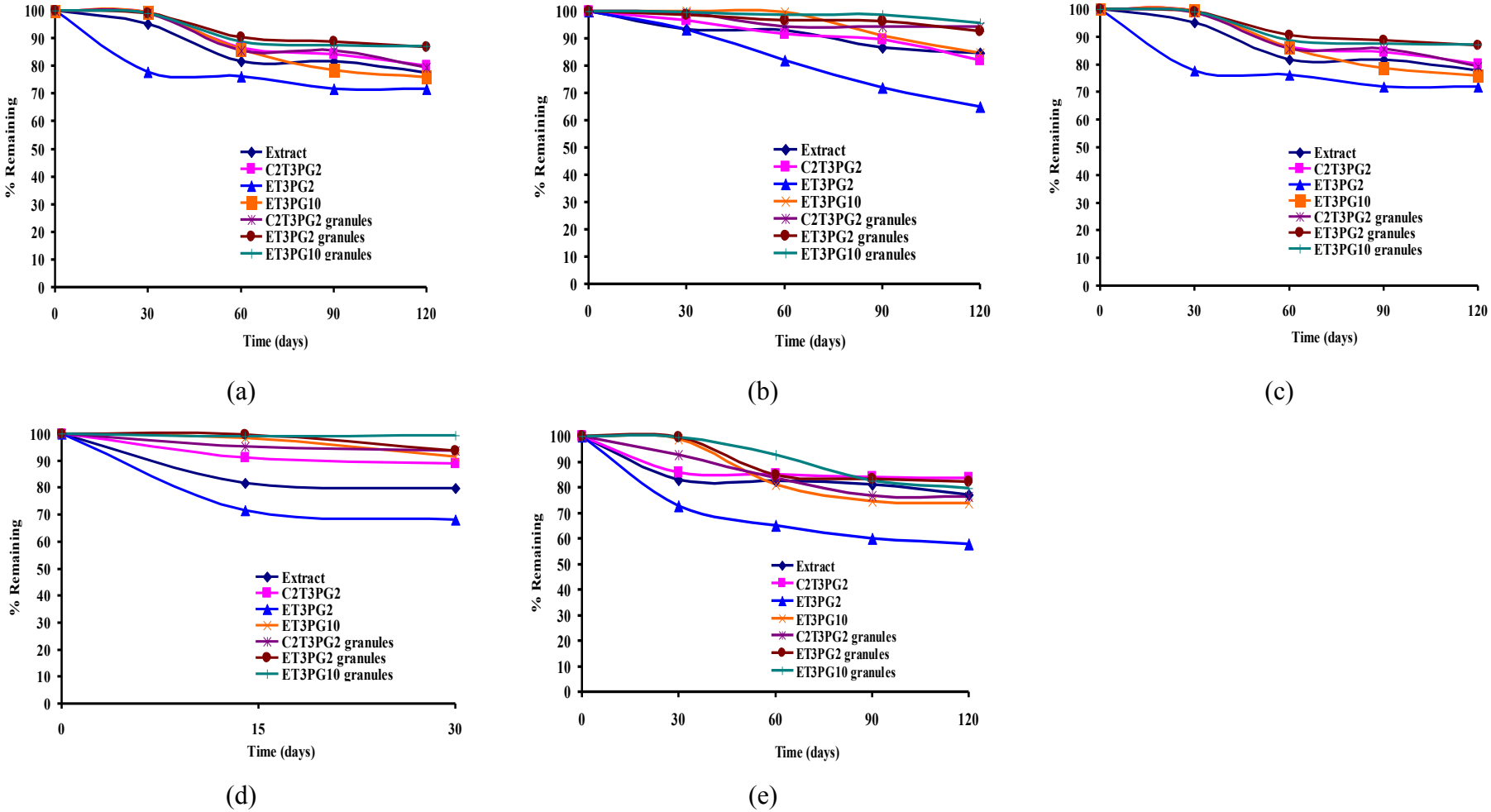


Figure 20.1 Percentage remaining of **vitexin** in the extract, self-emulsifying liquids and granules after storage for 4 months: (a) in refrigerator; (b) 25 °C; (c) 30 °C, 75%RH; (d) 30 °C/ UV light, 75 %RH; (e) 40 °C, 75 %RH

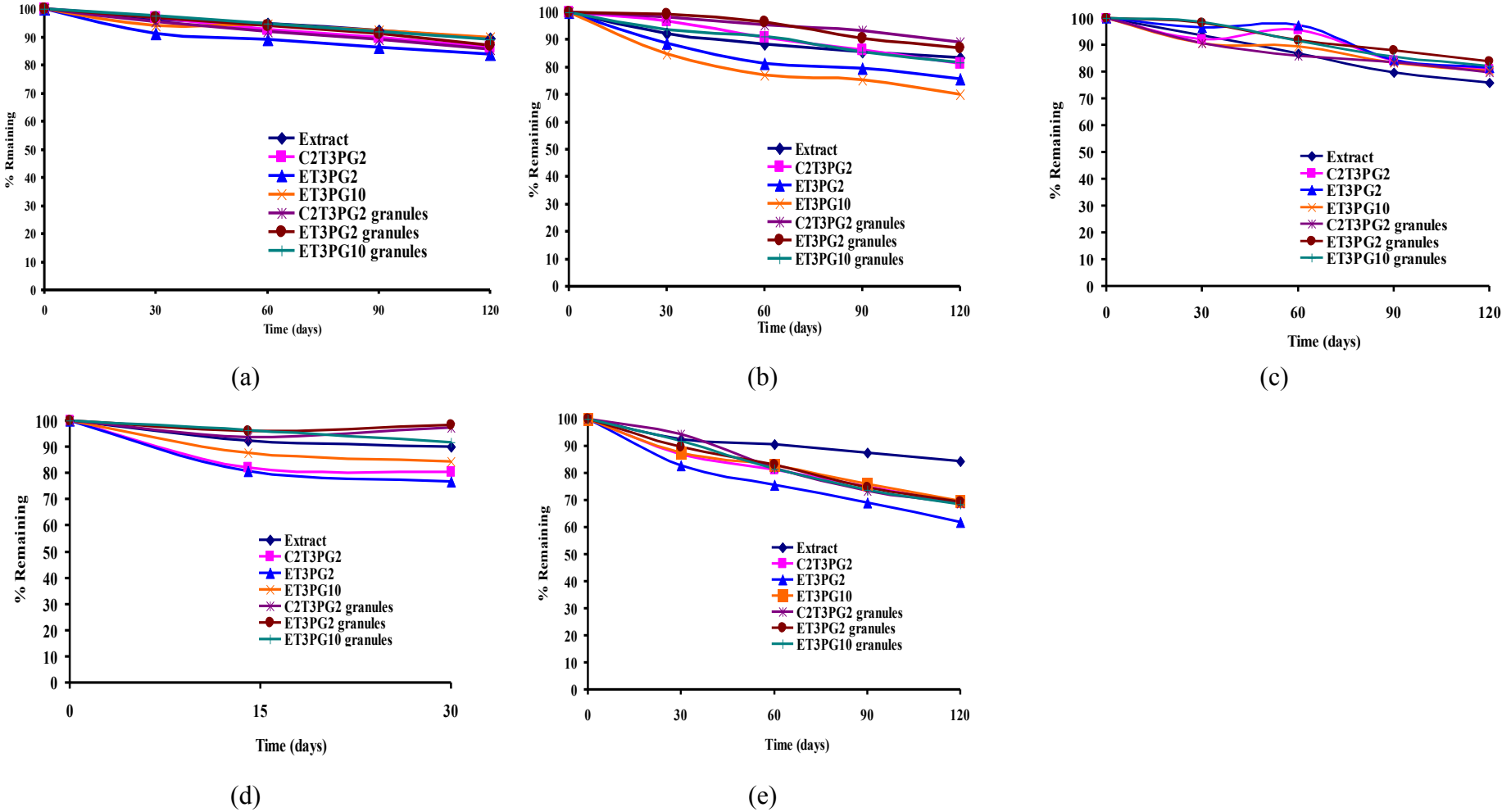


Figure 20.2 Percentage remaining of **total flavonoids** in the extract, self-emulsifying liquids and granules after storage for 4 months: (a) in refrigerator; (b) 25 °C; (c) 30 °C, 75%RH; (d) 30 °C/UV light, 75 %RH; (e) 40 °C, 75%RH

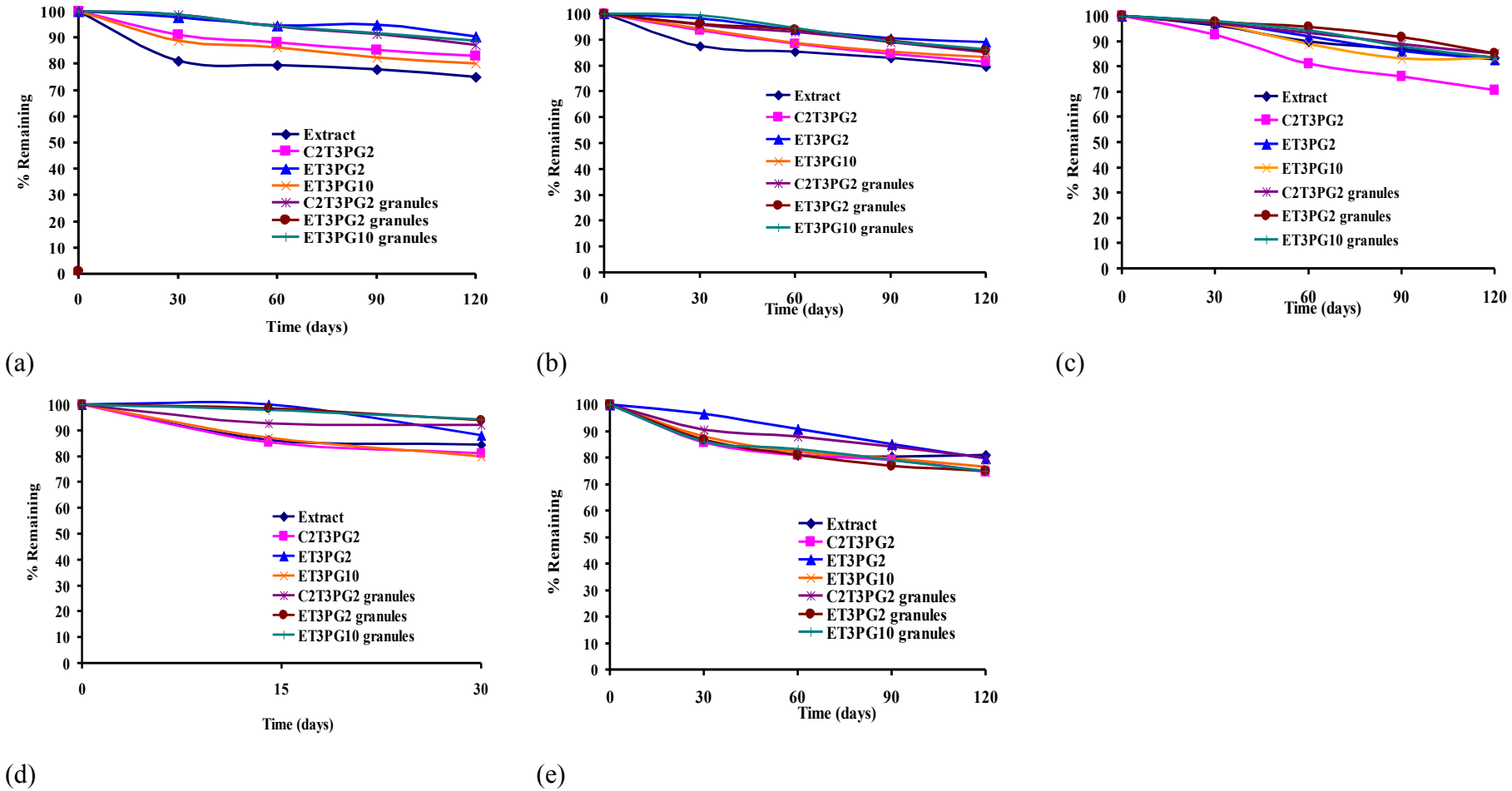


Figure 20.3 Percentage remaining of **total phenolic compounds** in extract, self emulsifying liquids and granules after storage for 4 months: (a) in refrigerator; (b) 25 °C; (c) 30 °C, 75%RH; (d) 30 °C/UV light, 75%RH; (e) 40 °C, 75%RH

When vitexin was monitored, the most stable formulation was ET3PG10 granules, while the unstable formulation was shown in ET3PG2 liquid (Figure 17). The ET3PG2 was also less stable than the extract. The component in SES liquid, probably, induced degradation of vitexin. However, adsorption onto the solid carrier clearly improved the stability of vitexin in SE liquids and the extract. The remaining amount of vitexin in ET3PG2 granules was 82.2%, while in the extract was 77 % and in ET3PG2 liquid was 57.7% after storage at 40 °C/75% RH for 4 months.

The storage temperature was also likely to affect the stability of vitexin. Vitexin in ETPG10 granules remained up to 95% after storage in refrigerator and at 25 °C, but was significantly reduced at 40 °C to approximately 80%.

When the stability of total flavonoids was investigated, the results showed that the total flavonoids remaining in the extract, SE liquid and solid formulations stored in refrigerator for 4 months were similar and more than 84%. The higher temperature induced instability of flavonoids in all formulations studied but less effect on flavonoids in the extract (Figure 20). The remaining flavonoids in the extract was more than in all SE formulations after storage at 40 °C/75% RH for 4 months. This supported the earlier discussion that the SE formulation could induce the degradation of the extract constituents.

Stability of phenolic compounds in the extract stored in refrigerator and 25 °C was less than that in the formulations. This, however, was not shown at higher storage temperature. In contrast to storage at the low temperature, the phenolic compounds in the extract was slightly more stable than in the SE formulations at 40 °C/75% RH for 4 months, which agreed with the flavonoids' results.

After storage for 4 months, the dissolution of vitexin in water remained similar pattern (i.e. SE liquid > extract > SE granules > extract granules). However, the dissolved vitexin was reduced when the formulations were stored at higher temperature than refrigerated. This might be attributed to less vitexin available for dissolution after storage at higher temperature.

CHAPTER IV
CONCLUSION

Passiflora foetida ethanolic extract which was found to be slightly soluble in water might have poor oral bioavailability due to limited dissolution in the GI tract. In this study, an attempt was made to improve *in vitro* dissolution of *Passiflora foetida* extract by formulation as self-emulsifying dosage forms. As the major constituents of *Passiflora foetida* leaves are C-glycosyl flavonoids, vitexin, flavonoids and phenolic compounds was determined as the markers for dissolution and stability behaviors of the extract. The results from this study could be concluded as the followings:

Loading capacity of *Passiflora foetida* in self-emulsifying liquids was 1:20 to 1:4, indicating soluble to freely soluble. Addition of co-solvent or co-surfactant could enhance the loading capacity.

Dissolution of the extract constituents determined as vitexin, flavonoids or phenolic compounds was predominantly improved by self-emulsifying liquid formulations, but the extent was decreased when the self-emulsifying liquid was adsorbed onto microcrystalline cellulose.

Liquid and solid self-emulsifying formulations were unstable at relatively high storage temperatures, 30 and 40 °C. However, the self-emulsifying granules were proved to be more stable than the liquid formulations.

Overall data suggested that the formulation composed of 25% ethyl oleate, 56.3% Tween 80, 18.8% propylene glycol (ET3PG10) was a potential self-emulsifying system for improve dissolution of *Passiflora foetida* ethanolic extract. Applying solid carrier into the self-emulsifying system must be well justified. Although solid formulation may improve stability, it may give an adverse effect to dissolution. Furthermore, the dosage forms of *Passiflora foetida* extract is recommended to be stored at relatively low temperature.

In future work, stability of the self-emulsifying formulation should be further investigated. The study of bioavalability and toxicity of *Passiflora foetida* extract self-emulsifying systems should also be carried out.

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APPENDIX A

I. Validation data of stability-indicating analytical method for vitexin

Table A-1 Accuracy (percentage recovery of vitexin) of the HPLC method, n =3

No.	Con (mg/ml)	Peak area	% Recovery	Mean	%RSD
1	0.05	1493061	97.9	98.3	0.4
		1502427	98.5		
		1502227	98.5		
2	0.5	18725178	99.3	99.2	0.6
		18597108	98.6		
		18804802	99.7		
3	1.0	33183737	91.4	89.4	1.9
		31996770	88.1		
		32266195	88.8		

Table A-2 Precision (repeatability and intermediate precision) of the HPLC method obtained by using 0.5mg/ml vitexin standard solution (n=6)

No.	Day 1	Day 2	Day 3
	Peak area	Peak area	Peak area
1	18617468	19343829	21474079
2	18609307	19339662	21483880
3	18611194	19352457	21553547
4	18674115	19209827	21732301
5	18621146	18876831	21365769
6	18619125	19320843	21588047
Average	18612656	19345316	21503835
SD	24308	185847	124116
%RSD	0.13	0.96	0.58

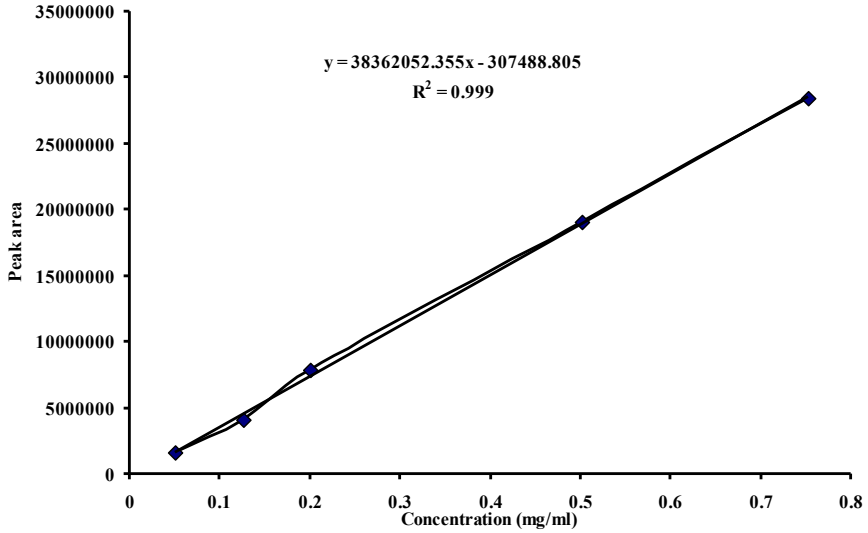
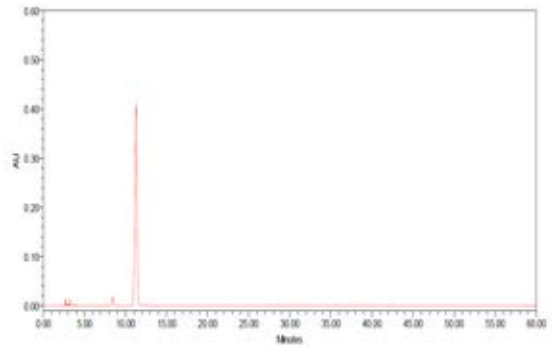
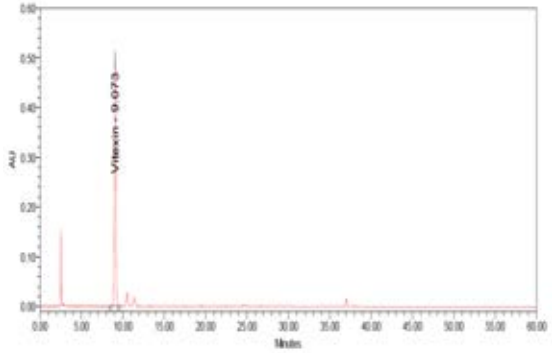


Figure A-1 Calibration curve of vitexin

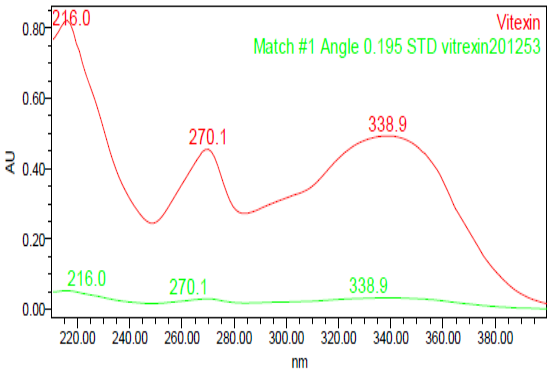


(a) HPLC chromatogram of vitexin after exposure to 1N HCl for 7 days

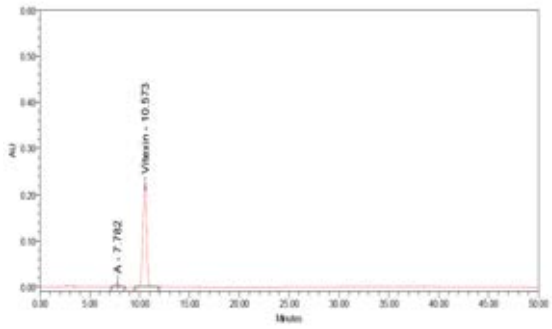
(b) UV spectrum of vitexin after exposure to 1N HCl for 7 days



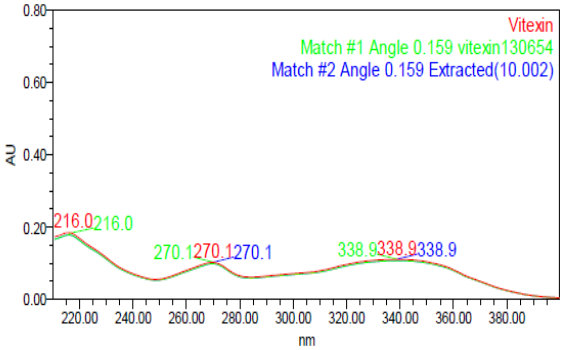
(c) HPLC chromatogram of vitexin after exposure to 1N NaOH for 5 h



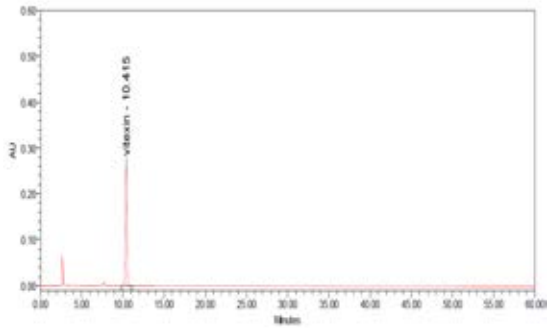
(d) UV spectrum of vitexin after exposure to 1N NaOH for 5 h



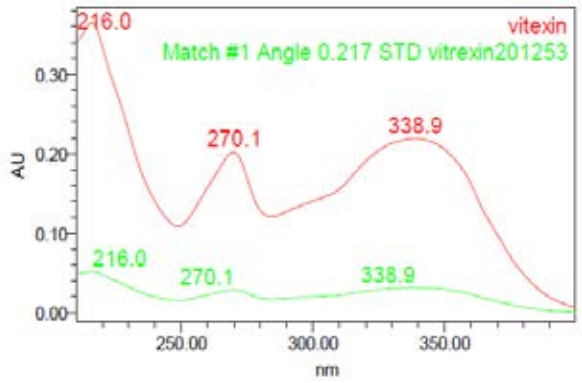
(e) HPLC chromatogram of vitexin after exposure to water for 7 days



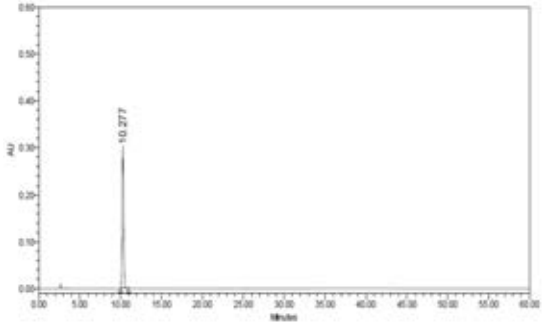
(f) UV spectrum of vitexin after exposure to water for 7 days



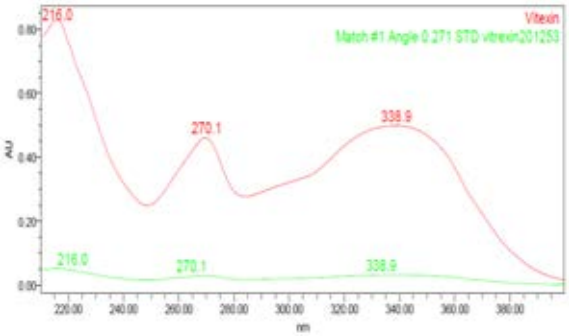
(g) HPLC chromatogram of vitexin after exposure to 30% H_2O_2 for 5 h



(h) UV spectrum of vitexin after exposure to 30% H_2O_2 for 5 h



(i) HPLC chromatogram of vitexin after exposure to UV light for 7 days



(j) UV spectrum of vitexin after exposure to UV light for 7 days

Figure A-2 HPLC Chromatograms and UV absorbance of vitexin after exposure to: (a-b) 1 N HCl for 7 days, (c-d) 1 N NaOH for 5 h, (e-f) water for 7 days, (g-h) 30 % H_2O_2 for 5 h, and (i-j) UV light for 7 days

II. Calibration curves of quercetin and gallic acid

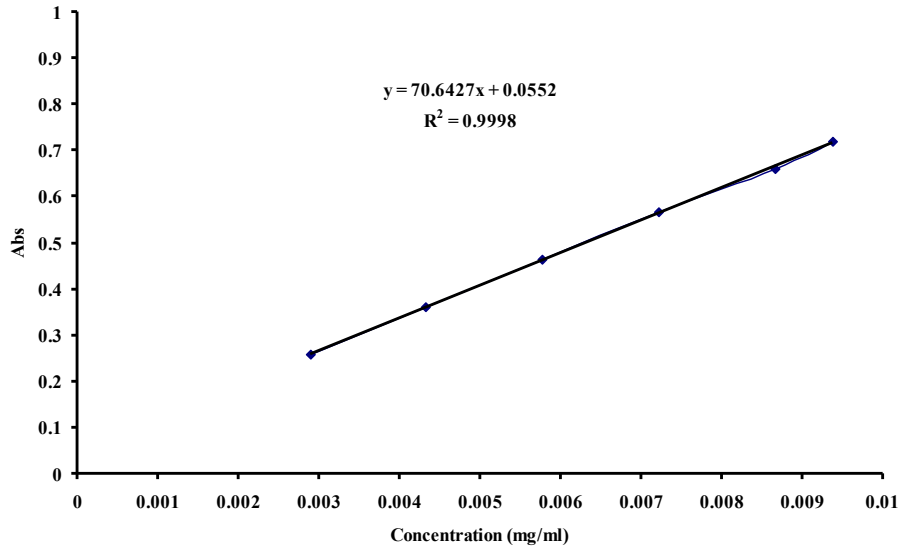


Figure A-3 Calibration curve of quercertin for determination of total flavonoids

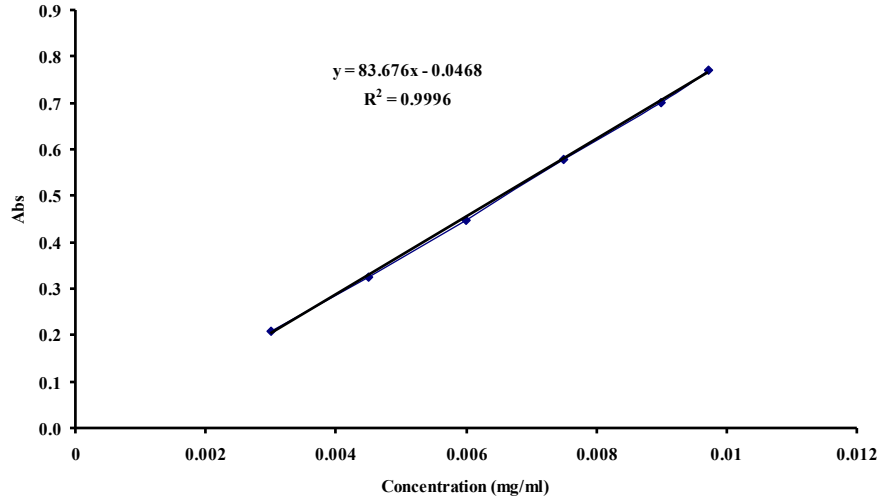
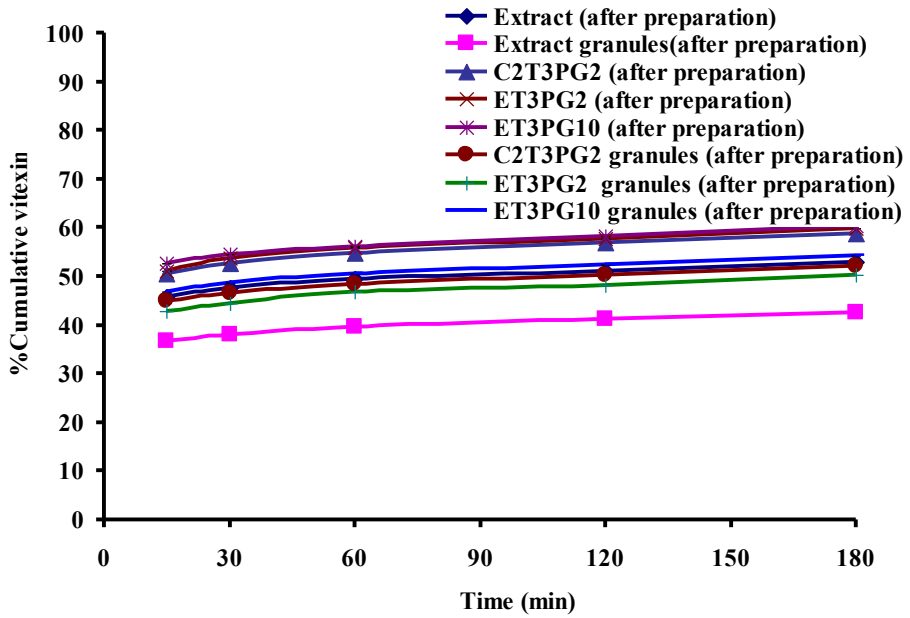
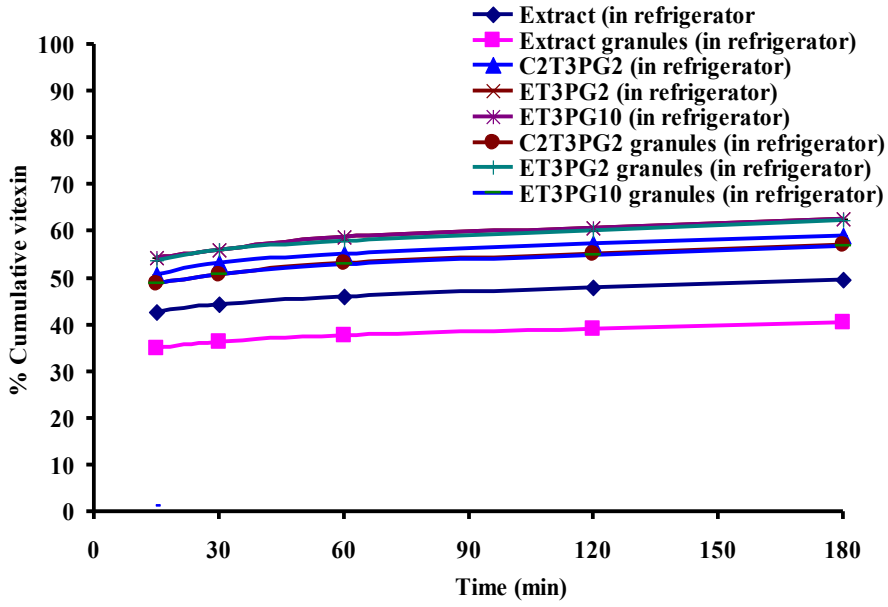


Figure A-4 Calibration curve of gallic acid for determination of total phenolic compounds

APPENDIX B

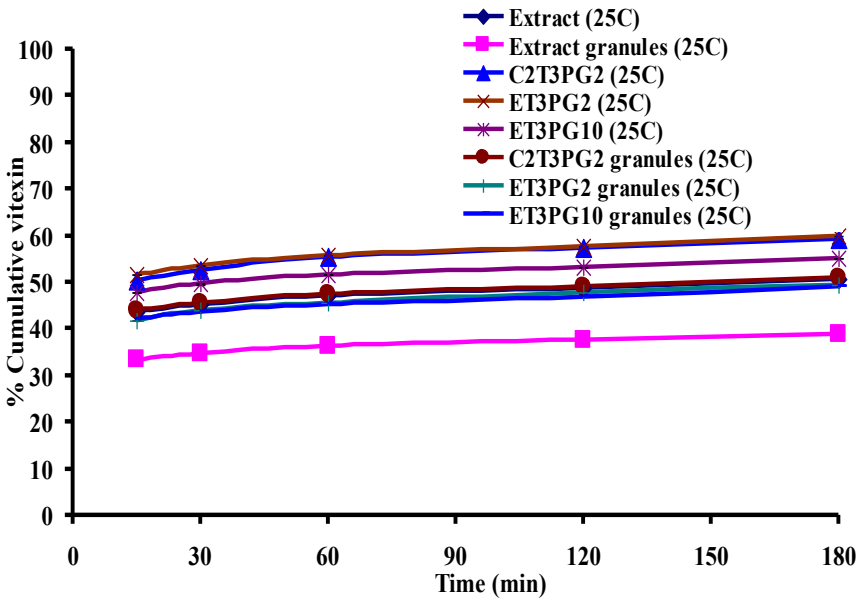


(a)

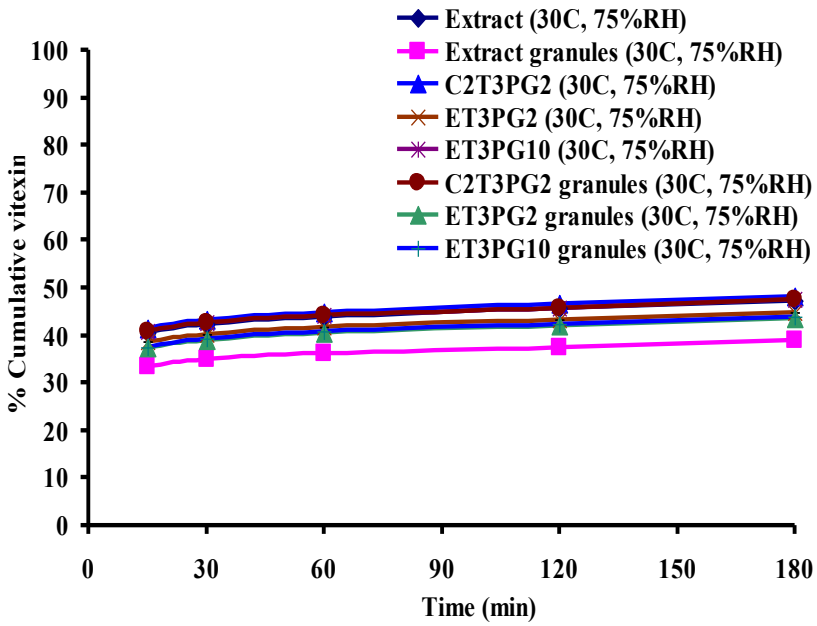


(b)

Figure B-1 Dissolution profiles of **vitexin** from the extract, the extract granules, self - emulsifying liquids and granules **in water**: (a) after preparation; (b) after storage in refrigerator for 4 months

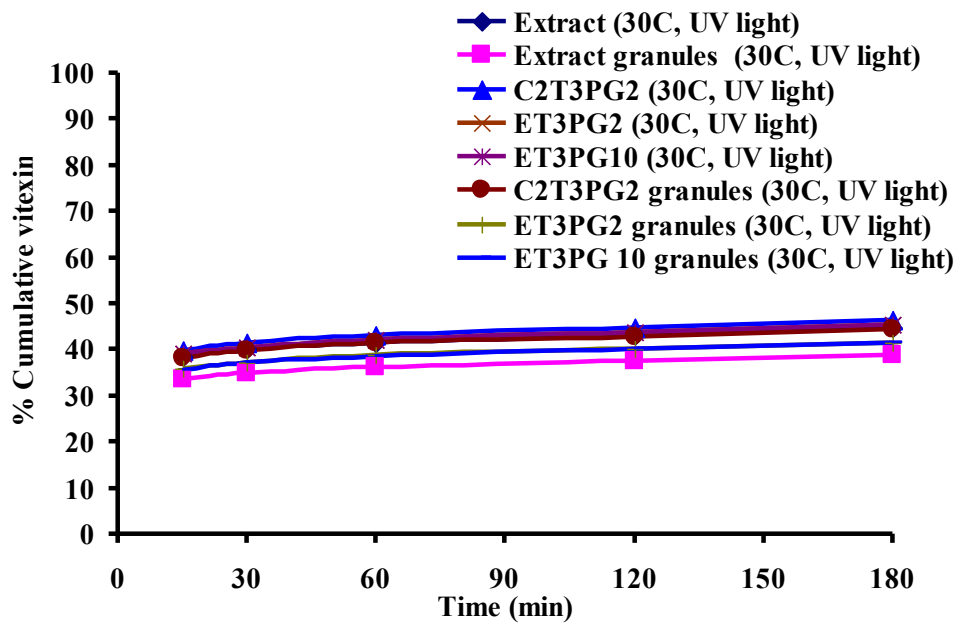


(c)

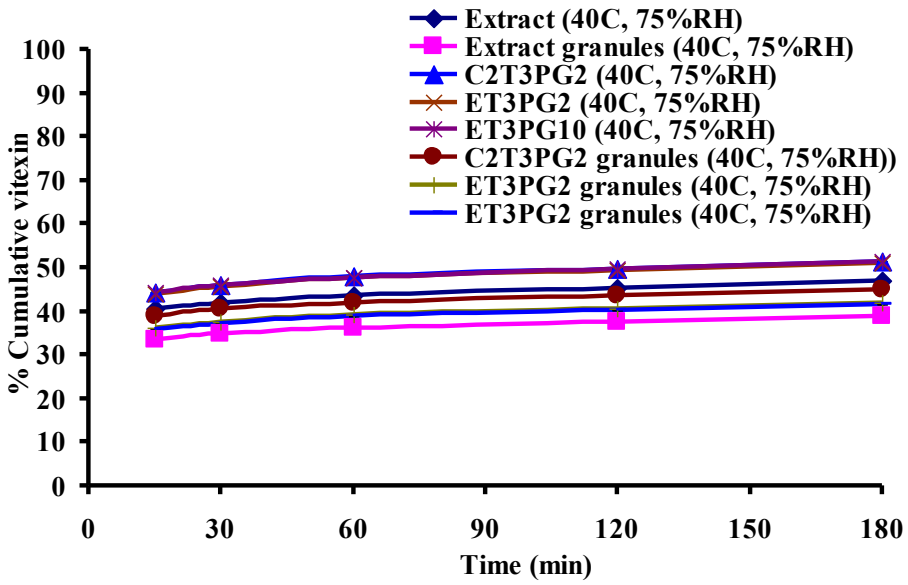


(d)

Figure B-1 (continue) Dissolution profiles of **vitexin** from the extract, the extract granules, self-emulsifying liquids and granules **in water** after storage at: (c) 25 °C; (d) 30 °C/75 %RH, for 4 months

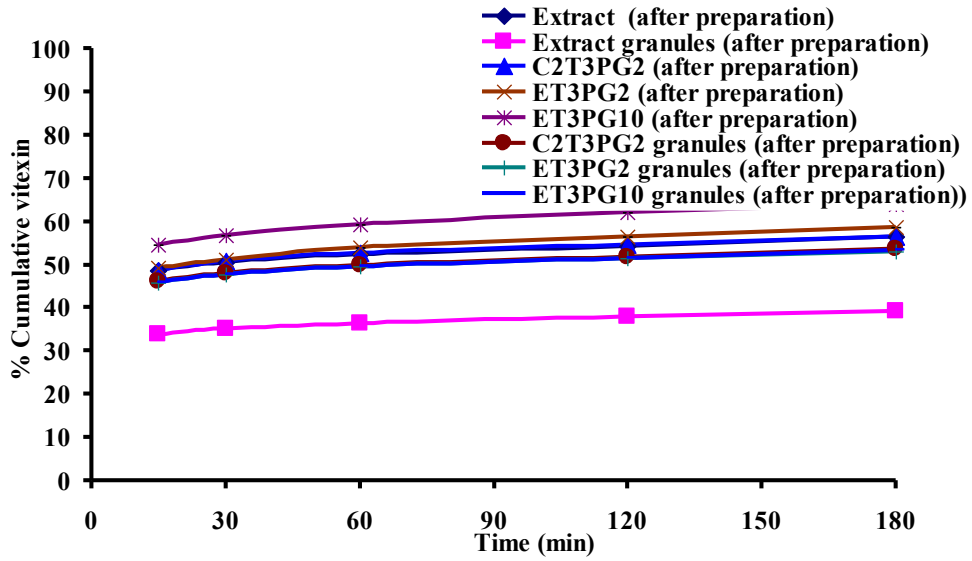


(e)

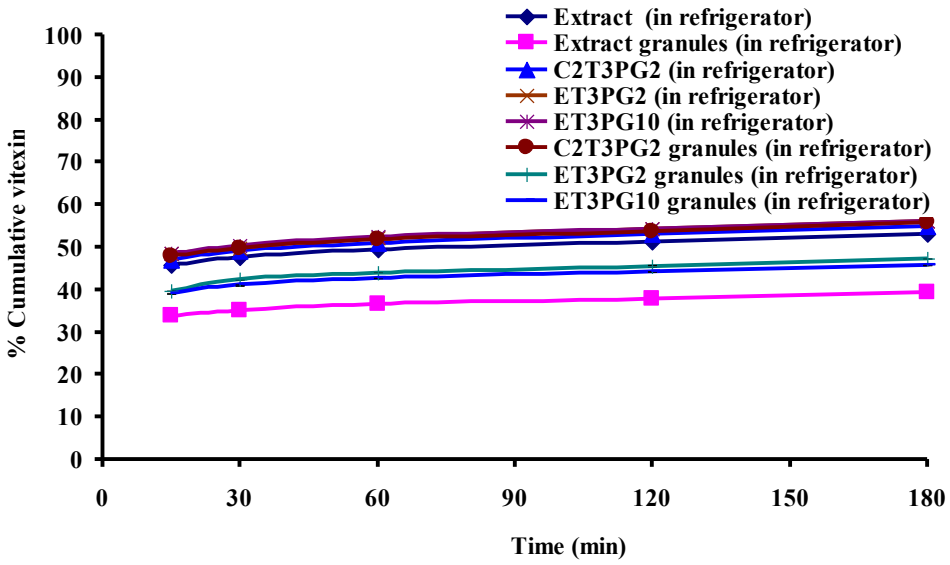


(f)

Figure B-1 (continue) Dissolution profiles of **vitexin** from the extract, the extract granules, self-emulsifying liquids and granules **in water** after storage at: (e) 30 °C/ UV light 75 %RH; (f) 40 °C/75 %RH, for 4 months

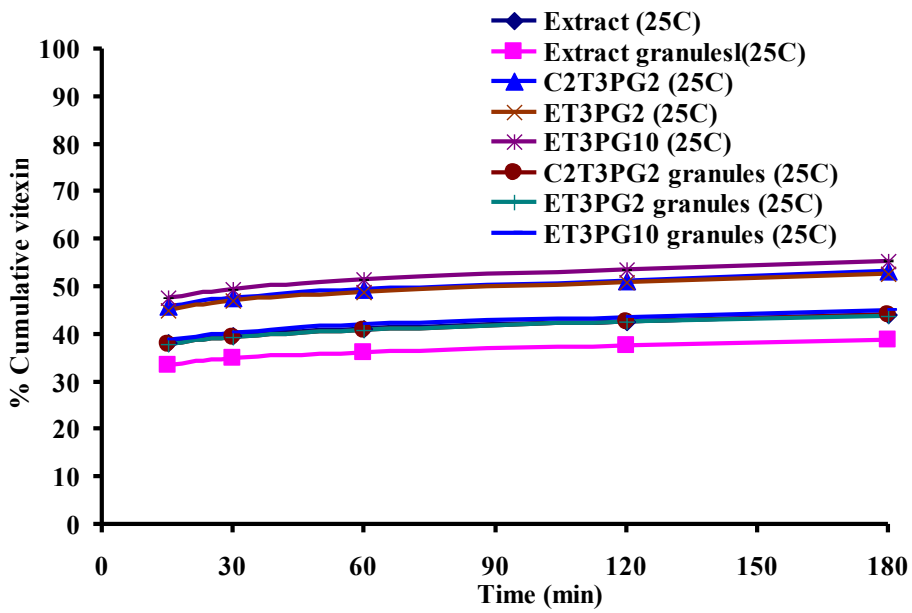


(a)



(b)

Figure B-2 Dissolution profiles of **vitexin** from the extract, the extract granules, self-emulsifying liquids and granules **in 0.1N HCl**: (a) after preparation; (b) after storage in refrigerator for 4 months



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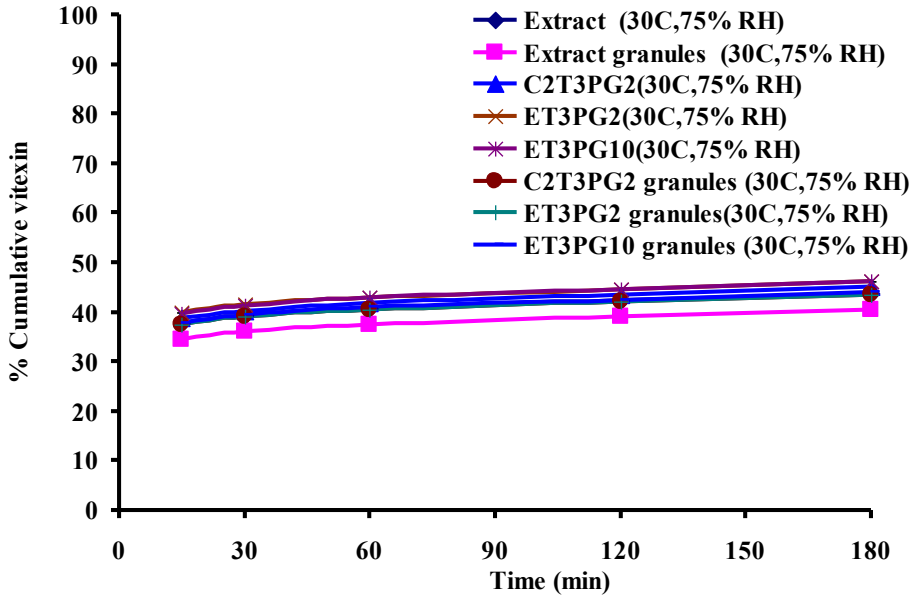
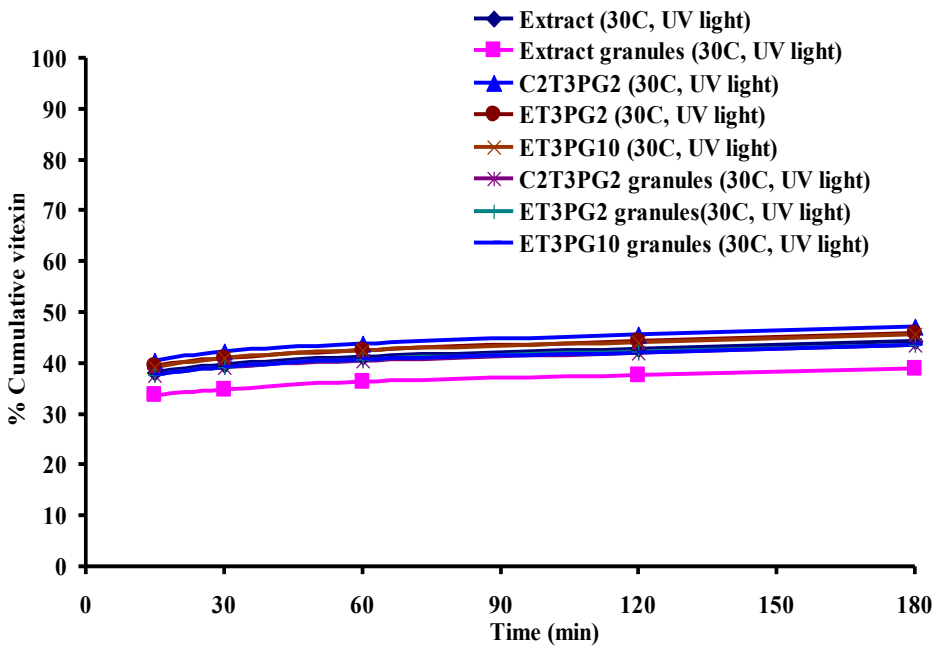


(d)

Figure B-2 (continue) Dissolution profiles of **vitexin** from the extract, the extract granules, self-emulsifying liquids and granules **in 0.1N HCl** after storage at: (c) 25 °C; (d) 30 °C/ 75 %RH for 4 months



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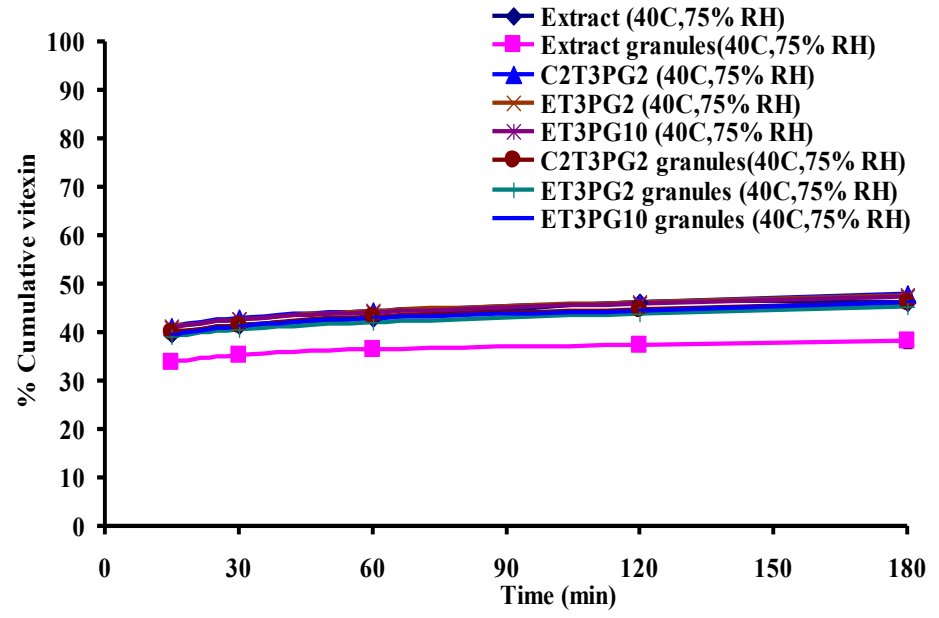
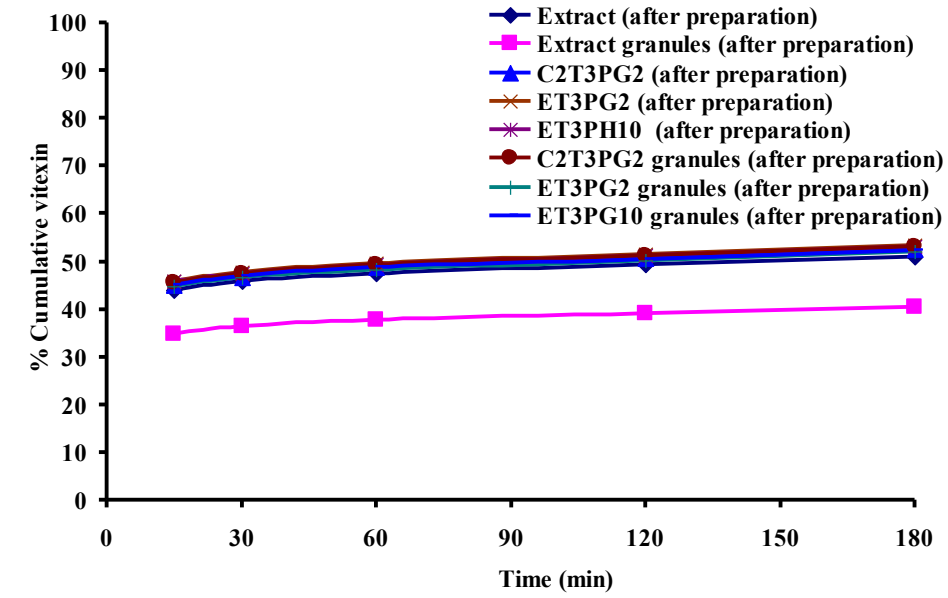
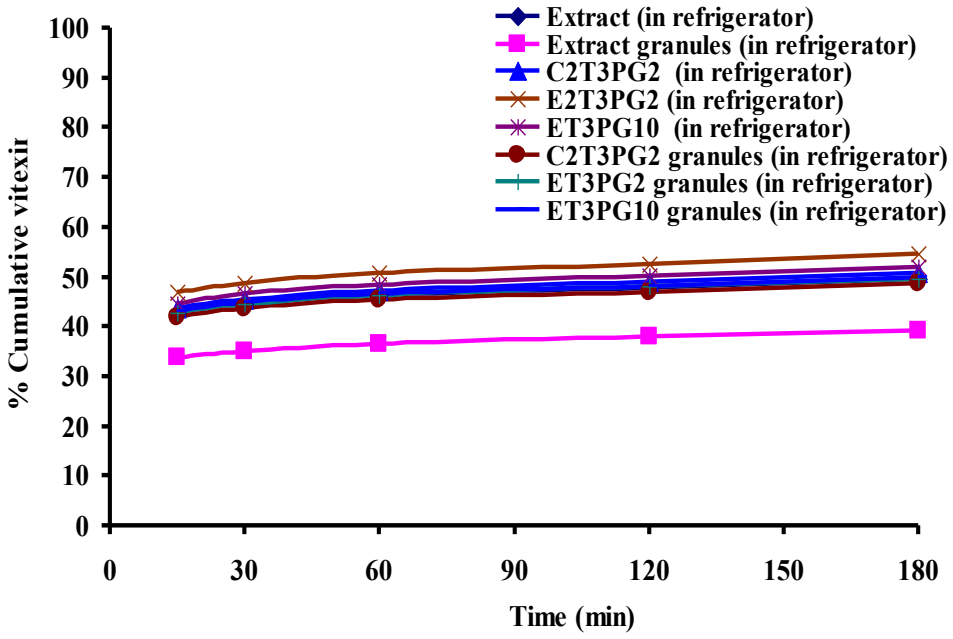


(f)

Figure B-2 (continue) Dissolution profiles of **vitexin** from the extract, the extract granules, self-emulsifying liquids and granules **in 0.1N HCl** after storage at: (e) 30 °C/UV light 75 %RH; (f) 40 °C/75 %RH, for 4 months.

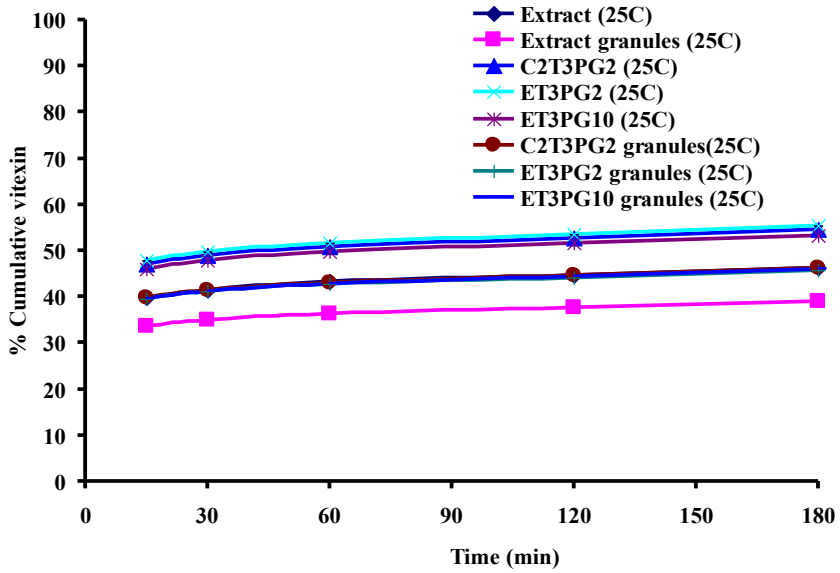


(a)



(b)

Figure B-3 Dissolution profiles of **vitexin** from the extract, the extract granules, self-emulsifying liquids and granules **in pH 6.8 phosphate buffer**: (a) after preparation; (b) after storage in refrigerator for 4 months



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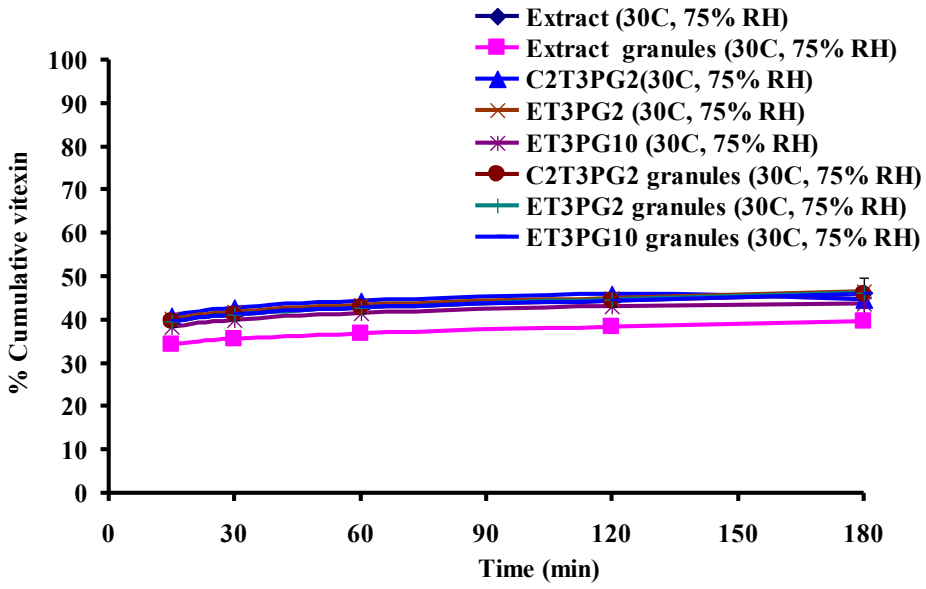
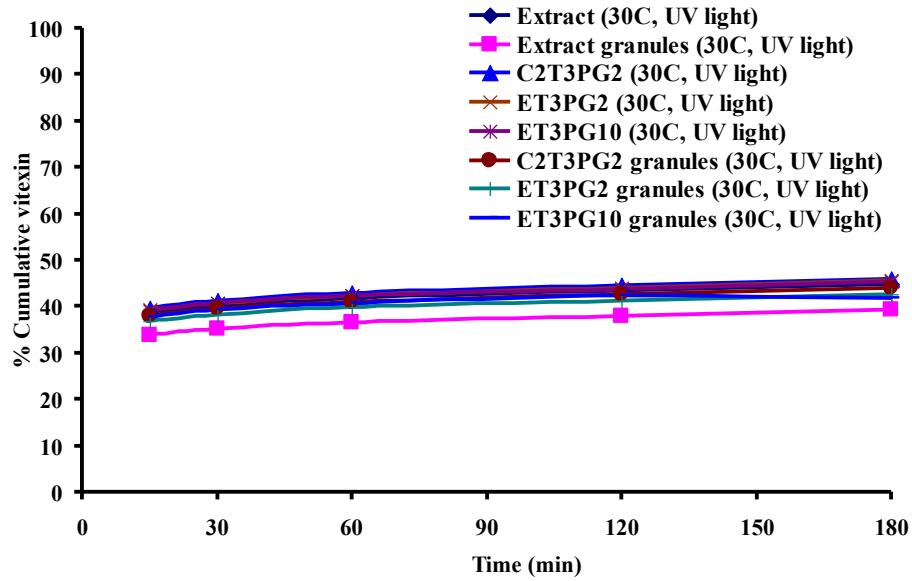
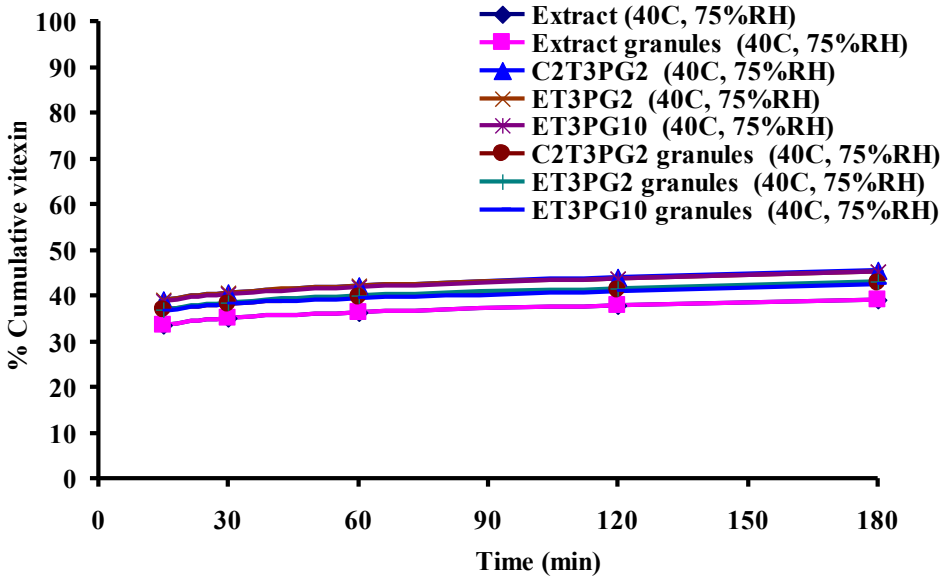


(d)

Figure B-3 (continue) Dissolution profiles of **vitexin** from the extract, the extract granules, self-emulsifying liquids and granules **in pH 6.8 phosphate buffer** after storage at: (c) 25 °C; (d) 30 °C/75 %RH, for 4 months

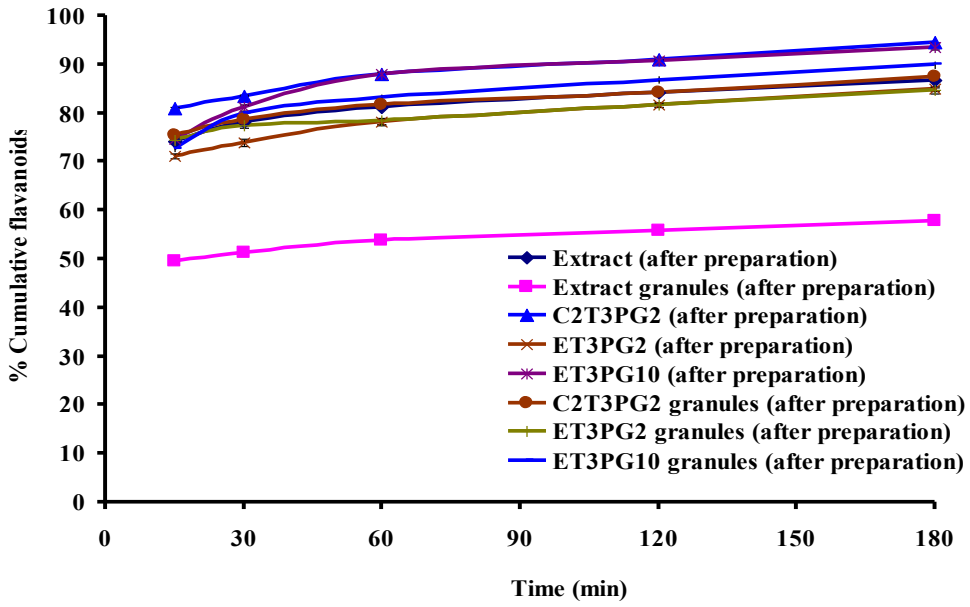


(e)

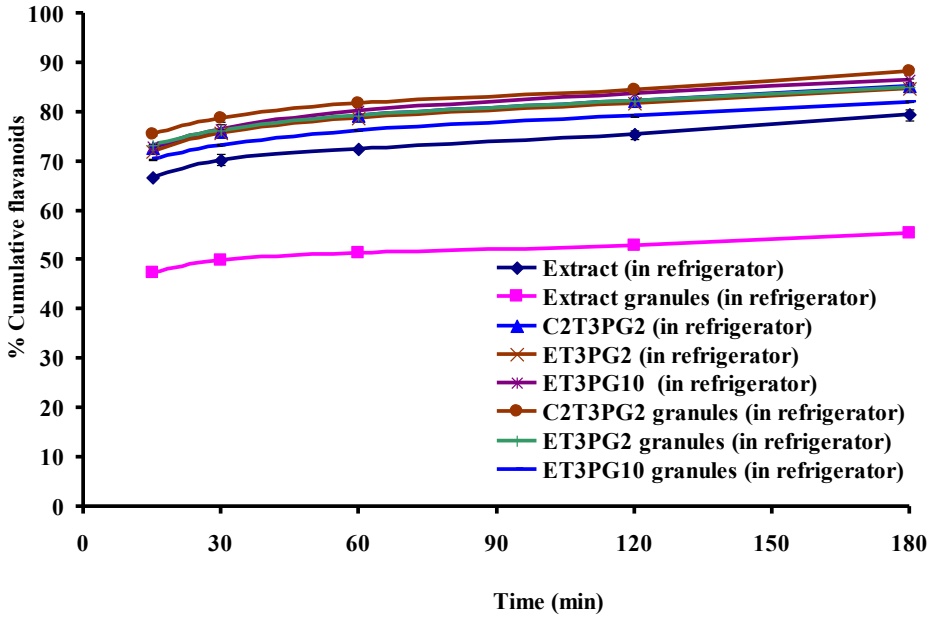


(f)

Figure B-3 (continue) Dissolution profiles of **vitexin** from the extract, the extract granules, self- emulsifying liquids and granules **in 6.8 phosphate buffer** after storage at: (e) 30°C/ UV light 75 %RH ; (f) 40 °C/75 %RH, for 4 months

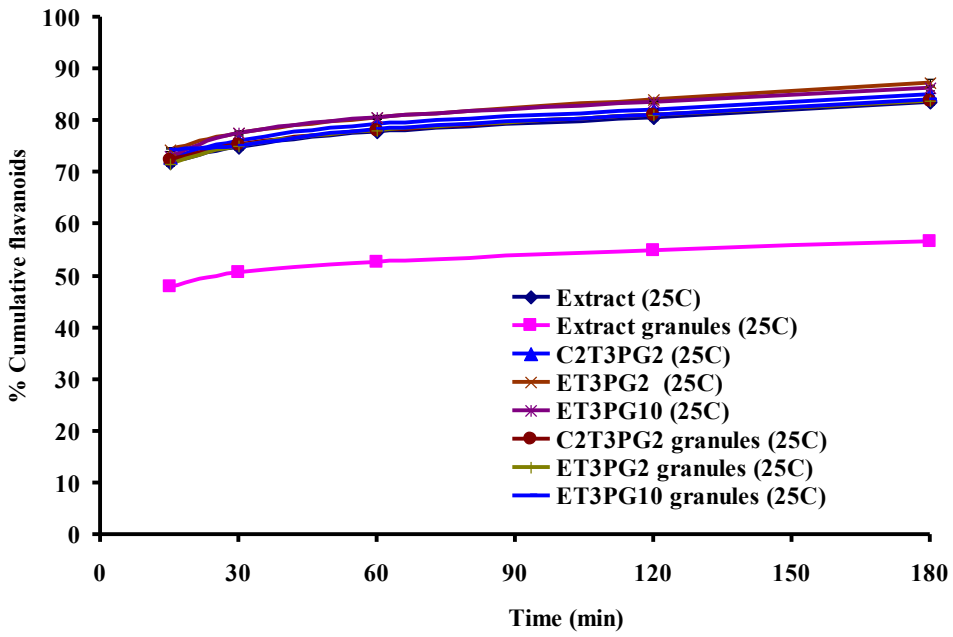


(a)

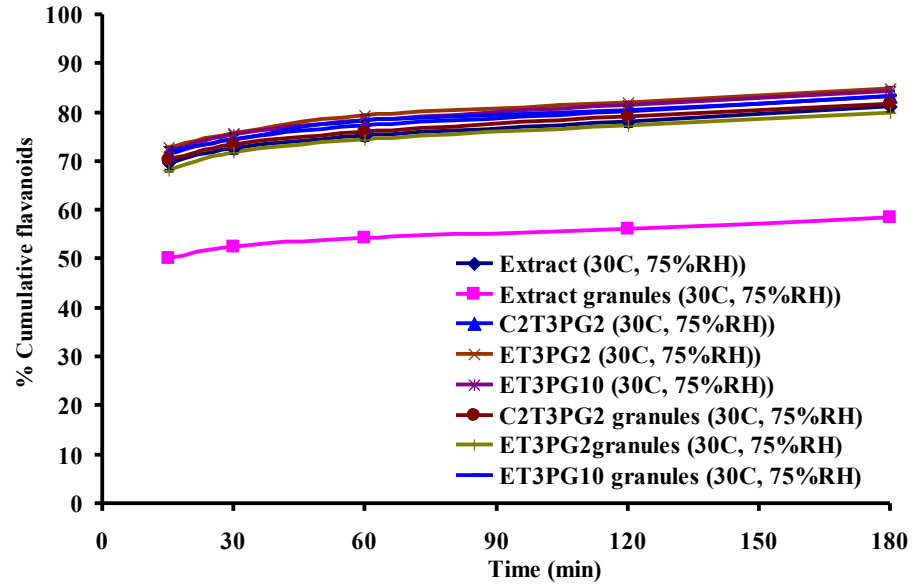


(b)

Figure B-4 Dissolution profiles of **total flavonoids** from the extract, the extract granules, self-emulsifying liquids and granules **in water**: (a) after preparation; (b) after storage in refrigerator for 4 months

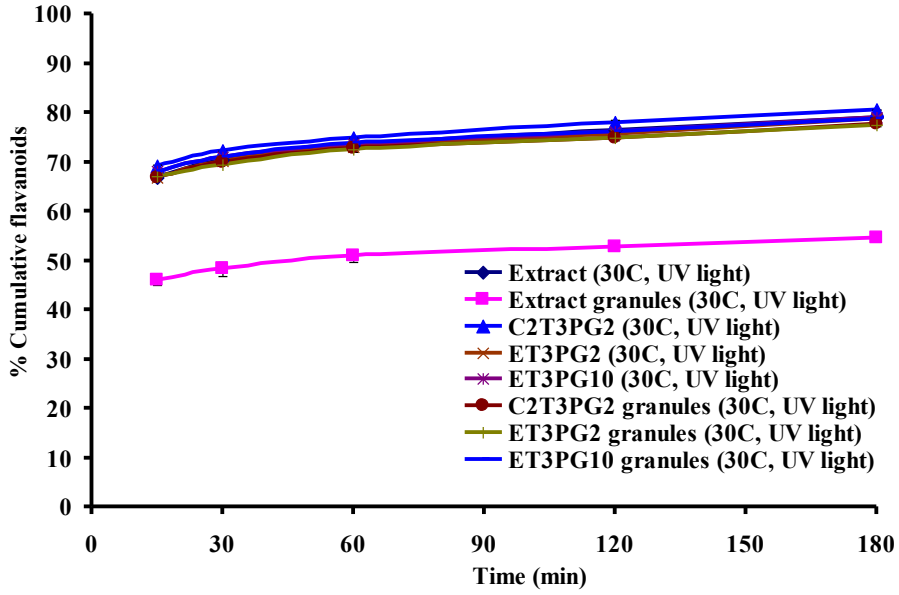


(c)



(d)

Figure B-4 (continue) Dissolution profiles of **total flavonoids** from the extract, the extract granules, self-emulsifying liquids and granules **in water** after storage at: (c) 25°C; (d) 30°C/75 %RH, for 4 months



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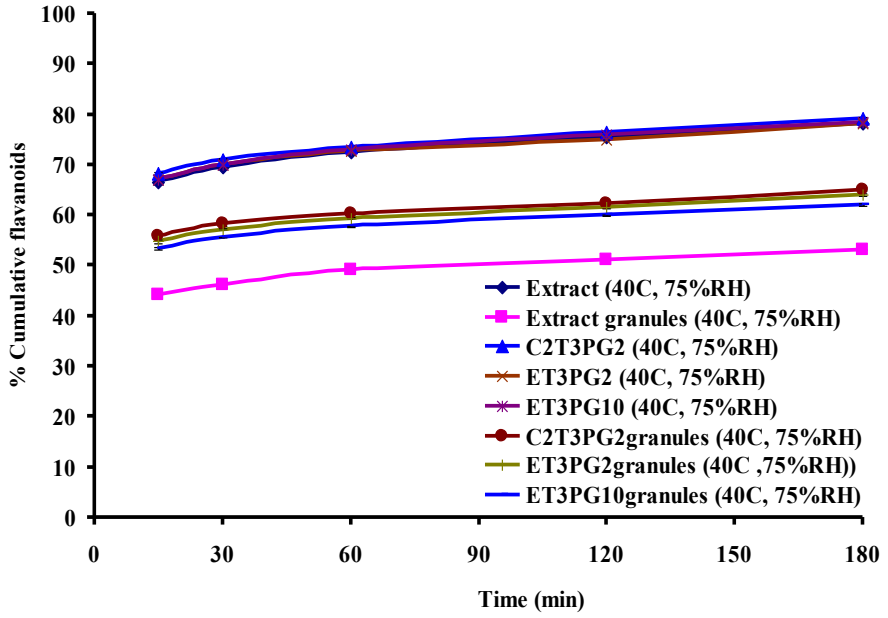
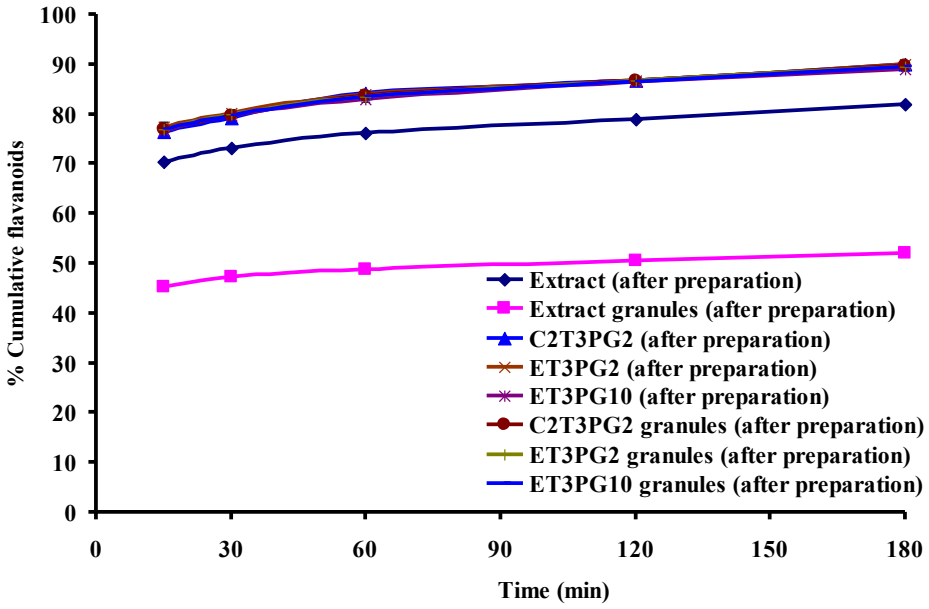
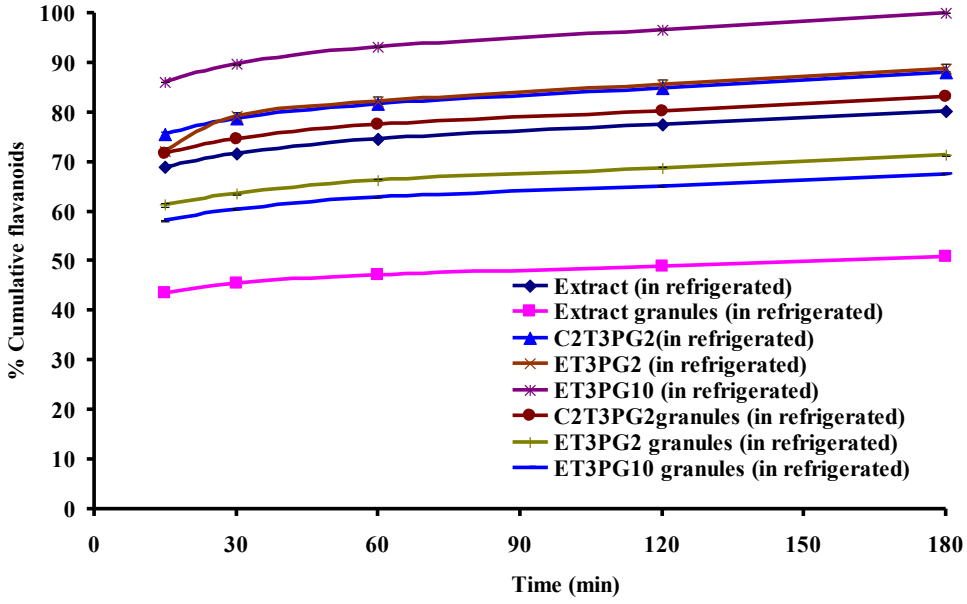


(f)

Figure B-4 (continue) Dissolution profiles of **total flavonoids** from the extract, the extract granules, self-emulsifying liquids and granules **in water** after storage at: (e) 30°C/UV light 75 %RH; (f) 40°C/75 %RH, for 4 months

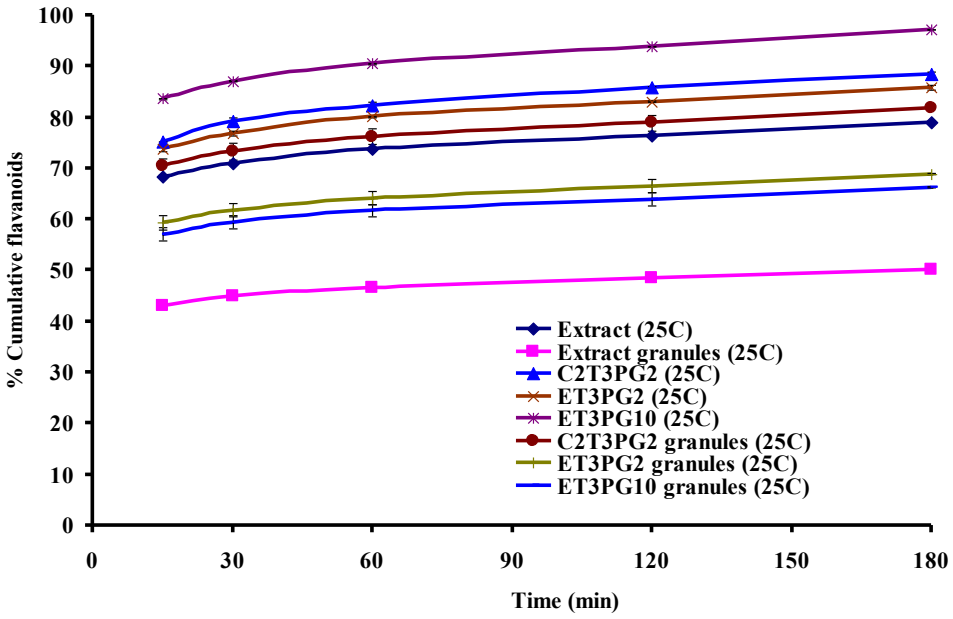


(a)

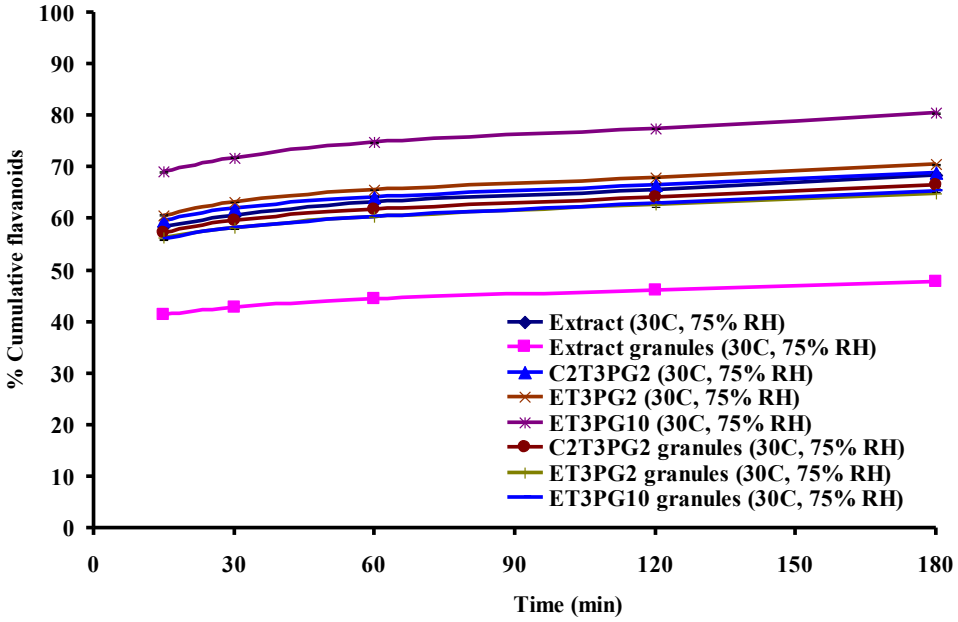


(b)

Figure B-5 Dissolution profiles of **total flavonoids** from the extract, the extract granules, self-emulsifying liquids and granules **in 0.1N HCl**: (a) after preparation; (b) after storage in refrigerator for 4 months

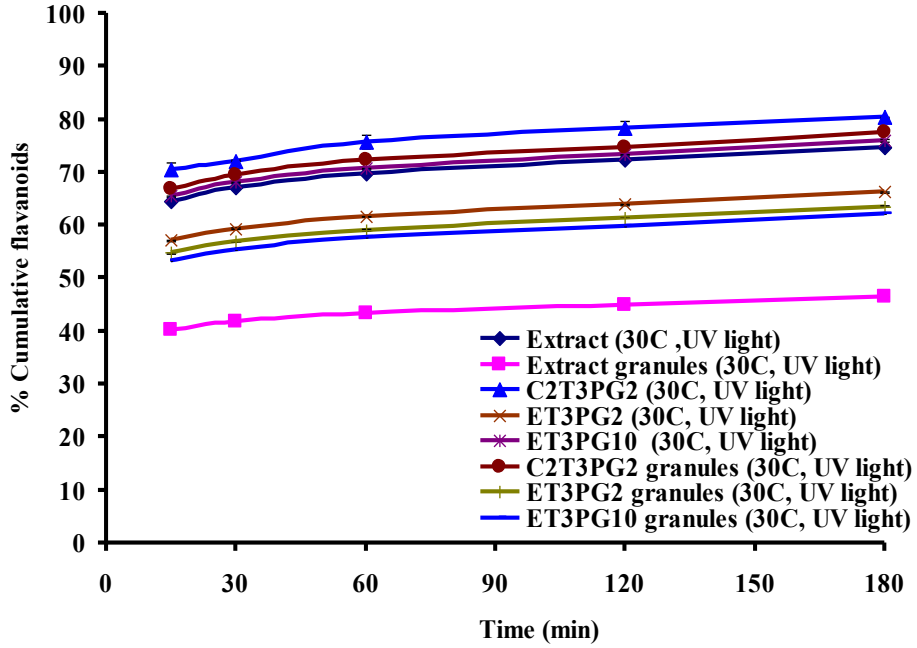


(c)



(d)

Figure B-5 (continue) Dissolution profiles of **total flavonoids** from the extract, the extract granules, self-emulsifying liquids and granules **in 0.1N HCl** after storage at: (c) 25°C; (d) 30°C/75 %RH, for 4 months



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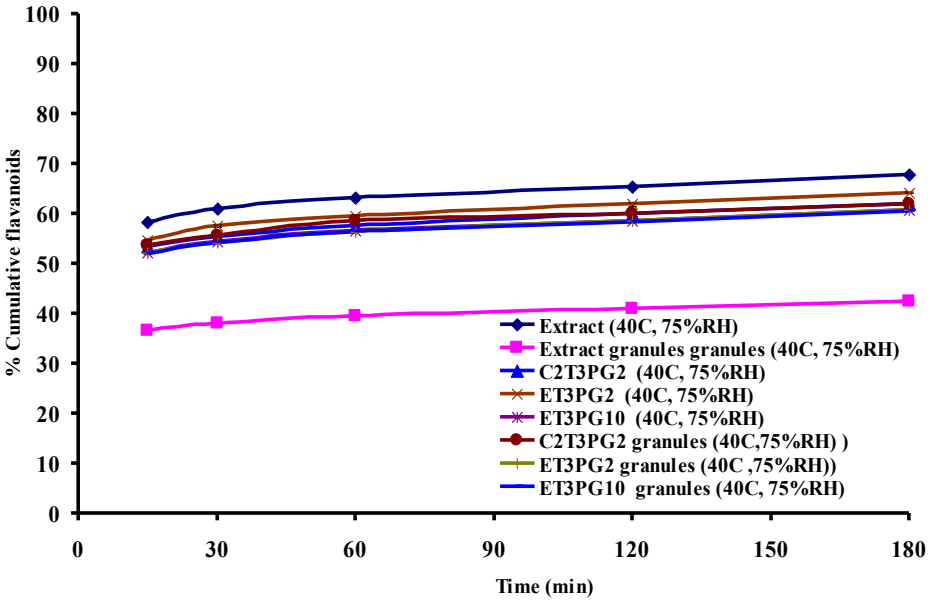
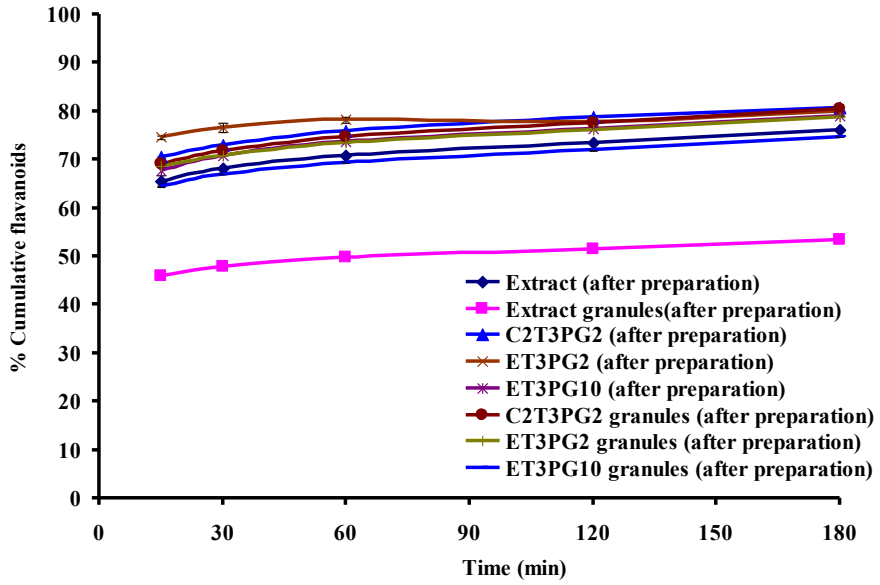
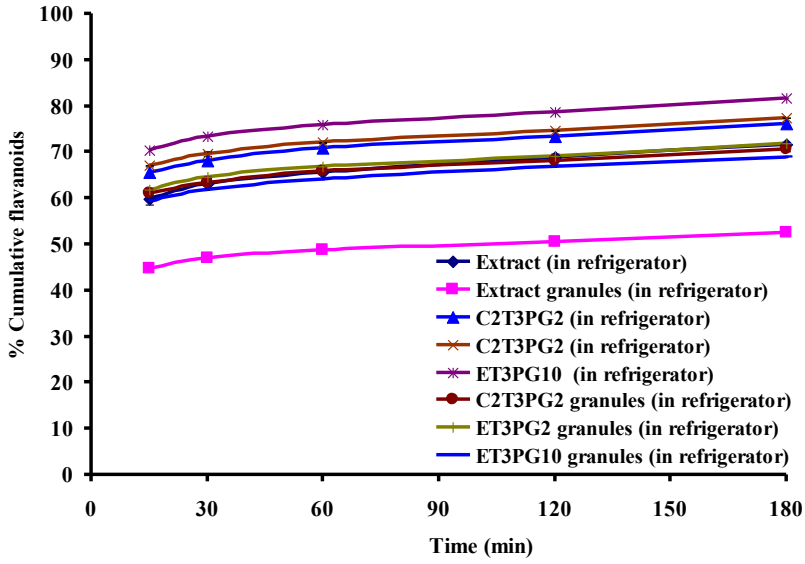


(f)

Figure B-5 (continue) Dissolution profiles of **total flavonoids** from the extract, the extract granules, self-emulsifying liquids and granules **in 0.1N HCl** after storage at: (e) 30°C/UV light 75 %RH; (f) 40 °C/75 %RH, for 4 months

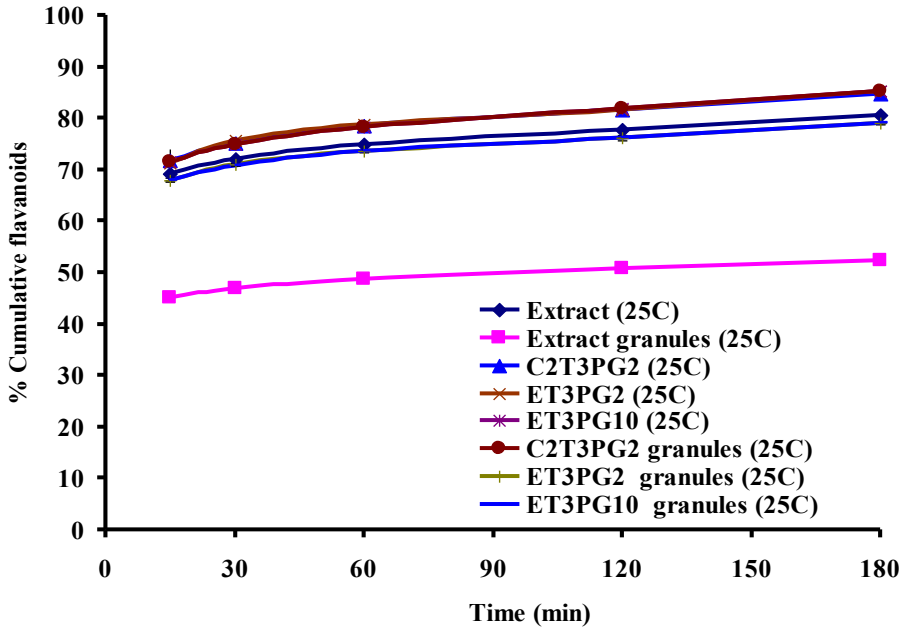


(a)

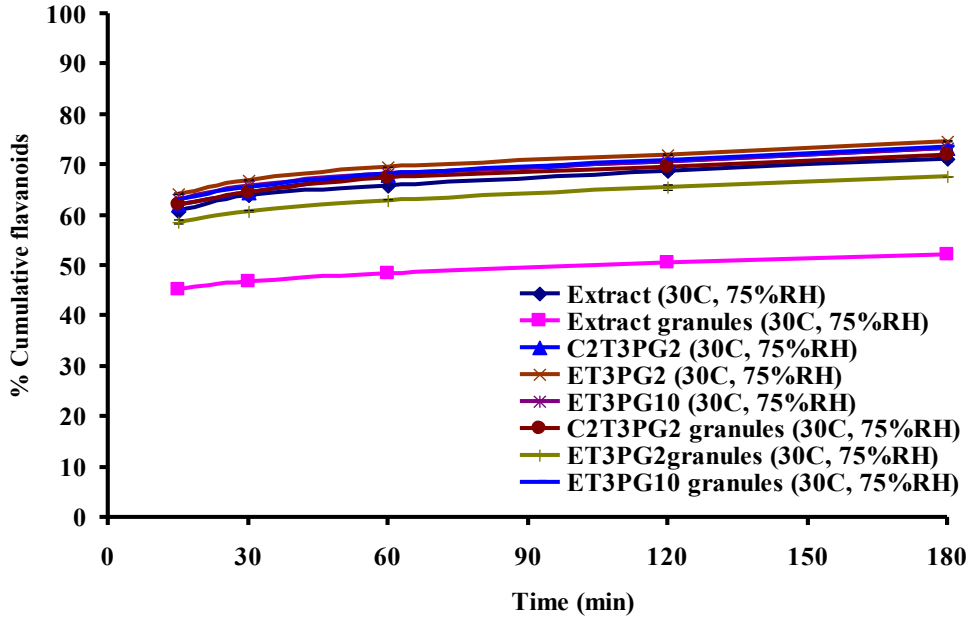


(b)

Figure B-6 Dissolution profiles of **total flavonoids** from the extract, the extract granules, self-emulsifying liquids and granules **in pH 6.8 phosphate buffer**: (a) after preparation; (b) after storage in refrigerator for 4 months

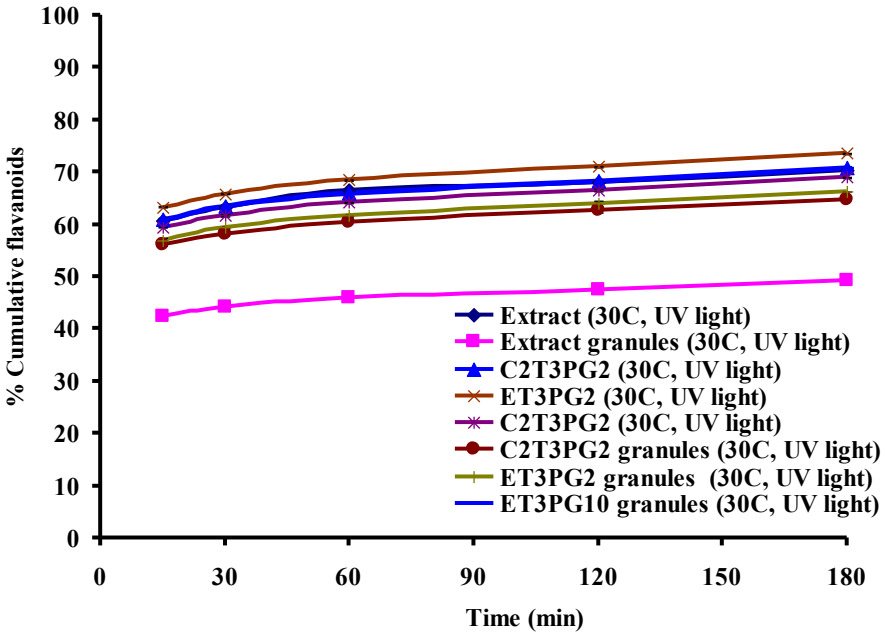


(c)

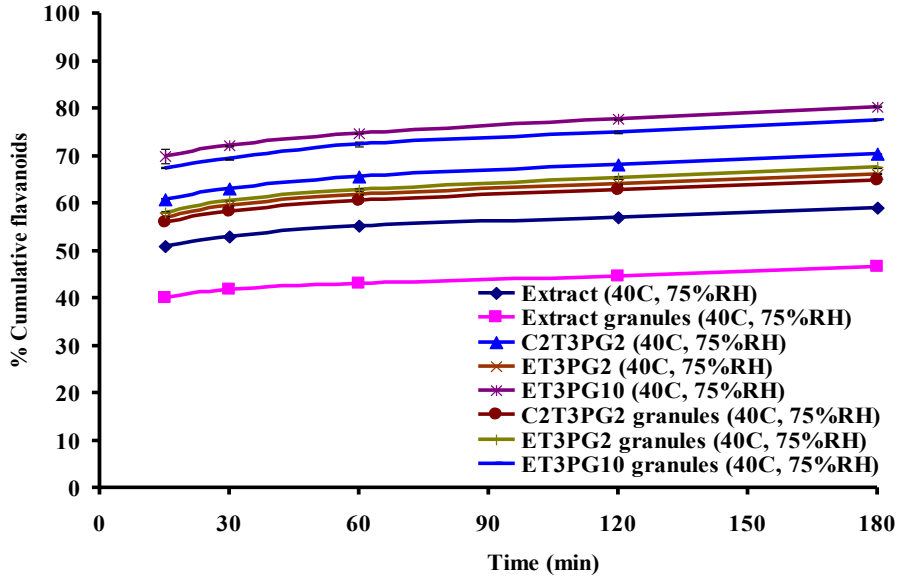


(d)

Figure B-6 (continue) Dissolution profiles of **total flavonoids** from the extract, the extract granules, self-emulsifying liquids and granules **in pH 6.8 phosphate buffer** after storage at: (c) 25°C; (d) 30°C/75 %RH, for 4 months

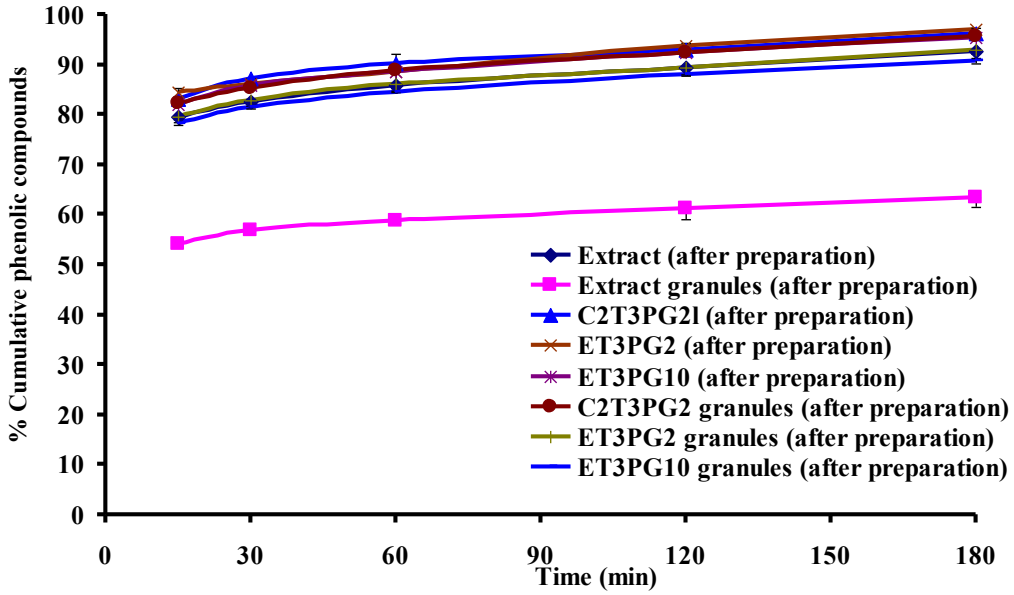


(e)

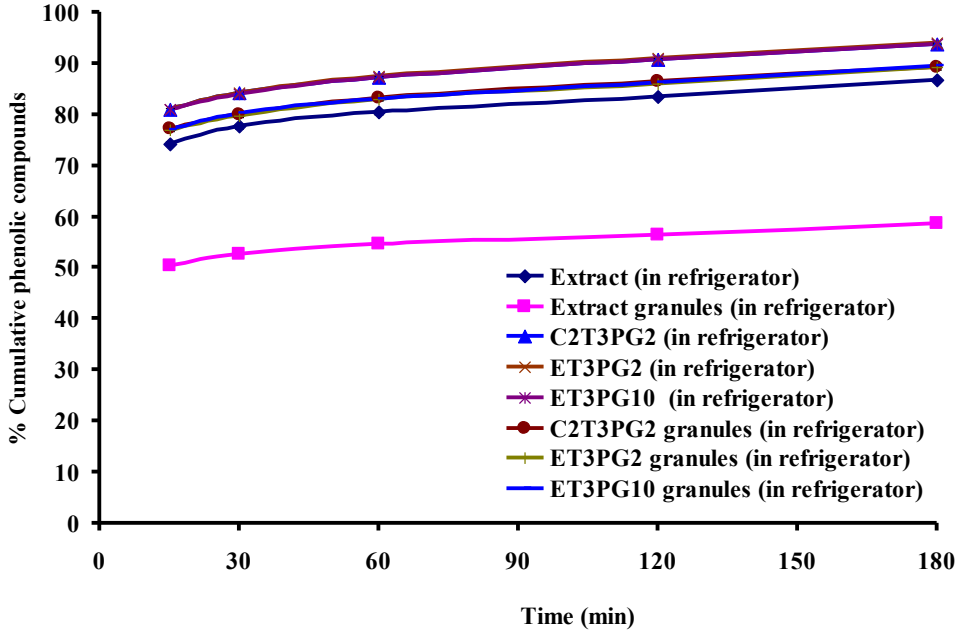


(f)

Figure B-6 (continue) Dissolution profiles of **total flavonoids** from the extract, the extract granules, self-emulsifying liquids and granules **in pH 6.8 phosphate buffer** after storage at: (e) 30 °C/UV light 75 %RH; (f) 40 °C/75 %RH, for 4 months

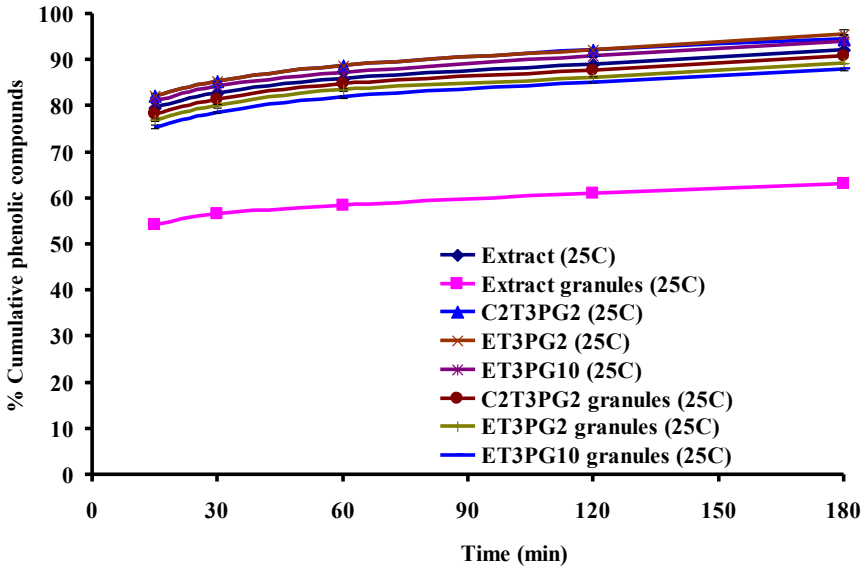


(a)

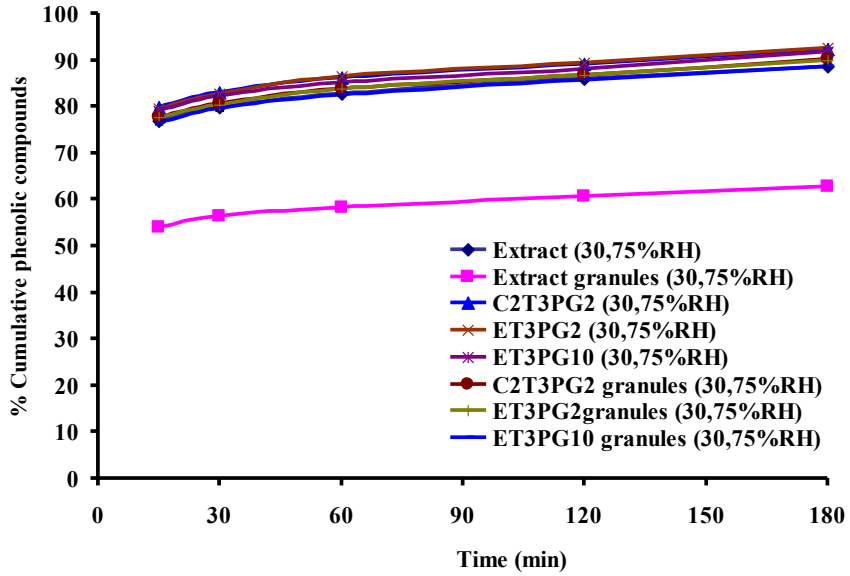


(b)

Figure B-7 Dissolution profiles of **total phenolic compounds** from the extract, the extract granules, self-emulsifying liquids and granules **in water**: (a) after preparation; (b) after storage in refrigerator for 4 months

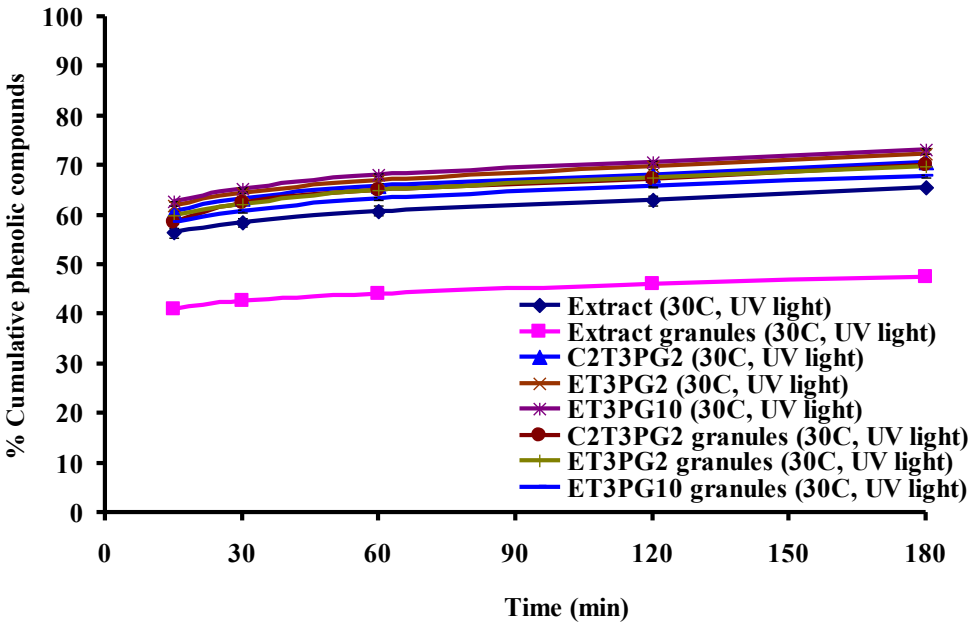


(c)

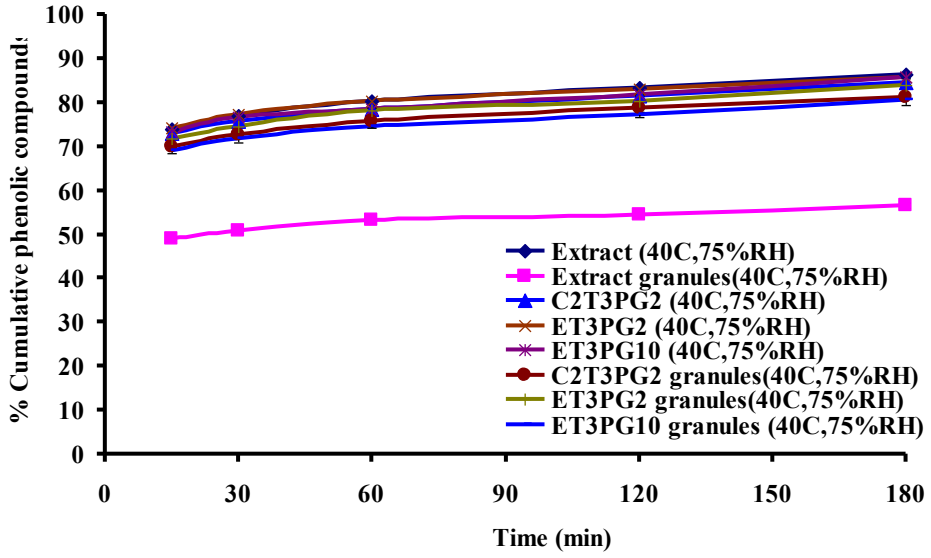


(d)

Figure B-7 (continue) Dissolution profiles of **total phenolic compounds** from the extract, the extract granules, self-emulsifying liquids and granules **in water** after storage at: (c) 25°C; (d) 30°C/75 %RH, for 4 months

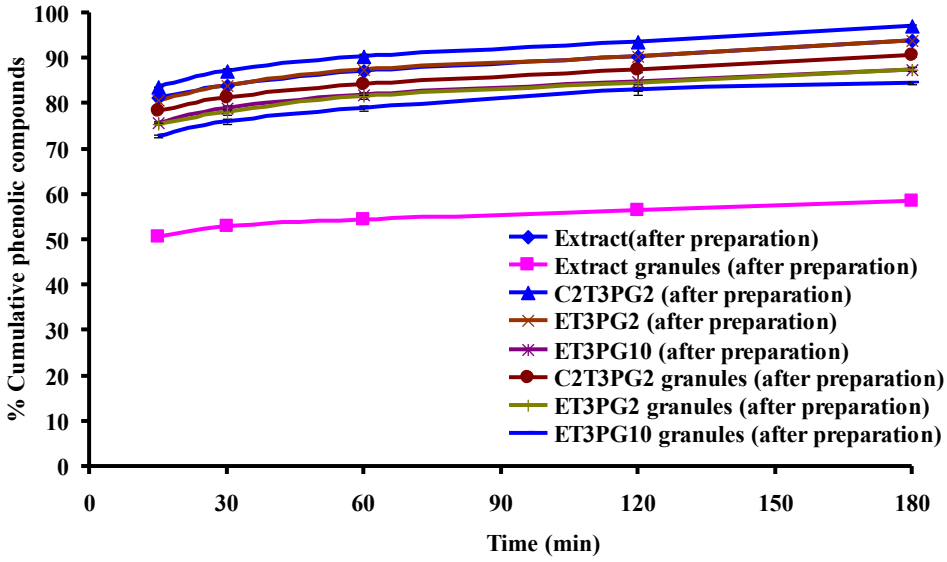


(e)

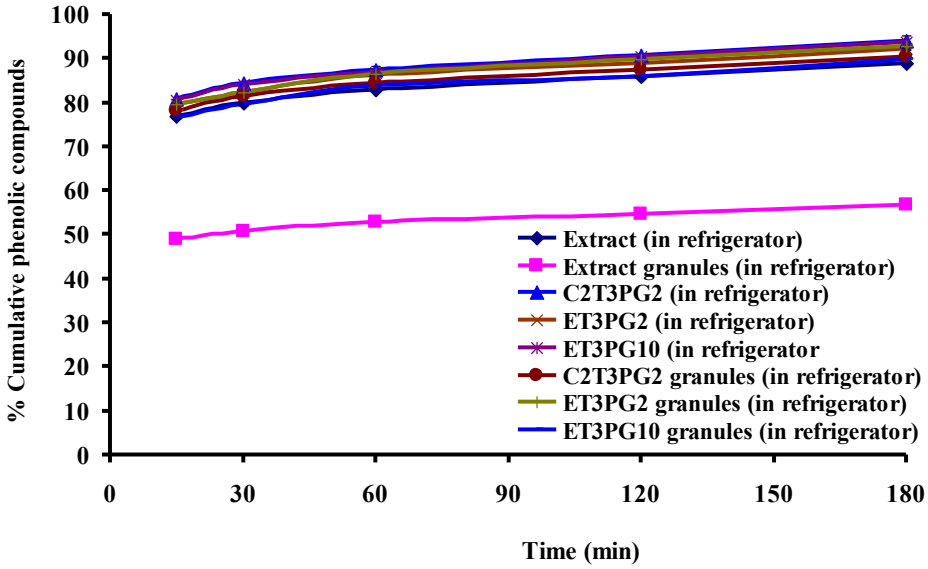


(f)

Figure B-7 (continue) Dissolution profiles of **total phenolic compounds** from the extract, the extract granules, self-emulsifying liquids and granules **in water** after storage at: (e) 30°C/UV light 75 %RH; (f) 40°C/75% RH, for 4 months

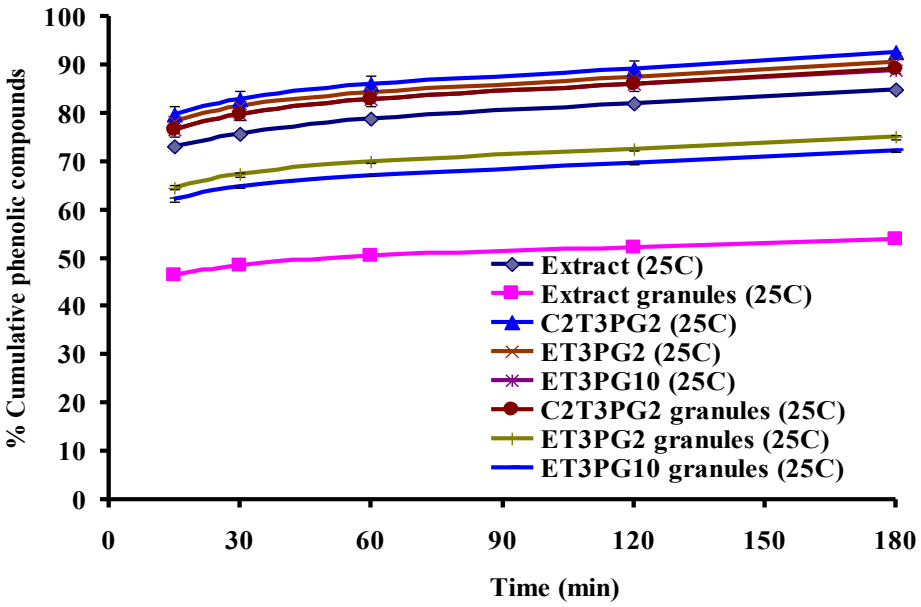


(a)

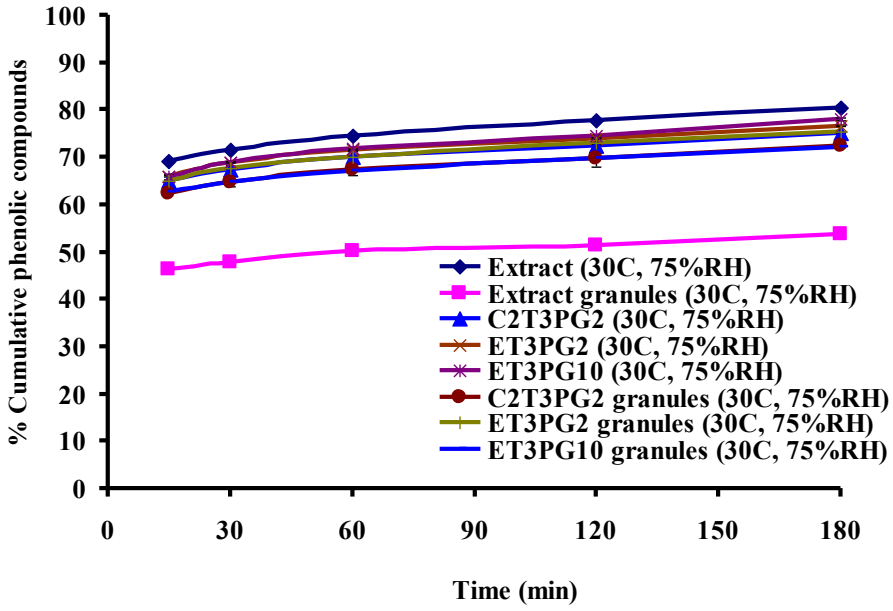


(b)

Figure B-8 Dissolution profiles of **total phenolic compounds** from the extract, the extract granules, self-emulsifying liquids and granules **in 0.1N HCl**: (a) after preparation; (b) after storage in refrigerator for 4 months

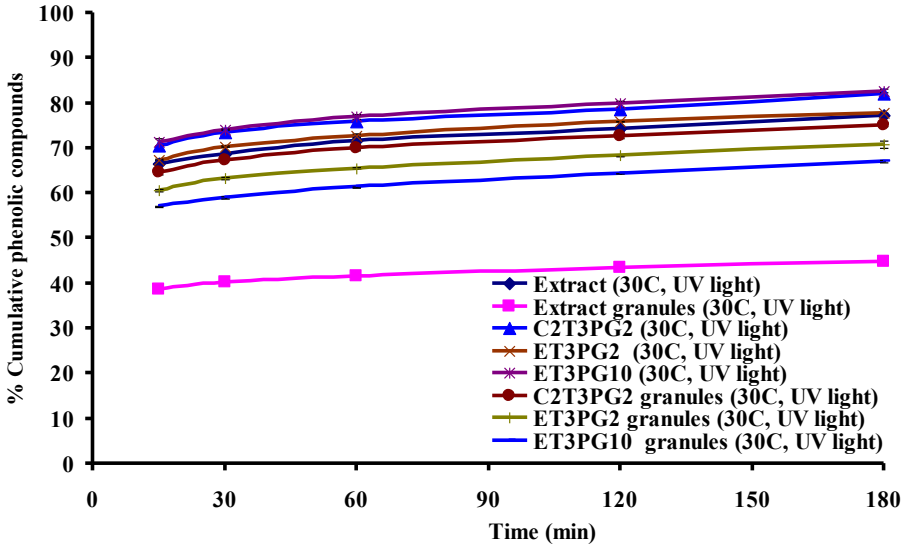


(c)

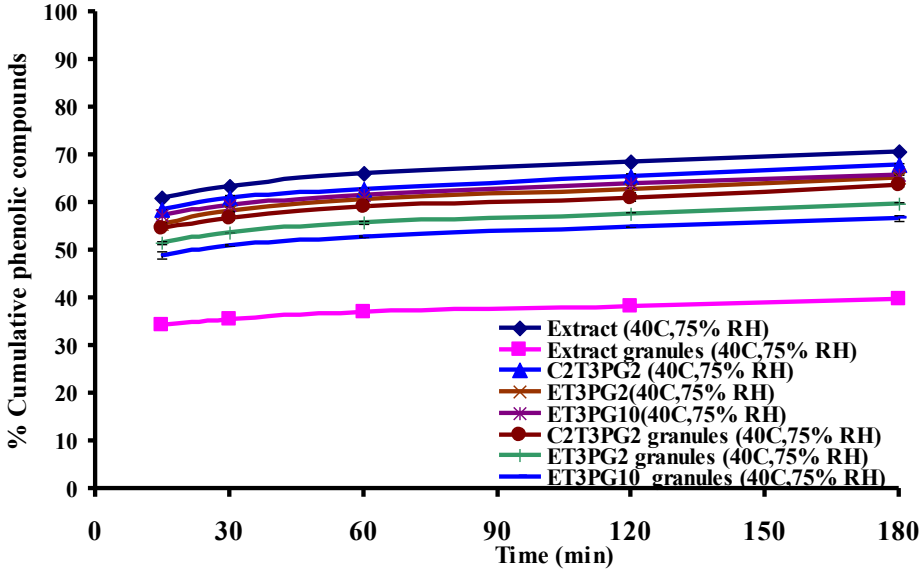


(d)

Figure B-8 (continue) Dissolution profiles of **total phenolic compounds** from the extract, the extract granules, self-emulsifying liquids and granules **in 0.1N HCl** after storage at: (c) 25°C; (d) 30°C/75 %RH, for 4 months

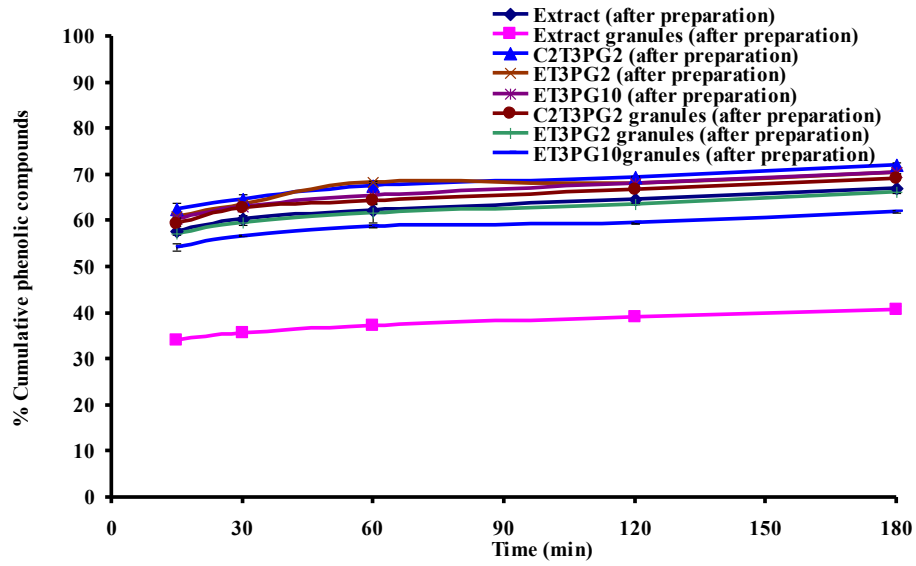


(e)

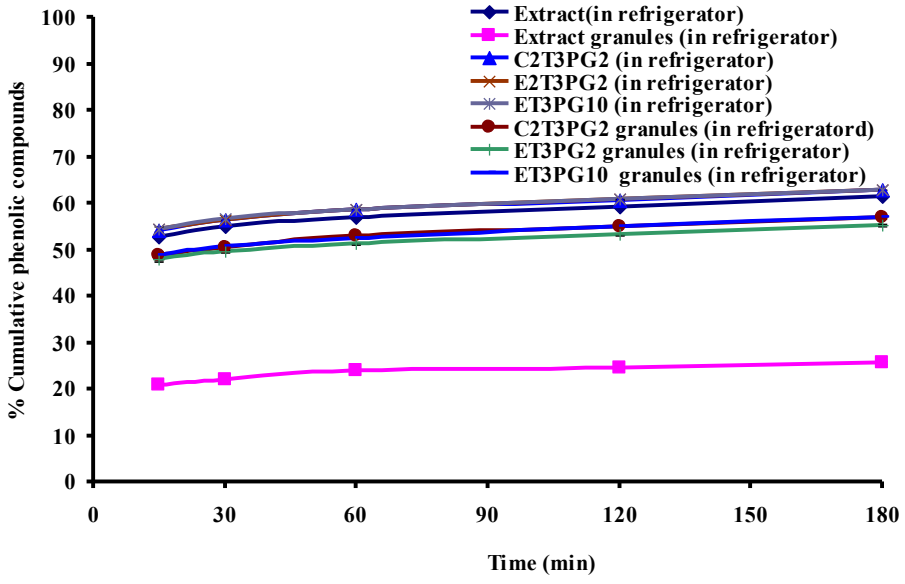


(f)

Figure B-8 (continue) Dissolution profiles of **total phenolic compounds** from the extract, the extract granules, self-emulsifying liquids and granules **in 0.1N HCl** after storage at: (e) 30°C/UV light 75 %RH; (f) 40°C/75% RH, for 4 months

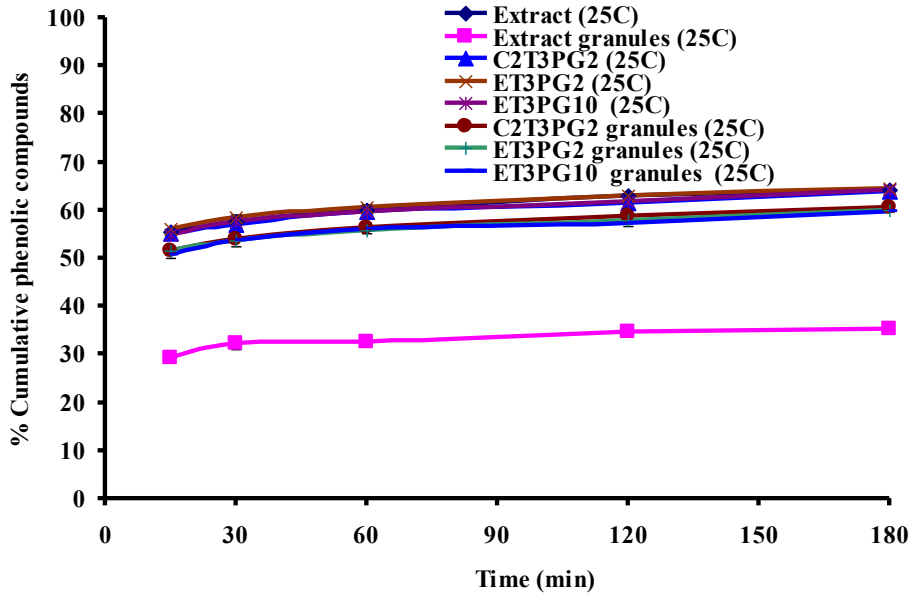


(a)

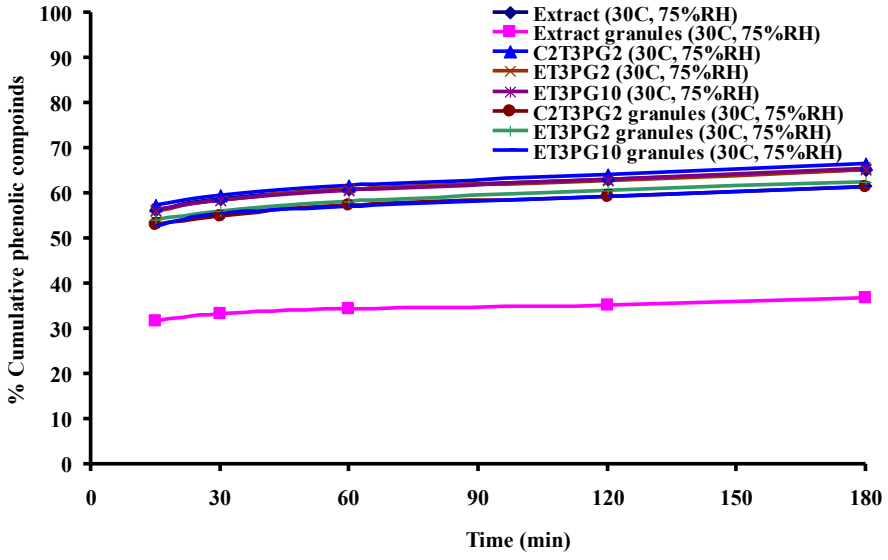


(b)

Figure B-9 Dissolution profiles of **total phenolic compounds** from the extract, the extract granules, self-emulsifying liquids and granules **in pH 6.8 phosphate buffer**: (a) after preparation; (b) after storage in refrigerator for 4 months

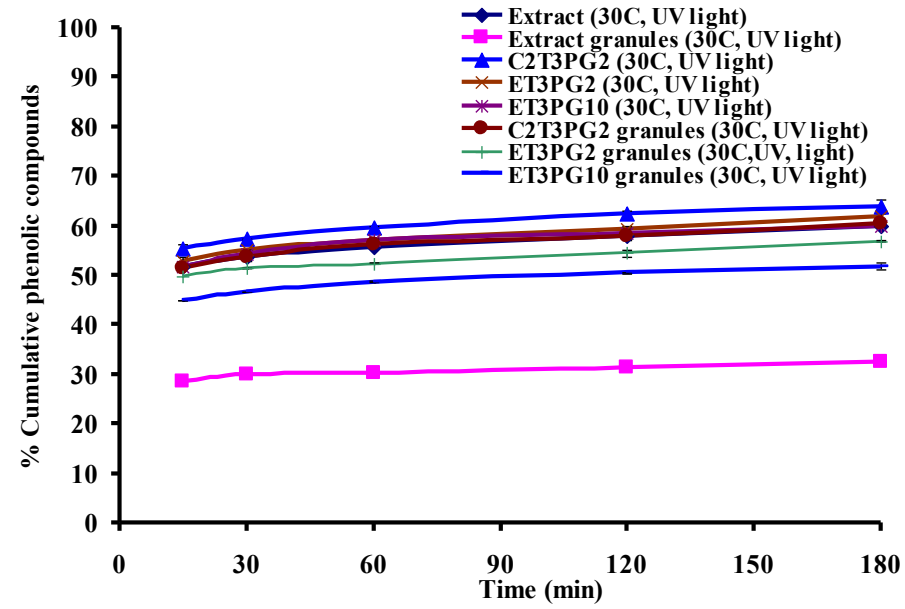


(c)

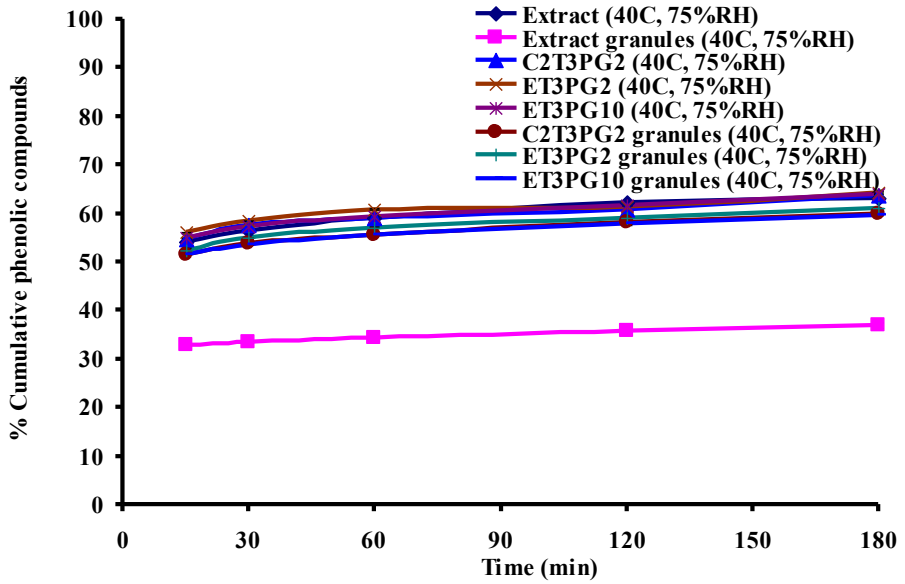


(d)

Figure B-9 (continue) Dissolution profiles of **total phenolic compounds** from the extract, the extract granules, self-emulsifying liquids and granules **in pH 6.8 phosphate buffer** after storage at: (c) 25°C; (d) 30°C/75 %RH, for 4 months



(e)



(f)

Figure B-9 (continue) Dissolution profiles of **total phenolic compounds** from the extract, the extract granules, self-emulsifying liquids and granules **in pH 6.8 phosphate buffer** after storage at: (e) 30°C/UV light 75 %RH; (f) 40°C/75 %RH, for 4 months

BIOGRAPHY

Miss Tharntip Wachirasakwong was born on May 14, 1980. She received her Bachelor of Science in Pharmacy degree in 2004 from Faculty of Pharmacy, Rangsit Universy, Thailand. After graduation, she has been working at Bureau of Drug and Nacrotic, Department of Medical Sciences, Ministry of Public Health, Nonthaburi.