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Original article

Development of raft-forming liquid and chewable tablet formulations incorporating quercetin solid dispersions for treatment of gastric ulcers

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ABSTRACT

Gastroretentive raft-forming formulations were developed in liquid and chewable tablet dosage forms to achieve prolonged delivery of quercetin in the stomach. The formulations contained a solid dispersion of quercetin and polyvinylpyrrolidone (PVP K 30) at a 1:10 w/w ratio to improve the solubility of the flavonoid. The formulations also contained sodium alginate as a gel forming agent, calcium carbonate as a calcium source and carbon dioxide producer and hydroxypropyl methylcellulose K100M as a drug release retarding polymer. The chewable tablets incorporated mannitol as a diluent. Both liquid and chewable tablet formulations exhibited rapid floating behaviour (lag time < 1 min) and long floating duration (>24 h) in 0.1 N HCl. The optimized liquid formulation showed superior characteristics based on high raft strength (10.4 g) and sustained release of quercetin (93 % over 8 h) whereas the optimized chewable tablet formulation exhibited lower raft strength (7.2 g) and lower drug release (79 % in 8 h). The optimized liquid and chewable tablet formulations were found to induce anti-inflammatory activity in cell culture using RAW 264.7 cells macrophages and enhance the migration of human gastric adenocarcinoma (AGS) epithelial cells *in vitro*, indicating wound healing potential for treatment of gastric ulcers.

1. Introduction

Gastric ulcers develop in the mucosal lining of the stomach giving rise to burning pain, discomfort of the stomach, heartburn and indigestion. In the worst cases, complications may lead to excessive bleeding and stomach cancer which can be life-threatening. The main causes of gastric ulcers are related to *Helicobacter pylori* infections and the excessive use of non-steroidal anti-inflammatory drugs (NSAIDS). Although, conventional therapeutic regimens based on antibiotic and antisecretory agent, for example the triple therapy with amoxicillin, metronidazole, and omeprazole, are effective in the treatment of gastric ulcers, side effects including headache, stomach pain, and nausea-vomiting are unavoidable and compromise their therapeutic benefits. In recent years, several studies have reported that herbal medicines including *curcuma longa* and *centella asiatica* are effective in treating gastric ulcers and have been proposed as alternative or adjunct treatment for gastric ulcers with fewer side effects, drug interactions and lower cost than conventional drugs (Bi et al., 2014).

Quercetin is a plant flavonoid that is commonly found in many fruits (e.g. cranberries, cherries, grapes), wine and black or green tea. Quercetin has been reported to possess abundant pharmacological properties, including anti-inflammatory, antibacterial and anticancer activity. The effect of quercetin in gastric ulcers has been demonstrated both *in vitro* and *in vivo*. Quercetin prevented indomethacin-induced NF-kB activation and IL-8 production in Caco-2 cells. Their *in vivo* study performed in the rat gastric and ileal mucosa also supported the gastroprotective effect of quercetin by preventing indomethacin-induced ICAM-1, P-selectin expression (Mota et al., 2009). González-Segovia et al. found that quercetin was effective in treating peptic ulcers induced by *H. pylori* in the gastric mucosa of guinea pigs, by reducing both the inflammatory response and lipid peroxidation (González-Segovia et al., 2008).

Although quercetin has demonstrated promising therapeutic effects against gastric ulcers and high safety profile, the flavonoid exhibits poor bioavailability following oral administration due to

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low aqueous solubility and rapid elimination from the body. Several techniques have been used to improve the solubility of quercetin, including particle size reduction by micronization (Loh et al., 2015), and self-emulsifying strategies (hu et al., 2007). Among these, solid dispersion (SD) which involves dispersion of a poor aqueous solubility drug in a high solubility hydrophilic polymer matrix in the solid state is one of the most promising approaches. SDs enhance drug solubility by various mechanisms, including particle size reduction, achieving dispersion at the molecular level, enhancing wettability and porosity, as well as by changing a drug's crystalline state to the amorphous form (Vasconcelos et al., 2007).

Numerous studies have shown that solid dispersions produced from various hydrophilic polymers can enhance the solubility of quercetin (Otto et al., 2013; Setyawan et al., 2017). However, the therapeutic efficacy of the SD is likely to be limited by rapid elimination of quercetin via faeces and urine (Cai et al., 2013). The average terminal half-life of quercetin is 3.5 h (Li et al., 2016); thus frequent dosing is required to sustain therapeutic activity. Raft-forming gastroretentive drug delivery systems are generally produced as liquid dosage forms, which are designed to float on the stomach contents following oral administration and prolong drug release. Gelling polymers including alginic acid, guar gum and pectin are usually mixed with alkaline bicarbonates or carbonates as a gas generating agent, which liberates carbon dioxide on contact with the gastric acid. Gas bubbles are trapped in the gel, resulting in a reduction in density such that the raft floats on the stomach content. The raft also acts as a physical barrier to prevent the reflux of gastric content into the esophagus, making it a useful drug delivery system for the treatment of gastroesophageal reflux disease (GERD) (Prajapati et al., 2013).

Recently, raft-forming systems based on liquid formulations for controlled delivery of herbal medicines to the stomach for treatment of gastric ulcers have attracted increasing attention. The poorly water soluble compounds curcumin and the glycoside-rich centella extract, when incorporated in liquid, raft-forming formulations showed superior curative effect on gastric ulcers compared to unformulated compounds and the anti-ulcer drug, lansoprazole (Kerdsakundee et al., 2015; Wannasarit et al., 2020). However, the raft-forming liquid dosage forms display several limitations compared with the solid dosage forms including lower stability due to chemical reaction and microbial contamination, more stringent packaging requirements and less convenient portability (Prajapati et al., 2013). The chewable tablets are considered as a suitable solid dosage form for raft-forming formulation. Chewable tablets are designed to be chewed in the mouth prior to swallowing and are not expected to swallow intact. After swallowing, the resulting powder can readily form raft in the stomach. Therefore, the powdered tablets are used in the physicochemical characterization of rafts to simulate the mastication process according to the previous report (Prajapati et al., 2012).

This study aimed to develop and characterize raft-forming liquid and chewable tablet formulations incorporating quercetin solid dispersion for treatment of gastric ulcers. The anti- inflammatory effect and gastric wound healing potential were assessed in cell culture using Raw 264.7 and AGS cells, respectively.

2. Materials and methods

2.1. Materials

Quercetin (95.4% quercetin) was obtained from Guangzhou Phytochem Sciences Inc. (Guangzhou, China). PVP K 30, PVP K 90 and mannitol were sourced from PC Drug (Bangkok, Thailand). Sodium alginates (low and high viscosity types, viscosity values 45 cps and 8001000 cps respectively (1 %w/w solution; 25 °C)) were supplied by High Science Limited Partnership (Songkhla, Thailand). Calcium carbonate was purchased from LOBA Chemie Pvt. Ltd. (Mumbai, India) and sodium bicarbonate was obtained from RCI Labscan (Bangkok, Thailand). All other reagents were of analytical grade.

2.2. Methods

2.2.1. Preparation of quercetin solid dispersions

Solid dispersions of quercetin with PVP K 30 and PVP K 90 were prepared by solvent evaporation. Quercetin was combined with each polymer in the ratio of 1:3, 1:5, 1:8 and 1:10 and dissolved in ethanol to yield clear solutions. The solvent was evaporated using a rotary evaporator (Hei-VAP Value, Heidolph Instruments, Schwabach, Germany) at 40 °C to obtain the dried mass and pulverized with a mortar and pestle prior to sieving (250 μm mesh) to obtain a powder. In the case of physical mixtures, the same quercetin and polymer ratios were weighed and mixed simply using a mortar and pestle before passing through a 250 μm sieve to obtain fine powders. Both solid dispersions and physical mixtures were kept in tightly closed containers at 25 °C and protected from light until used for further investigation.

2.2.2. Analysis of quercetin content of solid dispersions

Accurately weighed samples equivalent to 100 mg quercetin were dissolved in 15 mL methanol and stirred to obtain clear solutions. The solution was adjusted to 100 mL in a volumetric flask and filtered through a 0.45-μm nylon membrane filter. The amount of quercetin in the samples was determined using high performance liquid chromatography (HPLC) system (Agilent 1100 series, California, USA). Chromatographic separations were achieved using a Vertisep^{TM} C18 5 μm column (4.6 × 150 mm) (Ligand Scientific, Bangkok, Thailand). The mobile phases consisted of methanol and 0.1% ortho phosphoric acid (65:35% v/v) at a flow rate 1 mL/min and column temperature 35 °C. The UV detector was set at 370 nm. All formulations were determined in triplicate.

2.2.3. Solubility of quercetin solid dispersions

The solubilities of quercetin powder, quercetin solid dispersions and physical mixtures were measured in 0.1 N hydrochloric acid (pH 1.2) and distilled water, respectively. An excess amount of quercetin powder, solid dispersion, and physical mixture was added to tubes containing 1 mL of 0.1 N hydrochloric acid and distilled water. The tightly closed tubes were properly mixed by vortex (Vortex-Genie 2, 50 Hz model, Scientific Industries, Inc., USA) at maximum frequency for 10 min to facilitate solubilization. The resulting homogeneous mixtures were placed in a shaking water bath (Heto Lab, Scientific Promotion, Bangkok, Thailand) maintained at room temperature for 48 h before centrifuging at 6,000 rpm for 10 min (Kubota 5922B/N, Kubota, Tokyo, Japan). The supernatants were collected and diluted with methanol before filtering through a 0.45 μm membrane filter. The filtrates were analyzed to determine the amount of solubilized quercetin by HPLC. All experiments were conducted in triplicate.

2.2.4. Release behaviour of quercetin from solid dispersions and physical mixtures

The release of quercetin from solid dispersions was compared with that from physical mixtures and pure quercetin powder. The study was carried out in 0.1 N HCl (pH 1.2) solution and distilled water at 37 ± 0.5 °C using a USP-type-II dissolution apparatus (EDT 08Lx, Electrolab, Mumbai, India) and 50 rpm rotation speed. The solid dispersions and physical mixtures containing the equivalent of 500 mg qurecetin together with 1000 mg of pure quercetin powder were taken into vessels containing 900 mL of dissolution

medium respectively. Aliquots (5 mL) were withdrawn at determined time intervals (5, 10, 15, 30, 45, 60, 90 and 120 min), and replaced with an equal volume of fresh dissolution medium. The samples were filtered using a 0.45 μm pore size filter to remove particulates and analyzed to determine the dissolved quercetin using HPLC. All experiments were performed in triplicate. The cumulative percentage of quercetin in the release medium for each formulation was calculated and plotted versus time to generate release profiles.

2.2.5. Physicochemical characterization of quercetin solid dispersions

2.2.5.1. Scanning electron microscopy (SEM). SEM (Quanta 400, FEI, Czech Republic) was employed to reveal the surface morphology of quercetin powder, PVP K30, quercetin solid dispersions and physical mixtures. Samples were coated with gold (SPI supplies, West Chester, USA) prior to examination at 20 kV voltage and 12 mA current.

2.2.5.2. Powder X-ray Diffraction (PXRD). PXRD studies were performed to identify the presence of crystalline or amorphous forms in quercetin powder, solid dispersions and physical mixtures. PXRD patterns were obtained at room temperature using an X-ray diffractometer (Xpert MPD, Philips, Natherlands) operated at 40 kV, 30 mA current, using a scan speed 1 s/step over a 2 theta range of 5–90° and step size of 0.05°.

2.2.5.3. Fourier Transform Infrared Spectroscopy (FTIR). FTIR (Vertex 70, Bruker, Ettlingen, Germany) studies were performed using quercetin powder, solid dispersions and physical mixtures to identify chemical bonding, functional groups, and the existence of quercetin/polymer interactions. Each samples (equivalent to 10 mg of quercetin) was mixed and pressed into KBr disks before scanned over the range 4000 to 400 cm^{-1}.

2.2.6. Preparation of raft-forming liquid formulations incorporating quercetin solid dispersions

The raft-forming liquid systems were prepared using different concentrations of high viscosity sodium alginate, hydroxypropyl methylcellulose K 100 M (HPMC K 100 M) and calcium carbonate ($CaCO_3$) as listed in Table 1. The solution of sodium alginate (1–2 % w/v) were prepared by dissoving sodium alginate in deionized water. Then quercetin solid dispersion (1.1 %w/v) and HPMC K 100 (0.25–1% w/v) were added, respectively. Finally, 5 % w/v calcium carbonate was dispersed well in the mixture with continuous stirring. The uniform dispersion was finally adjusted to a volume of 100 mL with distilled water.

2.2.7. Physicochemical characterization of raft-forming liquid formulations

2.2.7.1. Viscosity measurements. The viscosity of raft-forming liquid formulations was measured using a Brookfield DVIII ultra viscometer (Ametek Brookfield, Massachusetts, USA) fitted with spindle No. 64 at a temperature of 25 ± 1C°. Sample viscosity was measured in triplicate at set spindle rotation speeds of 50 revolution per minute (rpm).

2.2.7.2. Density of formed rafts. Ten ml of the raft-forming liquid was added to 35 mL of 0.1 N HCl (pH 1.2) contained in a 50 mL measuring cylinder. A cylinder used for raft formation was pre-weighed (W1). The total weight of the cylinder and contents was obtained after raft development for 30 min (W2). The weight of raft formation was calculated from the formula: (W2 -W1). The final volume of the raft gel (V) was observed from the increasing scale on the cylinder. The volume and weight of the gel was used to calculate the density of the formed gel (D = (W 2-W1)/V). Each formulation was tested in triplicate.

2.2.7.3. Raft strength. Raft strength was determined using a texture analyzer (TA. XT plus, Stable microsystems, London, United Kingdom) to assess the ability of the raft to withstand peristaltic movement *in vivo*. An L-shaped stainless steel wire probe was positioned vertically in a 250 mL glass beaker containing 150 mL of 0.1 N HCL (pH 1.2) maintained at 37 °C, such that the probe did not contact the beaker base. The raft-forming liquid dosage form was transferred to the beaker and left for 30 min to form a raft around the probe. The wire probe was subsequently pulled vertically through the raft at a rate of 5 mm/s and the maximum force (g) was recorded as a measure of raft strength.

2.2.7.4. Floating time and in vitro release studies. The release behavior of quercetin from raft-forming liquid formulations was investigated under sink-conditions using a USP-type-II dissolution apparatus (EDT 08Lx, Electrolab, Mumbai, India) as mentioned in Section 2.2.4 but using 200 mL of 0.1 N HCl (pH 1.2) as the release medium. Individual samples (10 mL) were injected into the release medium contained in separate glass vessels of the dissolution apparatus using a disposable syringe. The time required for the formed raft to float to the surface of the release medium was recorded as 'floating lag time'. The total flotation time was also measured. Samples (5 mL) of release medium were withdrawn and replaced with fresh medium after 30, 60, 120, 180, 240, 300, 360, 420 and 480 min. The samples were filtered through 0.45 μm pore size filter and the concentration of dissolved quercetin was determined by HPLC. A plot of % cumulative release of quercetin against time was constructed to generate release profiles. The release experiments were performed in triplicate and the data were reported as a mean ± S.D.

Table 1
Physical characteristics of raft-forming liquid formulations.

Formulations	Composition(%w/v)		Viscosity (Cps)	Raft strength (g)	Floating lag time (s)	Density (g/cm^3)
	Alg	HPMC				
F1	1.0	0.0	595.87 ± 79.88	9.6 ± 0.2	8.46 ± 0.92	0.69 ± 0.05
F2	2.0	0.0	4,714.66 ± 31.37	11.2 ± 0.2	14.13 ± 2.20	0.49 ± 0.02
F3	1.0	0.25	1,187.75 ± 13.86	9.2 ± 0.2	11.83 ± 1.95	0.52 ± 0.08
F4	1.0	0.5	1,805.61 ± 54.10	9.1 ± 0.2	14.68 ± 2.03	0.59 ± 0.08
F5	1.0	1.0	2,019.57 ± 48.49	5.6 ± 0.3	10.83 ± 1.71	0.49 ± 0.07
F6	2.0	0.25	5,122.91 ± 35.98	9.8 ± 1.2	6.39 ± 1.11	0.49 ± 0.03
F7	2.0	0.5	7,042.50 ± 135.20	10.4 ± 1.0	6.74 ± 1.01	0.46 ± 0.02
F8	2.0	1.0	8,926.10 ± 191.96	10.4 ± 0.6	7.24 ± 2.54	0.50 ± 0.03

$CaCO_3$ concentration of all formulations = 5 %w/v.
SD concentration of all formulations = 1.1 %w/v.

2.2.8. Preparation of raft-forming chewable tablet formulations incorporating quercetin solid dispersions

Raft-forming chewable tablets were produced by direct compression composed of gelling agent (low viscosity sodium alginate), alkalizing agents (calcium carbonate and sodium bicarbonate), release retarding agent (HPMC K100M) and quercetin/PVP solid dispersion(ratio1:10). Briefly, Quercetin/PVPk30 solid dispersion and other ingredients as listed in Table 2 (except magnesium stearate) were weighed and mixed with a geometric dilution method using a mortar and pestle to obtain a homogeneous mixture. Then, the mixtures were further blended with magnesium stearate as a lubricant before being compressed by a single-punch tablet machine (Yeo Heng Co., LTD, Bangkok, Thailand) with a 16 mm diameter flat punch.

2.2.9. Physicochemical characterization of raft-forming chewable tablet formulations

2.2.9.1. Tablet hardness and thickness. Mean tablet hardness and thickness were obtained by measurements of 10 tablets for each formulation using a two-parameter tablet tester (EBT-2PRL, Electrolab, Mumbui, India). Hardness was expressed as the tablet breaking force (kg/cm^2) while tablet thickness was recorded in mm.

2.2.9.2. Tablet friability. Tablet friability was determined using a Roche friabilator (EF 2 W, Electrolab, Mumbui, India).Dust-free samples (10 tablets) were weighed and placed in the drum which was rotated at 25 rpm for 4 min. The tablets were reweighed and sample weight loss was taken as a measure of friability. A weight loss of not>1.0 % was considered as an acceptable friability property.

2.2.9.3. Raft strength. Raft-forming chewable tablets were ground in a mortar. The obtained powdered tablet equivalent to a unit dose (20 mg) of quercetin was tested as described in Section 2.2.7.

2.2.9.4. In vitro release studies. Raft-forming chewable tablets were ground in a mortar. The obtained powdered tablet equivalent to a unit dose (20 mg) of quercetin was transferred to 0.1 N HCl (pH 1.2) release medium in a USP-type-II dissolution apparatus. Release testing was performed as described in Section 2.2.7

2.2.10. Effect of raft-forming formulations on cell viability

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide) assay was performed to determine the effect of free quercetin, quercetin/PVP solid dispersion, raft-forming liquid and tablet formulations on the viability of Raw 264.7 cells and AGS cells. RAW264.7 cells were maintained in supplemented Dulbecco's modified Eagle's medium (DMEM) while AGS cells were grown in Kaighn's Modification of Ham's F-12 Medium (F12K medium). Quercetin, and equivalent doses of quercetin-loaded formulations (quercetin/PVP solid dispersion, raft-forming liquid and tablet formulation) were dissolved in small amount of ethanol and diluted in culture medium to obtain various concentrations (1, 2, 3, 4, 5, 10, 15, 20 μg/mL). Both cell types were seeded in 96-well plates at a density of 1x10^5 cells per well and allowed to adhere for 24 hr at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was replaced with fresh medium containing test samples in an individual well of each 96-well plate and incubated under the same conditions for 24 hr. The culture medium containing the test samples was removed and replaced with 100 μL of fresh medium. MTT solution (50 μL) was added to each well and the plates were incubated for a further 4 hrs. Finally, the supernatant was removed and the formazan crystals which were produced by viable cells were dissolved with 100 μL of dimethyl sulfoxide (DMSO). The absorbance of the formazan solution was read spectrophotometrically at 570 nm. The percentage of viable cells was calculated with the following equation:

$$\%\text{viability} = [(OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank})] \times 100$$

Where OD sample, OD blank, OD control were the absorbances at 570 nm of the sample, blank and control wells, respectively.

2.2.11. Investigation of in vitro anti-inflammatory activity of raft-forming formulations using the nitric oxide (Nitrite / Nitrate) assay

RAW264.7 cells at a density of 1 × 10^6 cells/well in 96-well plates were treated with free quercetin, quercetin/PVP solid dispersion, raft-forming liquid and tablet formulations with or without 1 μg/mL lipopolysaccharide (LPS) for 24 hr. The quercetin content of the test samples was equivalent to15 μg/mL of culture medium in an individual well of each 96-well plate. Cells that were not exposed to LPS or quercetin-containing samples were utilized as a negative control, and cells treated with LPS alone were utilized as a positive control. Following incubation for 24 hr, the supernatant (100 μL) was transferred to separate 96 well plates for measurement of NO. Due to the short half-life of NO, nitrite (NO^-) a stable NO metabolite was used for indirect measurement of NO content. Griess reagent (100 μL) was added to the supernatant samples to react with nitrite, producing an azo-compound which absorbs light at 540 nm. The concentration of nitrite was determined by comparison with a sodium nitrite standard curve (3–20 0 μg/ml) prepared in deionized water.

2.2.12. Cell migration in the 'scratch' model of wound healing

AGS cells were seeded in 6-well plates to reach 100 % confluent monolayers. A 'scratch' wound model was created in the mono-

Table 2
Composition of raft-forming chewable tablets.

Ingredients (mg)	Formulations										
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11
Solid dispersion	110	110	110	110	110	110	110	110	110	110	110
Sodium alginate	100	200	300	400	300	300	300	300	300	300	300
HPMC K100	0	0	0	0	25	50	100	25	25	25	25
$NaHCO_3$	100	100	100	100	100	100	100	100	100	100	100
$CaCO_3$	100	100	100	100	100	100	100	100	100	100	100
Mannitol	400	300	200	100	175	150	100	150	125	150	125
Menthol	10	10	10	10	10	10	10	10	10	10	10
Aspartame	5	5	5	5	5	5	5	5	5	5	5
Lemon flavor	20	20	20	20	20	20	20	20	20	20	20
Magnesium sterate	5	5	5	5	5	5	5	5	5	5	5
PVP K 30	0	0	0	0	0	0	0	25	50	0	0
PVP K 90	0	0	0	0	0	0	0	0	0	25	50
Total	850	850	850	850	850	850	850	850	850	850	850

layer by producing a cell-free line with a sterile 200 μL pipette tip and designated as time 0 h of wound repair. The negative control was provided by untreated cell monolayers which exhibited the normal capacity for cell migration. The cells were treated with sample groups consisting of free quercetin, quercetin/PVP solid dispersion, raft-forming liquid and powdered tablet formulations. The quercetin content of the test samples was equivalent to 15 μg/mL of culture medium in the 6-well plate. The cell monolayers were incubated at 37 °C in a humidified atmosphere with 5% CO2 to allow cell migration into the wound scratch area. The old medium were removed and then replaced with a fresh medium containing samples before viewing cell migration for the next 24 h. Images were acquired at the same position at 0, 24, 48, and 72 h using a microscope with digital camera (EVOS™ XL Core Imaging System, Thermo Fisher Scientific Inc., Massachusetts, USA) and scratch closure was measured using the software ImageJ version XX. The wound healing capacity of quercetin-containing formulations was determined by comparing the distance of cell migration toward the center of the scratch with the negative (untreated) control.

2.2.13. Statistical analysis

The data obtained were expressed as the mean ± standard deviation (S.D.). The data analysis was performed by Student's *t*-test and one-way analysis of variance (ANOVA). Statistical probability (p) values<0.05 was considered as significantly different.

3. Results and discussion

3.1. Solubility of quercetin solid dispersions and physical mixtures

The solubility of quercetin solid dispersion prepared using PVP K 30 and PVP K 90 respectively and their physical mixture was investigated in both distilled water and 0.1 N HCl (pH 1.2) . The solubility of quercetin powder was extremely low (0.76 ± 0.15 μg/mL in distilled water and 0.86 ± 0.03 μg/mL in 0.1 N HCl (pH 1.2), in agreement with other reports (Wang et al., 2016). The solubility of quercetin was improved by formulating physical mixtures with PVP K 30 and PVP K 90 but values were still confined to less than 1 mg/mL (Fig. 1). In contrast, the solubility of quercetin was increased significantly to around 10 mg/mL in HCl solution by production of solid dispersions with PVP K 30 or PVP K 90. The improvement in quercetin solubility when formulated as a solid dispersion is considered to result from the water solubility of PVP. When polymer chains with attached drug molecules were dissolved in the solution medium, PVP acted as the wetting agents facilitating solution medium to come in contact and penetrate into the pure drug resulting in increased the solubility of quercetin (Chen et al., 2020). The solubility of quercetin solid dispersions was enhanced significantly by increasing the content the hydrophilic carrier (1:10 > 1:8 > 1:5 > 1:3), which may be explained by the high dispersion or dilution of the quercetin phase and improved wettability (Lu et al., 2014). Moreover, PVP K30 was found to be the most effective carrier to employ in solid dispersions to increase the solubility of quercetin in both distiled water and HCl solution.

3.2. Release behavior of of quercetin solid dispersions and physical mixtures

The release behavior of quercetin from solid dispersions and physical mixtures with PVP K30 and PVP K90 in 0.1 N HCl solutions at 37 °C is shown in Fig. 2. The dissolution of unformulated quercetin powder was found to be restricted to 1.2 ± 0.2 % after 2 h. Quercetin release from physical mixtures with PVP K 30 and PVP K 90 was increased to around 13% over the 2 h time period. Solid dispersions of quercetin with PVP K 30 showed increasing quercetin release with increasing content of the hydrophilic carrier polymer, namely 20, 30, 70 and 100% for 1:3, 1:5, 1:8 and 1:10 quercitin : PVP ratios respectively (Fig. 2a). Solid dispersions formulated using PVP K 90 showed lower quercetin release (71% and 52%, for 1:10 and 1:8 ratios and zero release for 1:3 and 1:5 ratios, Fig. 2b). This behavior supports the findings of Knopp et al. that the increased viscosity of the diffusion 'pathway' through the high molecular weight polymer was expected to lead to a decrease in drug dissolution rate (Knopp et al., 2015). Quercetin solubility and release behavior were found to be enhanced in the present study by forming solid dispersions with PVP K30 at a 1:10 ratio. These formulations were subsequently characterized in detail using SEM, PXRD and FTIR prior to incorporation in raft-forming liquid and chewable tablet oral dosage forms.

3.3. Physicochemical characterization of quercetin solid dispersions

3.3.1. Scanning electron microscopy (SEM)

SEM revealed an acicular crystal morphology for quercetin powder with crystals having length 8–30 and width of approximately 2–10 μm (Fig. 3a). PVP K 30 used as a hydrophilic carrier in SDs was made up of spherical particles ranging from 20 to 40 μm in diameter (Fig. 3b). Physical mixtures of quercetin and PVP K30 (Fig. 3c) exhibited spherical particles characteristic of PVP K30 and the acicular structures corresponding to quercetin. In contrast the irregular particles of quercetin/PVP K30 solid dispersions

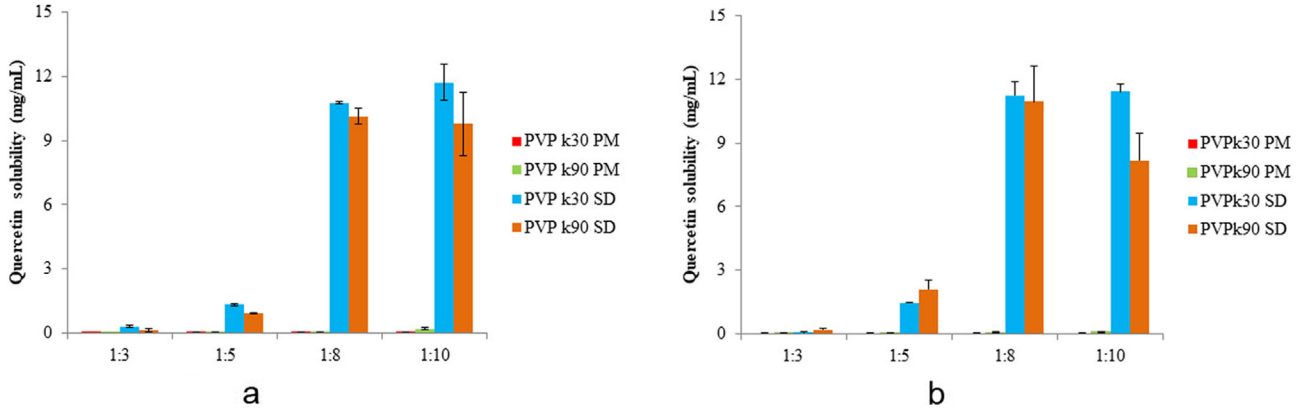

Fig. 1. The solubility of quercetin-solid dispersions (SD) and physical mixtures (PM) in (a) distilled water and (b) 0.1 N hydrochloric acid (pH 1.2). Bars represent mean ± SD (n = 3).

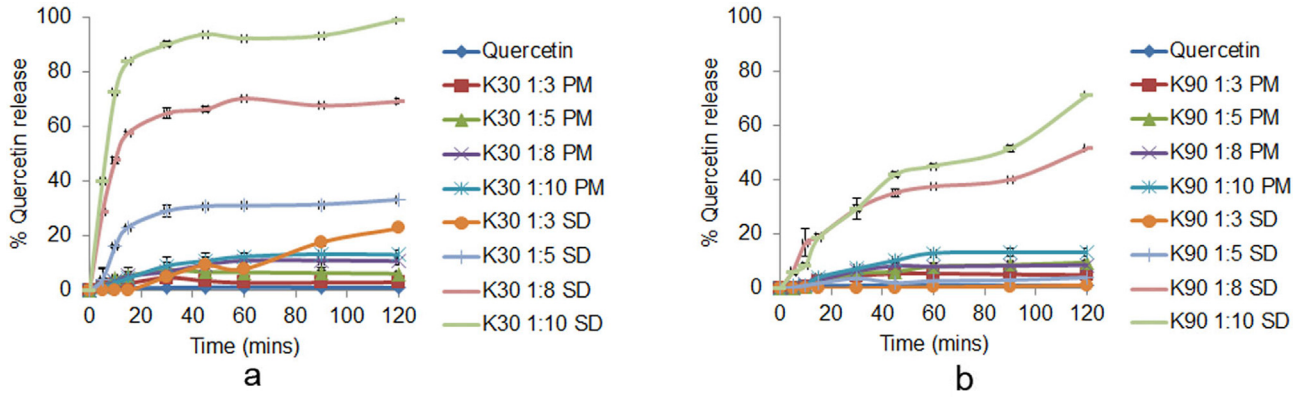

Fig. 2. Release profiles of quercetin solid dispersions (SD) and quercetin physical mixtures (PM) with (a) PVP K30 and (b) PVP K90 in 0.1 N hydrochloric acid (pH 1.2). Bars represent mean ± SD (n = 3).

with an absence of acicular crystals or spherical particles (Fig. 3d) indicated miscibility of both components of the SD.

3.3.2. Powder X-ray Diffraction studies (PXRD)

The crystallinity of quercetin powder was confirmed by PXRD. The powder diffraction pattern of quercetin (Fig. 4) showed the high-intensity diffraction reflections at 2θ values of 10.78, 12.46, 15.80, 16.15, 23.87, and 27.37. PVP K 30 showed broad reflections, indicating its existence in the amorphous form (YANG et al., 2017). The PXRD pattern of quercetin and PVP K 30 physical mixture revealed the presence of crystalline reflections at 2θ value of 22, for example and the broad doublet reflection characteristic of the amorphous PVP K30 component (Fig. 4c). Crystalline diffraction peals were not evident in the case of quercetin/PVP K30 solid dispersion (Fig. 4d) indicating conversion of quercetin to the amorphous form and providing an explanation for the increased solubility of the flavonoid in SDs compared with physical mixtures (Section 3.1). Similar findings were reported by de Mello Costa et al. for quercitin/PVP K25 solid dispersions (de Mello Costa et al., 2011).

Preparation of quercetin/PVP K30 solid dispersion by co-dissolution in ethanol, followed by solvent evaporation evidently resulted in high miscibility and strong intermolecular association between the flavonoid and PVP, which subsequently restricted crystallization of quercetin. FTIR analysis was performed to investigate the physicochemical nature of the interaction.

3.3.3. Fourier transform infrared spectroscopy

FTIR spectra of quercetin powder, PVP K 30, quercetin/PVP K30 solid dispersion and physical mixture are shown in Fig. 5. The FTIR spectrum of quercetin revealed prominent peaks at 3408 cm^{-1} and 3322 cm^{-1}corresponding to O-H stretching. The peak at 1663 cm^{-1} was due to C=O stretching in aryl group of trihydroxychromen-4-one part, while the aromatic ring C=C stretching produced three bands at 1610, 1562, and 1521 cm^{-1}. The peak at 1319 cm^{-1} was also attributed to C—H bending of the aromatic ring. The C—O stretching in the trihydroxychromen-4-one ring appeared at 1261 cm^{-1} and 1199 cm^{-1}, respectively. These findings are in agreement with the published data on quercetin (Catauro et al., 2015; Qi et al., 2015; Thanh Nguyen and Goycoolea, 2017). The FTIR spectrum of PVP K 30 showed characteristic peaks associated with the presence of a nitrogen heteroatom and carbonyl groups in the pyrrolidone ring of PVP similar to previous reports (-Tantishaiyakul et al., 1999). The stretching peak of C=O appeared at 1660 cm^{-1} while the peak at 1290 cm^{-1} was related to C—N stretching of amide groups. The FTIR spectrum of PVP K30 also displayed a broad peak at 3448 cm^{-1} due to OH stretching vibrations of absorbed water. Almost all peaks in the FTIR spectrum of physical mixture and solid dispersion corresponded with the spectrum of PVP K 30 due to the predominance of the polymer in the samples. However, there were important differences. The small peaks associated with C=C stretching of quercetin were observed at 1613, 1563, and 1521 cm^{-1}in physical mixture but were absent in the solid dispersion. The disappearance of typical peaks of quercetin in solid dispersion might be due to the complete incorporation of quercetin in PVP K30. Moreover, the O-H stretching peak in the FTIR spectrum of solid dispersion showed a broad peak at 3435 cm^{-1} compared with the corresponding peak at 3408 cm^{-1} and 3412 cm^{-1}in the quercetin powder and physical mixture spectrum respectively. The considerable shift of the O-H stretching peak in the solid dispersions spectrum to higher wave numbers indicated the occurrence of quercetin/PVP K30 intermolecular hydrogen bonds which prevent crystallization of quercetin (Li et al., 2013).

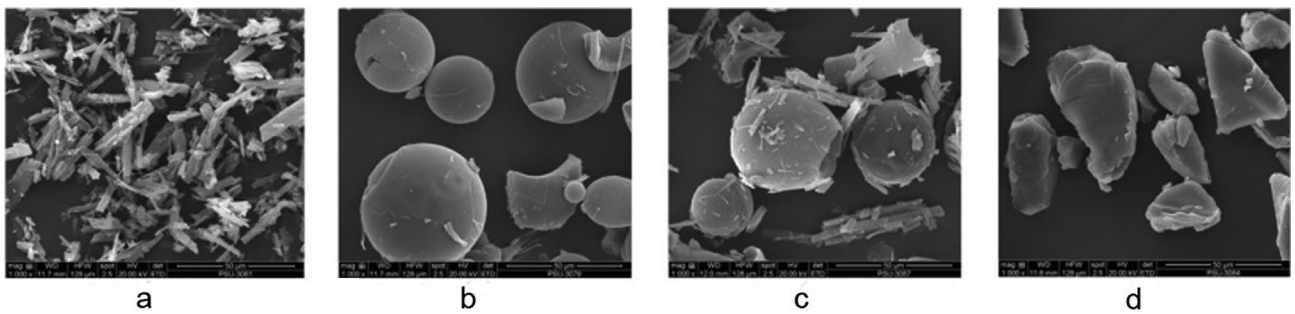

Fig. 3. The surface morphology of (a) quercetin powder , (b) PVP K30, (c) 1:10 quercetin-PVP K 30 physical mixture and (d) 1:10 quercetin -PVP K30 solid dispersion.

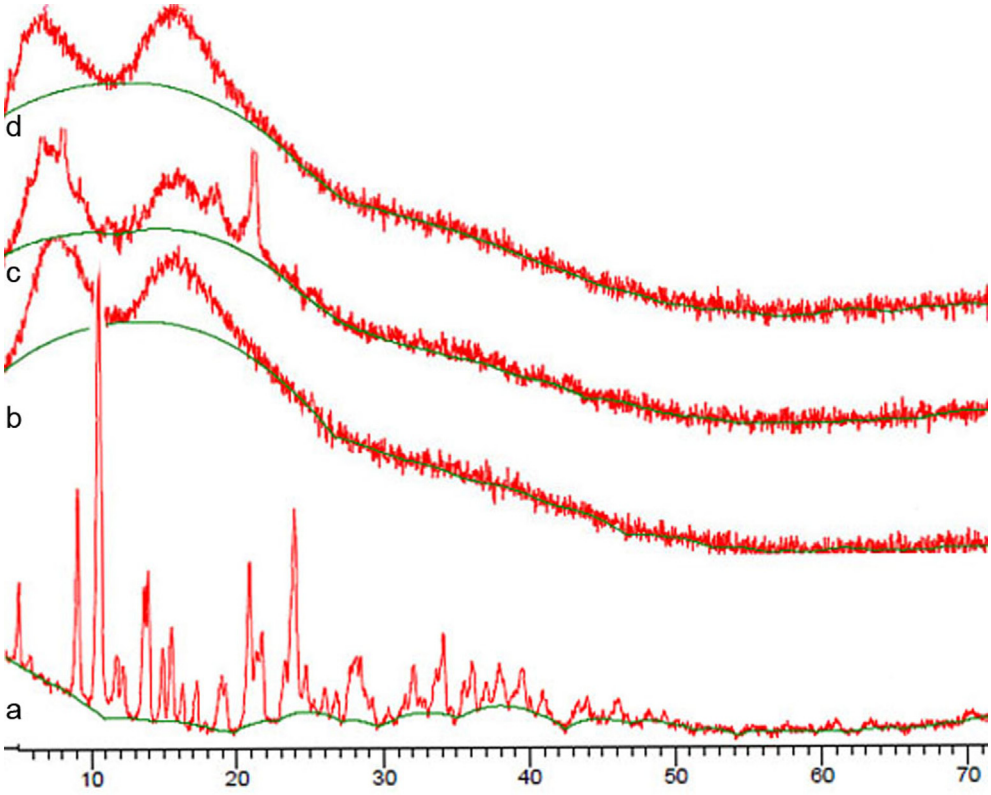

Fig. 4. Powder X-ray diffraction pattern of (a) quercetin powder, (b) PVP K30, (c) 1:10 quercetin-PVP K 30 physical mixture and (d) 1:10 quercetin-PVP K 30 solid dispersion.

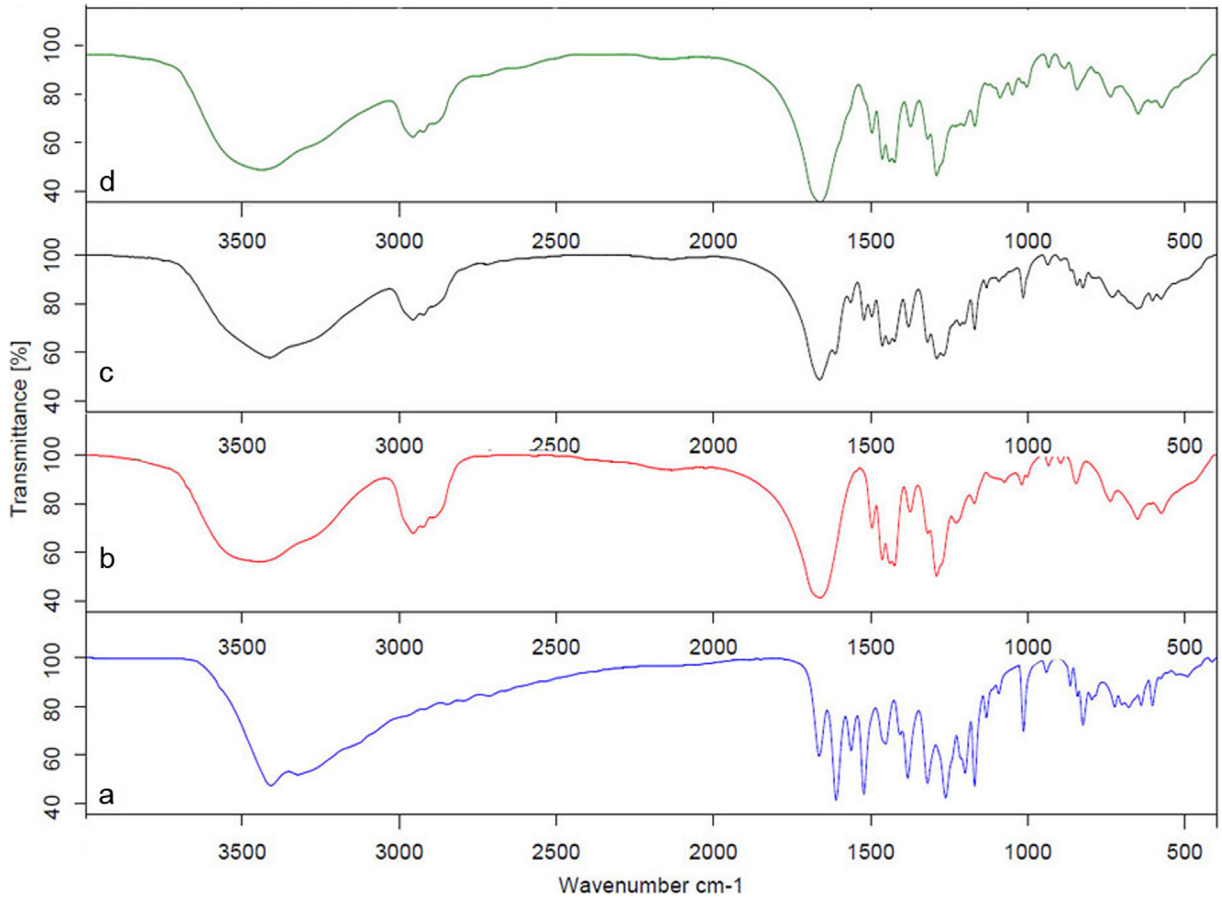

Fig. 5. Fourier transform infrared spectra of (a) quercetin powder, (b) PVP K30, (c) 1:10 quercetin-PVPK 30 physical mixture and (d) 1:10 quercetin-PVP K30 solid dispersion.

3.4. Physicochemical characterization of raft-forming liquid formulations

3.4.1. Viscosity measurements

The viscosity values of the raft-forming liquid formulations are presented in Table 1. Viscosity increased with an increase in the sodium alginate concentration (F1 > F2, $p < 0.05$) and HPMC concentration (F5 > F4 > F3 and F8 > F7 > F6). The viscosity of all liquid formulations decreased with increasing shear rate Fig. 6, indicative of pseudoplastic or shear thinning behavior, but was more marked for liquid formulations, F4, F5, F7 and F8 which were formulated using higher HPMC concentration solutions (0.5 and 1% w/v). Shear thinning is a desirable property of liquid pharmaceutical formulations due to ease of pouring and redispersal of particulate sediments.

3.4.2. Raft density and buoyancy testing

The rafts formed by gastroretentive delivery systems are intended to float on the stomach content, leading to an increase in gastric residence time for sustained drug release. Exposure of raft-forming liquid formulations to acidic medium in the present study, resulted in liberation of divalent Ca^{2+} ions from the $CaCO_3$ excipient, which acted as a crosslinking agent in alginate hydrogel formation together with CO_2, which was entrapped within the gel matrix resulting in buoyancy. The density of all rafts formed from liquid formulations (Table 1) was consequently much less than 1.004 g/cm^3 (the density of gastric medium). Furthermore, the rapid floatation of all liquid formulations (lag time 6–15 s) is highly advantageous since they would move away quickly from the pyloric orifice instead of exiting into the small intestine (Singh et al., 2013).

3.4.3. Raft strength

Although raft-forming preparations floated on SGF for>24 h (Table 1), gastric residence time *in vivo* may be disturbed by raft breakage under peristaltic movement. The raft-forming liquid formulations contained sodium alginate solution and insoluble $CaCO_3$ which released Ca^{2+} on exposure to acidic medium. Alginate is converted to a hydrogel by crosslinking with divalent metal ions, such as Ca^{2+}, which bind to guluronic acid blocks of two adjacent alginate polymer chains giving rise to a 3-D gel network. The raft strength was found to increase from be 9.6 and 11.2 g, with increasing alginate solution concentration (Formulations F1 and F2) indicating a higher crosslink density within the hydrogel (Nahar et al., 2017). An increase of HPMC solution concentration in the liquid formulation reduced the resulting raft strength, particularly when combined with 1% alginate solution (F1, F5), indicating disruption of the gel network. This occurrence might be due to the interaction between HPMC and calcium cation (Ca^{2+}) that could be explained by the same reason in the previous study. Nokhodchi and Tailor found that there was the interaction between HPMC and aluminium cation (Al^{3+}) acted as a sustaining agent and a crosslink agent respectively in the in situ alginate gel formulations; thus the Al^{3+} was not able to crosslink with alginate, leading to weakened alginate gel (Nokhodchi and Tailor, 2004). However, the reduction of raft strength due to an increase of HPMC concentration was not significant when liquid formulations were produced using 2% alginate solution (F6, F7, F8). The raft strength of all liquid formulations tested in the present study (6–11 g) was still found within the range of commercial products (4–16 g) (Hampson et al., 2005).

3.4.4. In vitro release studies

The release profiles of quercetin from raft-forming liquid formulations in 0.1 N HCl (pH 1.2) are shown in Fig. 7. Formulations based on 1% and 2% alginate solution exhibited 'bust release' of around 90 and 80%, respectively in the first 30 min of exposure to the medium and complete release at 8 hr. The crosslinked alginate gel matrix was incapable of delaying diffusion of quercetin, following rapid dissolution from the quercetin/PVPK30 solid dispersion. Several studies have reported that the burst release effect may be reduced by increasing the concentration of polymer in the delivery system (Chakraborty et al., 2009; Mašková et al., 2019). However, the alginate solution concentration was limited to 2% w/v in the present study due to the resulting high viscosity. The addition of HPMC K 100 M to the liquid formulations to lower the rate of quercetin release, resulted in a major reduction of the burst release phase to around 50% (F5, F6, F7, F8), followed by gradual release of over 90% of the quercetin content by 8 h. Similar findings were reported by Abbas et al (Abbas et al., 2017). The delayed release may be explained by formation of a viscous HPMC gel on exposure to acidic medium which augmented alginate gel formation to form a high resistance barrier to quercetin diffusion (Sahoo et al., 2015). Increasing the HPMC content was found to be effective in reducing the release of quercetin from the raft-forming liquid preparations, particularly when combined with the higher (2%) alginate solution.

3.5. Characterization of raft-forming chewable tablets

3.5.1. Physical properties of chewable tablet formulations

Tablet weight, thickness, hardness, and friability of the different raft-forming chewable tablet formulations are shown in Table 3. All formulations exhibited consistency in weight and thickness.

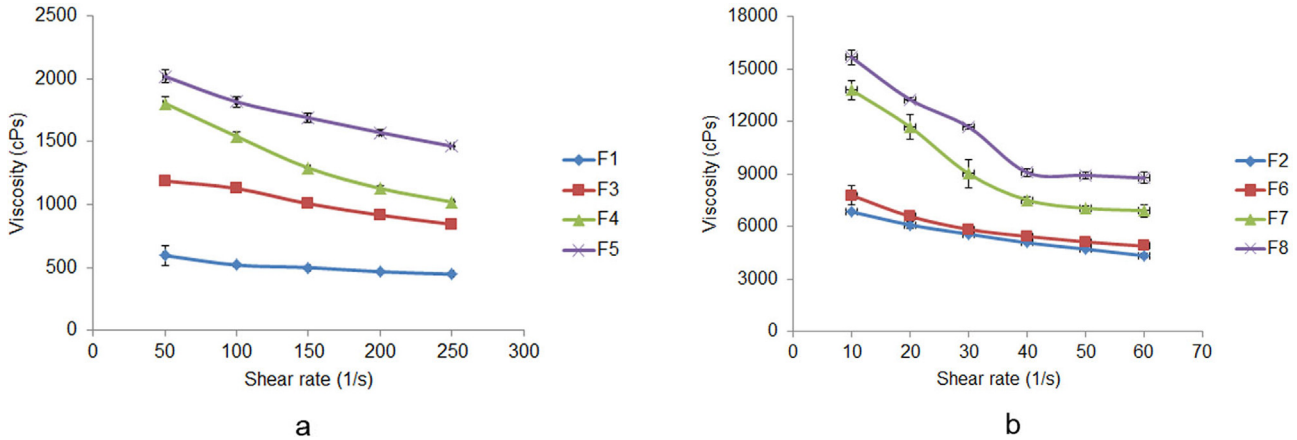

Fig. 6. Viscosity profiles of raft-forming liquid formulations (Composition F1, F2 etc listed in Table 1).

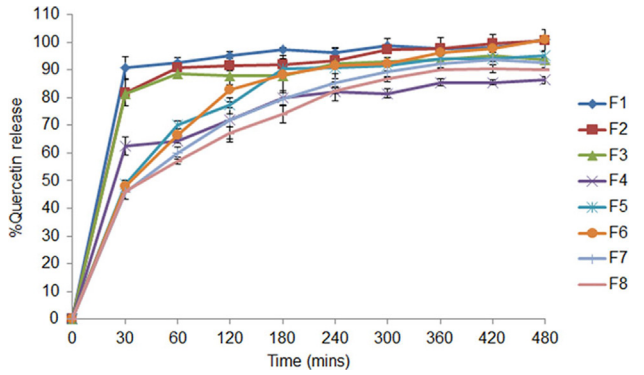

Fig. 7. Release profiles of quercetin from raft-forming liquid formulations in 0.1 N HCl (pH 1.2) Bars represent mean ± SD (n = 3).

Chewable tablets are required to possess adequate hardness to facilitate consumption yet avoid damage during packaging and transport. Famotidine raft-forming chewable tablets exhibited a hardness of 5–6 kg/cm^2 (Prajapati et al., 2012). Formulations based on sodium alginate and HPMC polymers (T1-T7) exhibited low hardness values below 4 kg/cm^2. However the addition of PVP K 30 to the tablet formulation at 25 mg and 50 mg respectively (T8, T9) or PVP K90 (T10 and T11) increased hardness to the acceptable range. The addition of PVP also improved friability properties. Friability for all the formulations containing PVP was in the range of 0.28–0.57% (Table 3).

3.5.2. Raft strength

The raft strength of powdered tablets was influenced by the content of sodium alginate and HPMC K 100 M similar to the raft-forming liquid systems. Raft strength increased significantly from 1 to 12 g with increasing sodium alginate content of 100 to 400 mg (T1-T4, Table 3), indicating formation of a progressively stronger hydrogel network. However, the addition of 100 g of HPMC K 100 M to the formulation (T3, T7) reduced the raft strength by approximately 25%, indicating disruption of the alginate gel network.

3.5.3. In vitro release studies

The presence of sodium alginate in the raft-forming powdered tablets at levels of 100, 200, 300 and 400 mg (T1-T4) resulted in a significant reduction of the burst release phase of quercetin from 93 to 81, 58 and 27 % respectively at 30 min (Fig. 8a). Gradual release of over 90% of the quercetin load occurred over 8 h from powdered tablets containing up to 300 mg sodium alginate, but release was restricted to around 60% for powdered tablets featuring the highest alginate content (400 mg).These findings are in accordance with previous studies (Rajinikanth et al., 2007) and may be explained by formation of alginate hydrogel of increasing density with increasing alginate concentration, which impedes diffusion of quercetin. Among the raft-forming powdered tablet formulation T3 resulted in the lowest burst release phase and complete release of quercetin in 8 h and was therefore selected for further development.

The incorporation of HPMC in the powdered tablet formulation at levels of 25, 50 and 100 mg (T5, T6, T7) resulted in similar release profiles but a significant reduction of the burst release phase of quercetin (at 30 min) to around 40% compared with 60% when HPMC was absent (T3, Fig. 7b). Powdered tablets containing HPMC released between 80 and 86% of the quercetin load at 8 h. The effect of HPMC K 100 M on the release profiles of powdered tablets was consistent with the study of raft-forming liquid systems (Section 3.4) and may be similarly explained by a combination of viscous gel formation by HPMC and alginate gel formation by Ca^{2+} crosslinking which impedes diffusion of quercetin molecules. The powdered tablet formulation containing sodium alginate and HPMC (T5) displayed low burst release in SGF (40%) followed by sustained quercetin release to a level of around 80% over the 8 h test period but unacceptably low hardness and friability (Table 3). The addition of 25 and 50 mg PVP K30 respectively to the formulation (T8, T9) resulted in increased burst release to above 60% (Fig. 8c), which may be explained by increased wettability of the powdered tablet, leading to enhanced dissolution of quercetin. In contrast, the addition of the higher molecular weight PVP K 90 to the T5 formulation (25 mg, T10, 50 mg, T11) reduced the burst release effect to around 30% and sustained release of quercetin to reach a level of around 70%. at 8 h. In summary, the release study recommended that the T10 formulation containing 300 mg of alginate (raft forming agent), 25 mg of HPMC (release retarding agent), and 25 mg of PVP K 90 (hardness modifier) as the most suitable for further development and evaluation using biological assays.

3.6. Effect of raft-forming formulations on cell viability

Cell viability (MTT) assays were performed to assess the concentration of raft-forming liquid and tablet formulations which would be tolerated by cells when administered for treatment of gastric ulcers. Raw 264.7 cells derived from murine macrophage cell line and AGS cells derived from human gastric epithelial cell line were used for assay and untreated cells served as controls. RAW 264.7 and AGS cell viability after 24 h exposure to test samples are shown in Fig. 9(a) and (b), respectively. Both types of cell displayed very high viability in excess of 99% when exposed to raft-forming liquid and tablet formulations containing quercetin equivalent concentrations up to 15 μg/mL. However, cell viability was reduced significantly to around 80% when RAW264.7 cells were exposed to raft-forming formulations containing the equivalent quercetin concentration of 20 μg/mL (Fig. 9a). These findings indicated that the maximum non-cytotoxic concentration of all

Table 3
Physical properties and raft strength of raft-forming chewable tablet formulations.

Parameters	Formulations										
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11
Hardness (kg/cm^2)	3.4 ± 0.3	2.3 ± 0.2	1.7 ± 0.2	1.3 ± 0.4	1.9 ± 0.1	1.8 ± 0.2	3.2 ± 0.7	6.3 ± 0.6	10.4 ± 0.7	6.1 ± 1.5	6.1 ± 1.6
Thickness (mm)	3.3 ± 0.0	3.2 ± 0.2	3.1 ± 0.0	3.0 ± 0.2	3.0 ± 0.0	3.1 ± 0.0	3.2 ± 0.2	3.1 ± 0.0	3.0 ± 0.0	3.0 ± 0.7	3.0 ± 0.1
Weigh (mg)	842.0 ± 2.3	848.0 ± 3.8	845.4 ± 2.7	840.0 ± 4.6	843.0 ± 5.7	852.0 ± 2.5	849.4 ± 3.2	843.2 ± 1.7	835.2 ± 4.5	841.6 ± 3.1	843.4 ± 2.2
Friability (%)	break	break	break	break	Break	break	break	0.54	0.28	0.41	0.57
Raft strength (g)	1.0 ± 0.0	3.07 ± 0.2	8.0 ± 0.4	12.0 ± 1.3	6.8 ± 1.0	6.8 ± 0.5	5.9 ± 0.9	10.2 ± 0.3	9.1 ± 0.7	9.8 ± 0.5	7.2 ± 0.1

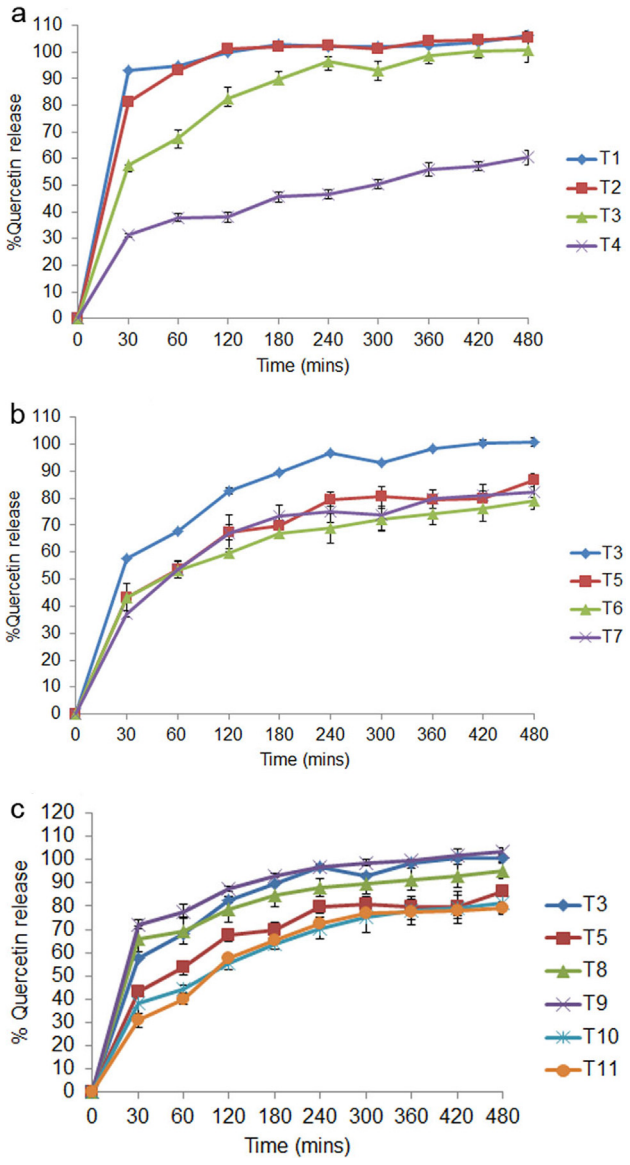

Fig. 8. Release profile of quercetin from raft-forming chewable tablets in 0.1 N HCl (pH 1.2). Bars represent mean ± SD (n = 3).

samples in RAW264.7 cells was found to be 15 μg/mL. This non-cytotoxic concentration was used to study in RAW264.7 cells to approve that the inhibition of NO synthesis by the quercetin was not simply due to cytotoxic effects. AGS cells similarly to RAW264.7 cells were sensitive to high concentration of quercetin. The viability of AGS cells was also reduced to around 70% (Fig. 9b) at the concentrations above 15 μg/mL. Samples containing the equivalent quercetin concentration of 15 μg/mL were chosen for wound healing studies by *in vitro* scratch assay to avoid the disturbance in AGS cells migration induced by high dose of quercetin.

3.7. Investigation of in vitro anti-inflammatory activity of raft-forming formulations

Overproduction of nitric oxide via inducible nitric oxide synthase is a widely recognised signal of inflammatory activity for development of gastritis and gastric cancer and its suppression by administered compounds forms the basis of assays of anti-inflammatory agents (Adebayo et al., 2019). The NO inhibitory activity of quercetin powder, quercetin-PVP K 30 solid dispersion, raft-forming liquid formulation (F7) and tablets (T10) was evaluated in RAW 264.7 cells stimulated using LPS. Test samples contained the equivalent of 15 μg/mL quercetin. NO levels of 2.78 ± 0.44 μg/mL and 25.51 ± 1.20 μg/mL were measured for cells which had not received LPS stimulation (negative control group) and LPS-stimulated cells (positive control), respectively. The secretion levels of NO following exposure of RAW 264.7 cells, which had not received LPS stimulus, to quercetin-containing samples were 2.57 ± 0.35 μg/ml, 2.49 ± 0.34 μg/ml, 2.35 ± 0.38 μg/ml, and 2.59 ± 0.26 μg/ml in pure quercetin, quercetin solid dispersion, raft-forming liquid (F7) and tablet (T10), respectively. No significant differences were measured between the negative control, quercetin powder, solid dispersion and raft-forming formulations, demonstrating an absence of inherent inflammatory properties of the quercetin-containing formulations. When LPS was combined with samples, In contrast, NO production was significantly decreased when LPS-stimulated RAW 264.7 cells were treated with quercetin powder (12.89 ± 1.33 μg/mL), quercetin solid dispersion (20.56 ± 0.89 μg/mL), raft-forming liquid (F7, 21.03 ± 1.10 μg/mL) and raft-forming tablet (T10, 18.76 ± 0.86 μg/mL), ($p < 0.05$), indicating anti-inflammatory activity. Interestingly, the unformulated quercetin powder sample exhibited the highest anti-inflammatory activity. The explanation for this behavior is as yet unclear but may involve the strong intermolecular association established between PVP K 30 and quercetin during formation of the solid dispersion reducing the bioactivity of the flavonoid. One or more of the excipients included in the liquid and tablet formulations (eg. sodium alginate, HPMC) may also impose an interference effect on quercetin's anti-inflammatory activity.

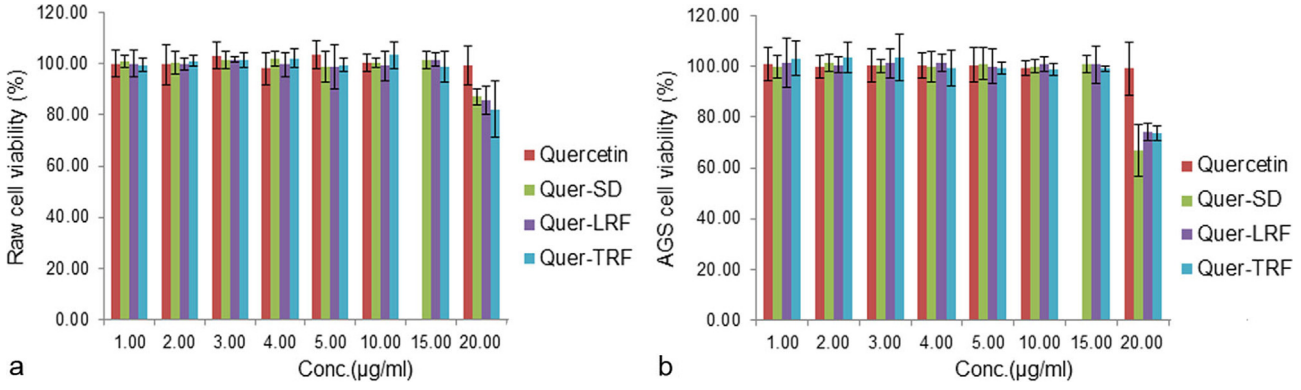

Fig. 9. Percentage cell viability of a) Raw 264.7 cells and b) AGS cells following exposure to quercetin powder, quercetin-PVP K30 solid dispersion, raft-forming liquid formulation (F7) and raft-forming tablet (T10). Cells were exposed to samples having equivalent quercetin concentrations of 1, 2, 3, 4, 5, 10, 15, 20 μg/mL. Data show the mean ± SD from four independent experiments (n = 4).

Table 4
Percentage wound closure in the AGS cell monolayer 'scratch' model due to treatment with quercetin powder, quercetin-PVP K30 solid dispersion, raft-forming liquid (formulation F7) and raft-forming tablet (formulation T10) 15 μg/mL equivalent dose of quercetin.

Samples	% Gap (wound) closure (n = 4, mean ± SD)			
	0 h	24 h	48 h	72 h
Negative control	0.00 ± 0.0	13.51 ± 3.7	34.59 ± 3.1	43.62 ± 3.3
Quercetin powder	0.00 ± 0.0	15.99 ± 2.3	51.98 ± 5.5	92.55 ± 8.6
Quercetin-PVP K30 solid dispersion	0.00 ± 0.0	21.07 ± 4.6	59.02 ± 3.5	88.40 ± 2.3
Raft forming liquid (F7)	0.00 ± 0.0	36.16 ± 9.9	79.43 ± 6.2	96.17 ± 7.7
Raft forming tablet (T10)	0.00 ± 0.0	11.26 ± 2.4	42.27 ± 5.6	71.65 ± 4.0

3.8. Cell migration in the 'scratch' model of wound healing

The wound healing potential of quercetin-containing, raft-forming liquid and tablet formulations was assessed using the 'scratch model' based on AGS cell monolayers. Wound closue resulting from cell migration was visualized and quantified using optical micrographs and expressed in terms of % gap reduction (Table 4). The result reveals gradual gap (wound) closure over time and highly efficient gap closure at 72 h for cell monolayers exposed to quercetin powder (93%), solid dispersion (88%), and raft-forming liquid formulation (96%) compared with untreated cell monolayers (control, 44%). Exposure of cell monolayers to raft-forming tablets resulted in lower gap closure at 72 h (72%), which may be explained by excipients in the more complex tablet formulation (Table 2) hindering cell adhesion and migration by modification of the substrate. The anti-inflammatory and wound healing potential of raft-forming liquid and tablet formulations demonstrated in the present study support *in vivo* findings of favorable healing properties of quercetin in acetic acid-induced gastric ulcers in rats (Suzuki et al., 1998).

4. Conclusions

Raft forming gastroretentive systems incorporating quercetin PVP K30 solid dispersions were developed in liquid and chewable tablet dosage forms for potential treatment of gastric ulcers. Both type of formulations exhibited rapid flotation (<1 min), long duration of floating (>24 h) and favourable raft strength on exposure to the acidic medium. Sustained release of >80% of the quercetin content was achieved from both types of formulation over 8 h in SGF at 37 °C. The raft-forming liquid and tablet formulations, at 15 μg/mL equivalent quercetin dose, maintained high viability of RAW 264.7 cells and AGS cells in culture, produced anti-inflammatory activity against LPS-stimulated RAW 264.7 cells and wound healing activity in the AGS cell monolayer 'scratch' model. These findings demonstrate the strong potential of both raft forming liquid and chewable tablet dosage forms containing quercetin solid dispersions for treatment of gastric ulcers.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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