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Original article

Development of apigenin loaded gastroretentive microsponge for the targeting of *Helicobacter pylori*

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ABSTRACT

The goal of the present work was to invent an apigenin-stacked gastroretentive microsponge to target *H. pylori*. The quasi-emulsion technique was used to prepare microsponges, which were then tested for various physicochemical properties, in-vivo gastric retention, and in-vitro anti-H. pylori study. The microsponge that demonstrated a comparatively good product yield (76.23 ± 0.84), excellent entrapment efficiency (97.84 ± 0.85), sustained in-vitro gastric retention period, and prolonged drug release were chosen for further investigations. The microsponge's SEM analysis showed that it had a spherical form, porous surface, and interconnected spaces. No drug-polymer interactions were detected in the FTIR investigation. Apigenin was found to be dispersed in the microsponge's polymeric matrix according to DSC & XRD investigations. Moreover, the microsponge in the rat's stomach floated for 4 h, according to the ultrasonography. The antibacterial activity of apigenin against *H. pylori* was nearly two folds more than the pure apigenin and had a more sustained release in the best microsponge, according to the in vitro MIC data, when compared to pure apigenin. To sum up, the developed gastroretentive microsponge with apigenin offers a viable alternative for the efficient targeting of *H. pylori*. But more preclinical & clinical studies of our best microsponge would yield considerably more fruitful results.

1. Introduction

Helicobacter pylori (*H. pylori*) is a gram-negative bacterium that infects the human gastric epithelium and leads to many gastric disorders including peptic ulcers and gastric cancer in a percentage exceeding 50% of the world's population (Bibi et al., 2017; Jaka et al., 2018). Previous studies suggest that the frequency of *H. pylori* infection amidst the population of Saudi Arabia is high (35%–67%) and the rate of prevalence is expanding with age (Eed et al., 2019). In 2017, clarithromycin-resistant *H. pylori* was listed as a high-priority pathogen by the World Health Organization (WHO) (Hu et al., 2020; González et al., 2019). Nowadays, antibiotics resistance is considered one of the main global issues in treating bacterial infections including *H. pylori* infection which has increased antibiotics resistance with a decline in eradication nearly to 60% in some countries (Jaka et al., 2018; Eed et al., 2019; González et al., 2019; Kasahun et al., 2020). Currently, choosing a potent therapy plan to eradicate *H. pylori* is difficult due to *H. pylori* resistance to levofloxacin, metronidazole, and clarithromycin. Following this further, due to clarithromycin resistance, standard triple therapy effectiveness against *H. pylori* is lower than 80% in many countries (Hu et al., 2017; Fiorini et al., 2020). In Saudi Arabia, metronidazole resistance can range from 60% to 80% which makes it one of the countries with the highest prevalence of metronidazole resistance (Jaka et al., 2018).

Abbreviations: APG, Apigenin; EGT, Eudragit; *H. pylori*, Helicobacter pylori; MIC, Minimum inhibitory concentration; USP, United States Pharmacopoiea; XRD, X-ray diffractometry; SEM, Scanning electron microscopy; FTIR, Fourier transform infrared spectroscopy; DSC, Differential scaning calorimetry.

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There are several regimens used to treat *H. pylori* infection (Gisbert, 2020). Clarithromycin triple therapy with either amoxicillin or metronidazole and proton pump inhibitor (PPI) is considered the first-line therapy (Chey et al., 2017). However, the clarithromycin-based regimen fails in more than 20–30% of cases to eradicate the infection (Gisbert, 2020). Bismuth-based quadruple therapy that includes bismuth, metronidazole, tetracycline, and PPI is an acceptable second-line treatment when clarithromycin-based treatment fails. Yet, the failure of eradication of *H. pylori* infection in Bismuth-based quadruple therapy reaches 20% of the cases and it is associated with more adverse events (Gisbert, 2020; Chey et al., 2017). Other regimens such as levofloxacin have a mean eradication rate of only 80% which is probably due to increased resistance of *H. pylori* (Gisbert, 2020).

Phytotherapy and plant-derived product have been used since ancient times, in which plant extracts were used for the treatment of several diseases (Sharifi-Rad et al., 2018). In addition, a broad range of secondary metabolites can be synthesized by plants which can include phenolic compounds metabolites such as flavonoids - found in fruits and vegetables- providing many biological activities (Parreira et al., 2014; Wang et al., 2019). As cure rates of *H. pylori* infection have decreased over time mainly due to antibiotic resistance; treating *H. pylori* infection by using an alternative approach such as phytotherapy instead of antibiotics has been thought to be a promising potential. Apigenin, chemically known as 4′, 5, 7,-trihydroxyflavone, is a naturally occurring yellow colored solid substance that belongs to the flavone class. Its molecular formula is $C_{15}H_{10}O_5$ and its molecular weight is 270.24. (Wang et al., 2019; Ali et al., 2016). According to Biopharmaceutics Classification System (BCS) apigenin has classified as Class II drug with low aqueous solubility and high permeability (DeRango-Adem and Blay, 2021). It has a high partition coefficient (log K 2.87.) which makes it able to penetrate biological membranes including the bacterial cell membrane and its high aqueous stability characteristic specially under acidic conditions (Li et al., 1997) makes it a suitable drug candidate to be used in the stomach area. Apigenin possesses multiple bioactivities including antioxidant, anti-inflammatory, anti-cancer, anti-viral, and anti-bacterial activities with a potent bactericidal effect against *H. pylori* infection (Yan et al., 2017). Furthermore, it shows an antibacterial action against three different *H. pylori* strains which include multi-drug resistance strains (Hu et al., 2020; González et al., 2019). Thus, the beneficial anti-*H. pylori* activity makes apigenin a considerable candidate for the eradication of *H. pylori* infection and consequently decreasing the progression *of H. pylori-induced* gastric cancer (González et al., 2019). Studies have shown that apigenin could act both systemically and locally. Upon oral administration apigenin undergo slow absorption from the GIT due to its poor aqueous solubility and the absorbed apigenin undergo phase I and phase II biotransformation in the liver generating major metabolites like luteolin. These metabolites are then immediately entered into the systemic circulation or recycled by local enteric/enterohepatic circulation resulting in biological response or systemic action (DeRango-Adem and Blay, 2021; Ali et al., 2017). As for local action is concerned, although the anti-*H. pylori* action demonstrated by the apigenin is likely multifactorial (Li., and Birt, 1996; Li et al., 1996), an increasing number of studies have successfully pinpointed the molecular targets of apigenin in *H. pylori*, unraveling its anti *H. Pylori* mechanism (González et al., 2019; Lee et al., 2013).

Microsponge is an advanced gastro-retentive drug delivery system of sponge microparticles consisting of highly pored microspheres and numerous interconnected voids ranging between 5 and 300 μm in diameter (Jafar et al., 2020; Chandra et al., 2017; Singhvi et al., 2019; Patel et al., 2016; Arathy and Sunil, 2020). Additionally, the microsponge system presents in different formulations including oral and topical forms, providing systemic and local effects (Arathy and Sunil, 2020). In oral application, the floating microsponges offer several advantages in which it enables as efficient drug targeting, prolonged drug release that reduces toxicity and allergic reactions, high drug loading capacity, self-sterilization, cost-effectiveness, improved solubility, and bioavailability with biocompatible and costless polymers (Arathy and Sunil, 2020; Arya and Pathak, 2014; Dharmasthala et al., 2018; Younis et al., 2019). Furthermore, the advantages of the microsponge system bridge are to promote patient quality of life and increase patient compliance (Lohot et al., 2020). The polymers commonly used in the formulation of microsponge are poly(ethyl acrylate), methyl methacrylate, & trimethyl ammonium-ethyl methacrylate chloride, in the ratios of 1:2:0.2 and 1:2:0.1, respectively, make up poly cationic polymers called Eudragit RS (EGT). The quaternary ammonium groups present in their structures decides their hydrophilicity characteristic of these polymers, i.e., higher the number of quaternary ammonium groups present higher would be the hydrophilicity of the polymer network. Only 5% of all eudragit polymers have these hydrophilic quaternary ammonium groups in their chemical structures and Eudragit RS polymers are one among them. Thus, Eudragit RS polymers remain practically insoluble in gastric acid fluid but permeable and could swell and form the matrix structure regardless of the pH in the region. Moreover, the polymer Eudragit RS 100 due to its low-density characteristics would make the microsponge float on the surface of the gastric acid fluid. These are thought to be the ideal properties of this polymer for use in the development of sustained release, floating matrix-type microsponge formulation (Tort et al., 2020; Thakral et al., 2013).

To reach successful targeting of *H. pylori* infection; especially in the case of metronidazole and clarithromycin-resistance, an effective novel therapy is needed. As described above apigenin has all the good qualities for its local action in the stomach such as slow absorption from the GIT, high stability in acidic environment, high partition coefficient, and most importantly its ability to bind to response regulator HsrA and inhibit its function in *H pylori* bacteria which is in the stomach mucosa. Moreover, its metabolite luteolin also exhibits the same functions as apigenin so, all these characteristics make this apigenin a most suitable candidate to be used to target and eradicate *H pylori* infection. Thus, the aim of this current study was to improve apigenin in a formulation that maintains suitable residence time in the stomach for effective targeted eradication of *H. pylori*. To the best of our knowledge, the optimization of apigenin into a floating microsponge system has not been studied yet. Therefore, developing apigenin floating microsponge using Eudragit RS 100 polymer as a potential therapy to achieve better targeting of the stomach infection caused by *Helicobacter pylori* is the principal goal of this study.

2. Material and methods

2.1. Material

Apigenin was purchased from Mesochem Technology. Beijing, China, Eudragit RS100 was procured from UFC Biotechnology, USA**,** Stabilizer polysorbate 80, and solvent acetone**,** were procured from SD fine chemicals Pvt. Ltd. in India**.** The *H. pylori* strain used in the study was ATCC43504 a reference strain which was procured from the America Type Culture Collection, USA. Bovine serum albumin, Brain Heart Infusion broth, and agar medium were purchased from Sigma Aldrich in the USA. For the in vivo study, Wistar albino rats were utilized. Other substances and additives used in the study were all the analytical research variety and were all obtained from the reputed sources.

2.2. Preparation of gastroretentive microsponge of apigenin

Quasi-emulsion method was employed to produce the apigenin (APG) gastroretentive microsponge formulations (Moran and Thomson 2020). Three steps were included in APG microsponge preparation. First, in a 100 ml capacity glass beaker, the organic phase was prepared by dissolving a fixed amount of APG, and fixed concentrations of Eudragit RS 100 (EGT) in a fixed volume of acetone. Second, in a 250 ml capacity glass beaker, the aqueous phase was prepared by dissolving stated amounts of emulsifying agent polysorbate 80 in distilled water (Table 1). Third, the organic phase was added to the aqueous phase in a dropwise manner with stirring at a rotation speed of 1500 rpm using an overhead stirrer (IKA Eurostar 20, Germany) and continued stirring for 90 min more. Lastly, the gastroretentive microsponges of APG found in the water phase were removed by filtration, they were then dried in the open air, and kept in a well-closed desiccator to be used for other investigations.

2.3. Physicochemical assessment of gastroretentive microsponge of apigenin

2.3.1. Product yield

For the estimation of the preparation method efficiency, known amounts of APG and EGT of all gastroretentive microsponges were accurately weighed using calibrated digital weighing machine, and the obtained weights were recorded, and they were read as theoretical weights. Then, the weights of prepared APG gastroretentive microsponges were obtained and they were considered as practical weights. Finally, to calculate the % product yield, the following formula was used:

$$\text{Product yeild}\,(\%) = \frac{\text{Actual weight of the gastroretentive microsponge}}{\text{Theoretical weight the drug and the polymer}\,(\text{APG}+\text{EGT})}\text{X}100$$

2.3.2. Estimating the drug content and entrapment efficiency

APG gastroretentive microsponge formulations weighing 10 mg of APG each were separately diluted with a small volume of acidic media having pH 1.2 in a 100 ml volumetric flask and the volume was then adjusted to 100 ml mark. After that, all the volumetric flasks were firmly closed, and then they were subjected to a sonication process for 12 h in a water bath at ambient temperature. Then, the media was filtered using Whatman filter paper and then suitably diluted with acidic media (pH 1.2) solutions, and absorbance for each sample was measured spectrophotometrically at a wavelength of 353 nm (Shimadzu 1700, Japan). Finally, % drug content and % entrapment efficiency of each APG gastroretentive microsponge formulations were calculated using the below-stated equations:

$$\text{Drug content}\,(\%) = \frac{\text{Actual quantity of APG in gastroretentive microsponge}}{\text{Weighed quantity of gastroretentive microsponge}} \times 100$$

$$\text{Entrapment efficiency}\,(\%) = \frac{\text{Actual quantity of APG in gastroretentive microsponge}}{\text{Theoretical quantity of APG in gastroretentive microponge}} \times 100$$

2.3.3. In-vitro gastric floating assessment

To assess the APG gastric floating microsponge formulations float ability the eight-basket Dissolution Test Station (USP XXIV), manufactured by Electrolab Pvt. Ltd. in India, was utilized as a USP type-2 dissolution study equipment. Dissolution flasks (made up of glass) were pervaded with a volume of 900 ml of acidic media (pH 1.2), rotated at 50 rpm and the temperature of 37 ± 0.5 °C was maintained throughout the experiment, and the quantities of the formulated APG gastroretentive microsponges which were ≅ 10 mg of APG were next transferred to the glass flasks of dissolution equipment containing acidic medium having pH 1.2. The lag time of floating, which is the duration of time required for microsponges to rise on the media surface, and the log time of floating, being the overall duration of floating, all were assessed by using a stopwatch.

2.4. In-vitro drug release study

Utilizing an eight-basket automated dissolution study equipment of USP type-2 manufactured by LOGAN Instruments Corp., Somerset, NJ, in the USA, pure APG and APG gastroretentive microsponges underwent drug release experiments in the laboratory environment. The gastric floating microsponges ≅ 5 mg of apigenin were wrapped in an overnight moistened cellophane membrane procured from Himedia Pvt Ltd. In India and then tied to the paddle in 900 ml of acidic release media (pH 1.2). The paddle rotated at a speed not exceeding 50 rpm with temperature maintained at 37 ± 0.5 °C to avoid damage occurrence of microsponge structure as a result of prolonged stirring. Samples of 5 ml were withdrawn at different time intervals and filtered. By using a spectrophotometer, the samples were analyzed at λmax of 353 nm for APG content and then a calculation of the cumulative percentage of APG that was released at every time period was made. This study was performed in triplicate. PCP Disso V3 Software (an excel based program developed in India by Pune college of pharmacy) was used to determine the mechanism through which APG was released from the microsponge formulations.

2.5. Scanning electron microscopy (SEM)

Images of APG, EGT, and APG gastroretentive microsponge (F-3 formulation) were captured using SEM (JSM 6360A, JOEL, Tokyo, Japan). The drug or the microsponge to be assessed was placed on the brass stub with the aid of double-sided tape and next, it was covered by the fine film of gold by an ion stumbler. Following that, the sample was examined using an electron microscope at various magnifications while being subjected to a 20 kV electric current. Pictures of all samples separately were then taken and preserved for analysis and interpretation. Mainly the surface mor-

Table 1
Formulation of gastroretentive microsponge of APG.

Composition*	Formulations				
	F-1	F-2	F-3	F-4	F-5
APG	250	250	250	250	250
EGT	375	375	375	375	375
Acetone	8	8	8	8	8
Polysorbate 80	0.3	0.45	0.6	0.75	0.9
Purified Water (QS)	100	100	100	100	100

Note: *APG, and EGT amounts are in milligrams; Purified water, Acetone, and Polysorbate 80 quantity is in mL.

phology of the APG gastroretentive microsponges was studied by using the captured gastroretentive microsponge images.

2.6. Fourier transform infrared spectroscopy (FTIR)

The FTIR study has been carried out to assess the drug and polymer compatibility that were used in the study. The samples pure APG, EGT, physical mixture, and APG-loaded microsponge have been evaluated to compare the spectral changes in the formulated microsponge with the pure samples. This assessment was conducted by using an infrared spectrophotometer (ATR-FTIR, Bruker Alpha, Germany). The scanning of samples was performed between 4000 and 400 cm^{-1}.

2.7. Differential scanning calorimetry (DSC)

Thermograms of APG, EGT, and APG gastroretentive microsponge (F-3 formulation) were recorded by using differential scanning calorimeter (DSC 214 Polyma NETZSCH. Germany) during this study. 4 to 8 mg of the material were weighed into an aluminum pan specially designed for the DSC, and after that the pan was tightly capped. Next the capped pans were moved to the differential scanning calorimetry apparatus to record the thermograms of the test samples. The nitrogen gas environment of 40 ml and 60 ml per minute and the temperature range was 0 °C to 400 °C and the heating rate maintained was 10 °C per minute for measuring the DSC thermograms of the samples.

2.8. X-ray diffraction study (XRD)

This work used an X-ray diffractometer to document the XRD patterns of APG, EGT, a physical mixture of APG/EGT, and an APG gastroretentive microsponge (F-3 formulation). CuKa radiation having a wavelength 1.54060 A^0 was utilized in the XRD equipment to measure the samples. Step scanning was performed on each sample between 0 and 700 at a scale of 2θ while diffraction peak intensities were recorded.

2.9. In vivo assessment of gastro-retention

The present animal protocol was approved with approval number IRB-UGS-2021–05-134 by the institutional review committee, at Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia. The Institute of Research and Medical Consultation Studies (IRMC) animal housing facilities were used as the source for the animals. Two Wistar albino (180–200 g of weight) were used in the gastroretentive experiment. Each rat was kept in a separate metabolic cage, with a temperature of 25 to 30 °C and a 12-hour light/dark cycle. To carry out the study, the selected animals were fasted for 18 and then divided and treated accordingly. The first animal was considered as a control and was given water, whereas the second animal was administered oral APG gastroretentive microsponge (F-3 formulation) having a dose of APG 10 mg/kg body weight of albino rats. After administration of water and APG gastroretentive microsponge (F-3 formulation), the rats were anesthetized, and ultrasound scanning was performed. To perform ultrasound scanning, the rats were prepared according to the standard protocol of scanning. A depilatory lotion was then administered to the shaved scanning area after the hairs on the rats were first clipped out (Mahant et al., 2020). To track the gastroretentive performance of the provided APG gastroretentive microsponge (F-3 formulation), ultrasound images of the stomach region were collected for the second animal at the fourth hour of administration utilizing ultrasonography equipment with model and make MyLabOneVET, in Italy. The first rat's empty stomach was photographed and marked as being at 0 h, while the second rat's stomach was photographed at the 4th hour after administering the APG gastroretentive microsponge.

2.10. In-vitro anti H. pylori activity

2.10.1. Determination of minimum inhibitory concentration (MIC)

Using a microdilution broth assay, an evaluation against the *H. pylori* ATCC43504 strain was conducted to determine MICs of apigenin and the chosen apigenin gastric floating microsponge formulation (F-3). The strain of *H. pylori* ATCC43504 was cultured on brain heart infusion broth (supplemented with 7% bovine serum albumin), while the test samples stock solutions were produced in 1% DMSO. The test samples were diluted twice in a serial manner using 96 well microtiter plates in salt water with phosphate buffer. 100 μL of Mueller-Hinton broth containing test agents with a range of 0.125 μg/mL to 32 μg/mL and 0.1 μL of *H. pylori* suspension (10^7 cfu/mL) were combined, and then the mixture went through an incubation for 5 days at 37 °C under a microaerophilic atmosphere (CO2 10%, O2 5%, N2 85%). The MICs of the test agents are the lowest concentrations that prevented bacterial growth which was evident in a turbid state.

2.10.2. Estimation of duration of inhibition of growth

A cell culture of *H. pylori* (ATCC43504) with dilution 10^7 cfu/mL was allowed to react with 2 × MIC of test agents diluted in 200 μL of Mueller-Hinton broth (CO_2 10%, O_2 5%, N_2 85%) and then the reaction mixtures were incubated at 37 °C for 96 h. The phase after which turbidity for bacterial growth become apparent was observed as the time period of an agent's activity.

2.11. Statistical analysis

In this study, a one-way analysis of variance test (ANOVA) was conducted to evaluate the obtained experimental data. Furthermore, to compare the standard apigenin versus the test samples of microsponge formulation, a Student's *t*-test was used, and the relevance rankings were indicated at a p-value of less than 0.05.

3. Results and discussion

3.1. Physicochemical assessment of gastro retentive microsponge of apigenin

For apigenin microsponge formulations, numerous trials showed varying drug content (DC), product yield (PY), and entrapment efficiency (EC). However, all formulations remained floating for more than 12 h. For the apigenin microsponge formulations, the PY, DC, and EE values ranged from 65.13 ± 0.27 to 76.23 ± 0.84, 39.38 ± 0.43 to 42.28 ± 0.35 and 90.60 ± 1 to 97.84 ± 0.84 respectively (Table 2). The values of product yield and the drug content were in increasing order from F1 to F3 formulations followed by a slight decrease in F4 and a sudden decrease in F5. This could be due to the emulsifier employed being non-ionic and molecules can associate away from the oil–water interface at the highest concentration of the stabilizer employed. Such an alternative hydrophobic region can dissolve some portions of a drug resulting in a reduction in production yield and drug content within the formulation F5 (Jelvehgari et al., 2006).

3.2. In-vitro drug release study

It has been observed the drug release for each formulation from F-1 to F-5 was in decreasing order as tween 80 amount was increasing. The reason could be because the drug is released from the polymer matrix after its pores are entirely opening and the

Table 2
Physicochemical assessment of apigenin stacked gastro retentive microsponge.

(%)Parameters*	Gastroretentive microsponge formulations				
	F1	**F2**	**F3**	**F4**	**F5**
Product Yield	68 ± 1	70.37 ± 0.74	76.23 ± 0.84	75.34 ± 0.99	65.13 ± 0.27
Drug content	39.39 ± 0.43	41.41 ± 0.70	42.54 ± 0.37	42.29 ± 0.35	40.25 ± 1
Entrapment efficiency	90.60 ± 1	95.27 ± 1.6	97.84 ± 0.85	97.27 ± 0.83	92.59 ± 2.3
In-Vitro floating(h)	12	12	12	12	12

* Every assessment was done in triplicate, and the findings were presented as mean SD (n = 3).

time needed for polymer pores to open is directly proportional to stabilizer/surfactant concentration (Mahant et al., 2020; Osmani et al., 2015). The slight decrease in release rate with increased tween 80 amount was from 36.35% to 22.70% for formulations F-1 to F-5 after 12 h of the study (Fig. 1). Similar results were reported in a similar study published recently in a well-known journal (Shahzad et al., 2018). The in vitro release model was fitted to the in vitro release profiles to understand the drug release process. The data was used to study the best linear fit for the following equations (Costa and Sousa Lobo, 2001)

1) Zero order
2) First order
3) Matrix (Higuchi matrix)
4) Peppas-Korsmeyer equation
5) Hixson-Crowell equation

%R = Kt.
Log % unreleased = Kt/ 2.303.
% R = Kt0.5.
Amount of drug released at time t.
---------- = Kt n.
Amount of drug released at time '∞'.
(% unreleased) 1/3 = Kt.

Where 'n' is the diffusion coefficient, which is suggestive of transport mechanism.

Higuchi's diffusion kinetics might be the most accurate way to characterize the release of APG from all microsponges. Diffusion exponent results revealed that all microsponges had (n) values that were less than 0.5. This shows that the porosity of the microsponges controls the Fickian diffusion based APG release mechanism from these microsponges.

3.3. Scanning electron microscopy (SEM)

The crystals of APG are visible in the micrograph of APG Fig. 2A. The micrographs of EGT Fig. 2B are revealing the amorphous nature of the polymer. Whereas micrographs of a physical mixture of APG/EGT showed APG particles adhered to the surface of the EGT revealing no evident interaction between both the drug and a polymer in a solid state Fig. 2C. The micrograph of the microsponge formulation Fig. 2D captured at large magnification power illustrated the broken spherical microsponge, uncovering its spongy matrix and confirming its inner polymeric construction. The APG was mostly completely disseminated in the microsponge's spongy matrix and was amorphized, with hardly a little quantity remaining in a partial crystalline position, according to the same micrograph, which also reveals a few APG particles that are clinging to the internal walls of the microsponge.

3.4. Fourier transform infrared spectroscopy (FTIR)

The natural product apigenin belongs to the flavone class. It is also known as 4, 5,7-trihydroxy flavone. The infrared spectroscopy showed various characteristic peaks confirming the presence of the flavone group. The phenolic hydroxyl group exhibited its characteristic peaks at 3274.65 cm-1, whereas the carbonyl group showed the stretching vibration at 1653.10 cm-1. The C-O-C peaks of the pyran ring were observed at 1354.18 cm-1 for the apigenin (Fig. 3A). The characteristic amine peaks of the carrier eudragit were observed at 3448.18 cm-1 (Fig. 3B). Taking into considera-

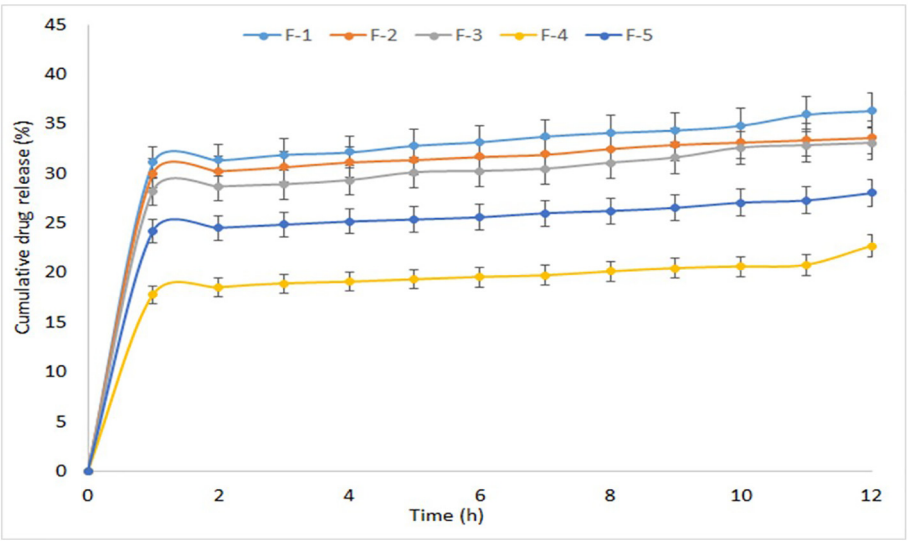

Fig. 1. In-vitro drug release profile of apigenin gastric floating microsponge formulations. * Every trial was done in triplicate, and the findings were presented as mean SD (n = 3).

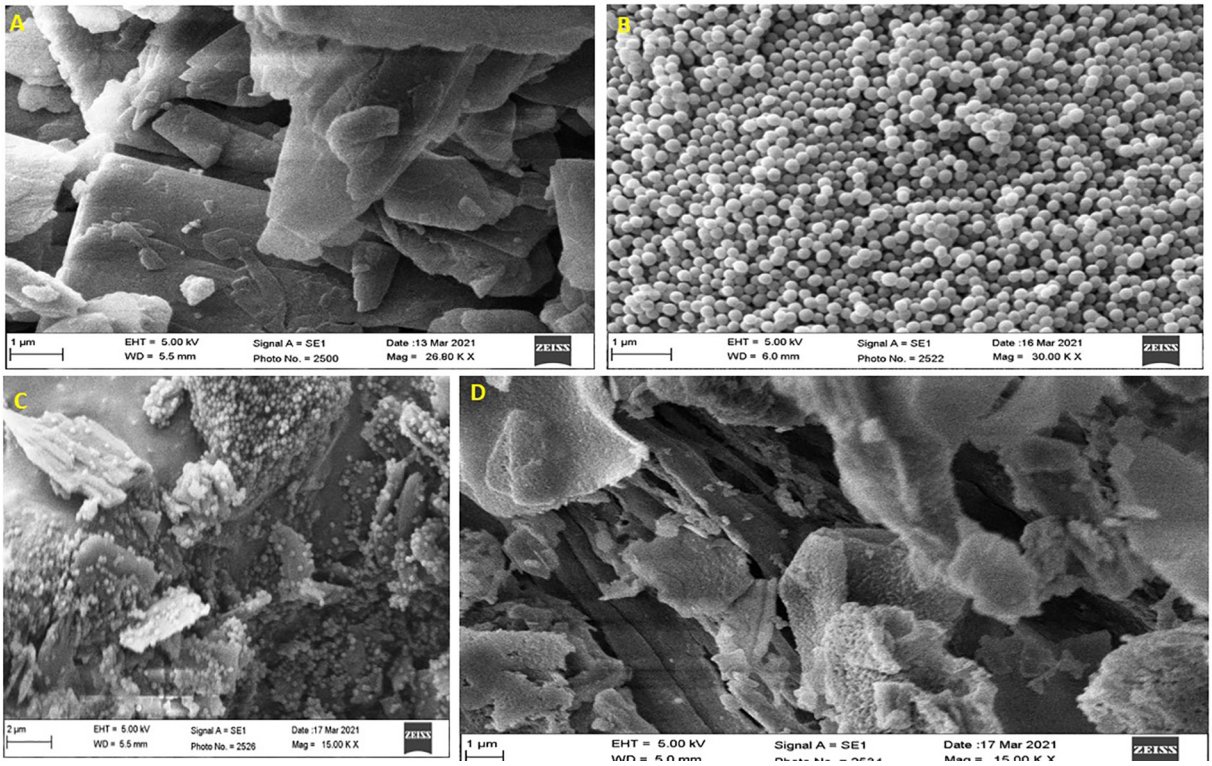

Fig. 2. SEM micrographs of APG (A), EGT (B), APG-EGT Physical mixture (C), Microsponge (D).

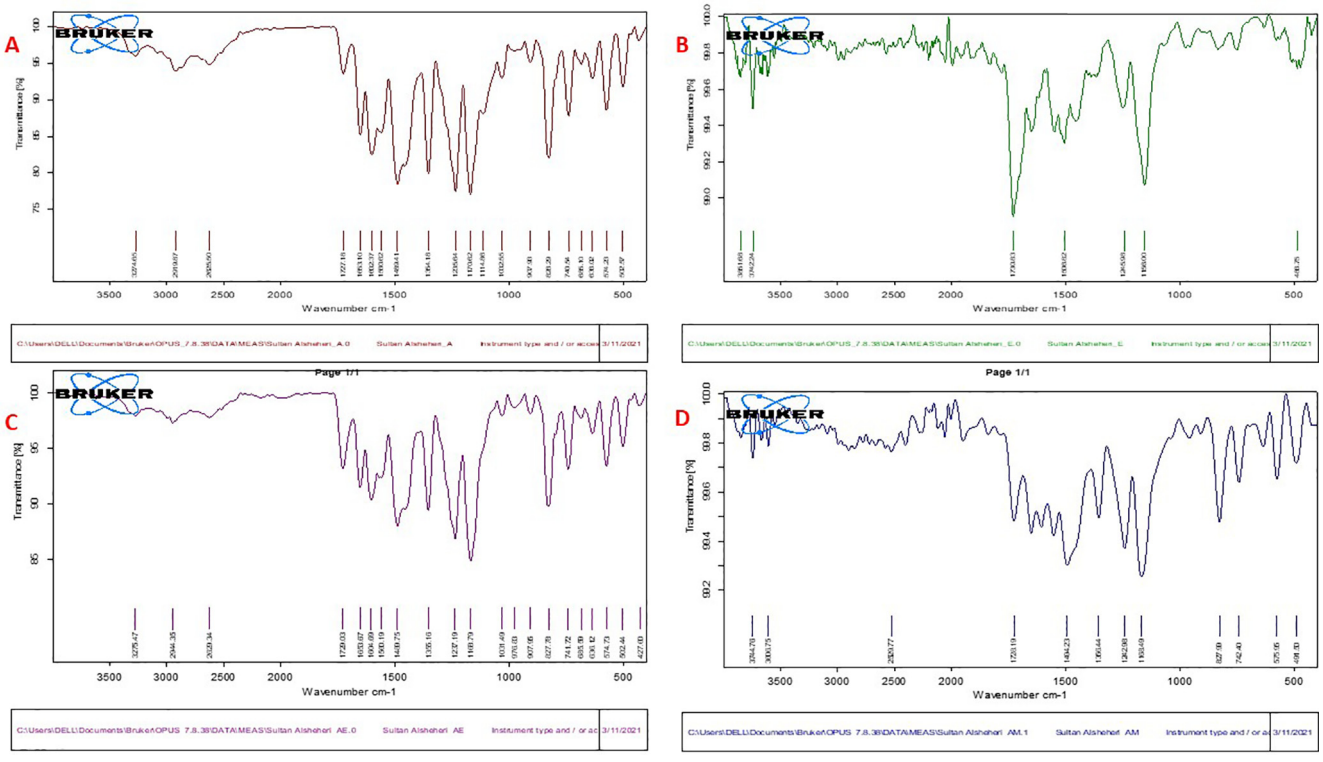

Fig. 3. FTIR Spectra of APG (A), EGT (B), APG-EGT Physical mixture (C), Microsponge (D).

tion, the physical mixture exhibited all the characteristic peaks of the apigenin with slight changes in the peak positions. The eudragit peaks for amines were missing in the physical mixture (Fig. 3C). FTIR spectra of the apigenin microsponge exhibited characteristic peaks of a phenolic hydroxyl group of pure apigenin at 3268.54 cm-1. The C = O group exhibited the stretching vibration at 1728.18 cm-1. The peaks for the pyran ring of the pure drug were also observed with minor changes at 1356.44 cm-1. It also

showed the amine peaks of the eudragit at 3436.10 cm-1 (Fig. 3D). Hence, concluding that there was no interaction of apigenin microsponge with the pure Apigenin and the carrier eudragit.

3.5. Differential scanning calorimetry (DSC)

The DSC tests were conducted on the APG, EGT, APG/EGT physical mixture, and the microsponge (Formulation-3) samples. APG and EGT's DSC thermograms, shown in Fig. 4, both manifested endothermic peaks at 366.3 °C & 130.9 °C, correlatively. These temperatures represent the crystalline and amorphous nature, as well as the melting points, of both materials. In each of the DSC thermograms of the physical mixture of APG and EGT, and microsponge formulation respectively, the characteristic endothermic peak of the apigenin was also observed, thus indicating that there was compatibility between the drug and the polymer used. Furthermore, a significant decrease in the melting point of the apigenin in the formulation could be because of the drastic decrease in crystalline behavior of the drug and it could be due to drug entrapment in the pores formed by the microsponge structure.

3.6. X-ray diffraction study (XRD)

APG's XRD spectrum revealed several distinct, strong peaks at 6.18°, 7.82°, 12.26°, and 17.31°, indicating that the substance is present in crystalline form. At diffraction angles between 5 and 40°, EGT did not exhibit any distinguishing peaks, indicating that they are amorphous in nature (Jafar et al., 2020). All the apigenin's distinctive peaks are present in the physical mixture with very minor variations. While the distinct APG diffraction peaks in the X-ray graphs of the APG microsponges showed a considerable intensity decrease, indicating a drug's partial solubility in the amorphous polymer(s) or semi-amorphization of the drug (Fig. 5). Results from FTIR and DSC investigations concur with those from XRD.

3.7. In vivo assessment of gastro-retention

In recent times, the use of ultrasonography techniques has gained popularity mainly due to high temporal resolution. It provides the potential basis for targeting the advanced drug delivery system such as gastro retentive. The other advantages of the ultrasonography technique include easy access, portability, and ease of operation. In the present study, the images obtained from ultrasonography images of intragastric administration of novel drug delivery formulation of APG to rats have provided interesting results. Fig. 6 illustrates how the gas bubbles are spread across the entire upper gastric region of the rat and it makes the whole area looks like a starry night in the ultrasonic image captured. The images of the second animal obtained at the 4th hour of administration of the formulation show the appearance of APG microsponge formulation (F-3) in the form of an accumulated viscous mass of the microsponges. These findings suggest that the best microsponge (F-3) has stayed in the upper GIT of the rat longer than the duration (1 to 2 h in rats) of gastric transit time, which could be because of the gastroretentive systems remain floating on the surface of the gastric juice as compared to the other part of the GIT and it is governed by the involuntary movements of the stomach. Hence, we can conclude that the in-vivo gastric retention study results of F-3 gastroretentive microsponge are in line with the reported scientific literature of gastric retention studies of microsponges until 8 h after X-ray assessments of the gastric regions of rats'(Younis et al., 2019).

3.8. In-vitro anti H. pylori activity

The foremost reasons for gastritis and peptic ulcer diseases are infections caused by *H. pylori* and conventionally used antibiotics against such infections are amoxicillin, clarithromycin, tetracycline, and metronidazole. But these therapies are not sufficient to completely eradicate *H. pylori* because of their lower stability at

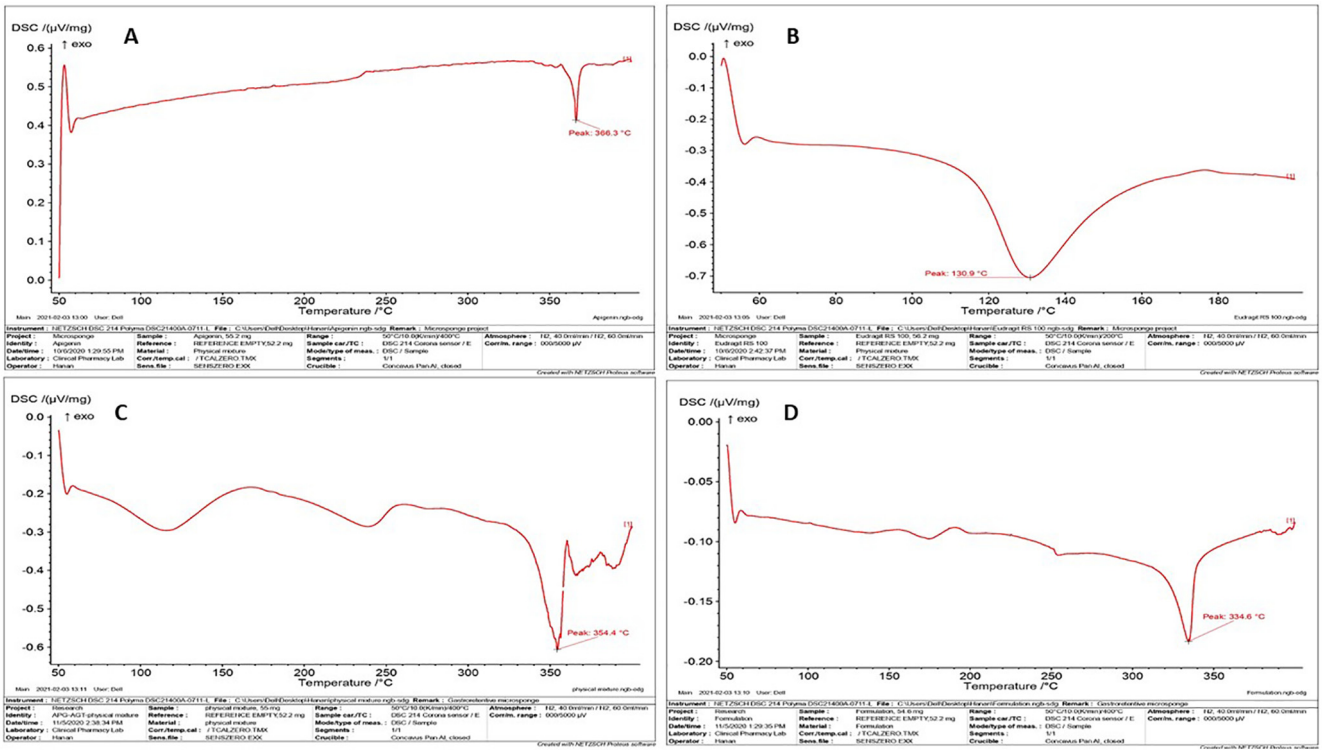

Fig. 4. DSC thermograms of APG (A), EGT (B), APG-EGT Physical mixture (C), Microsponge (D).

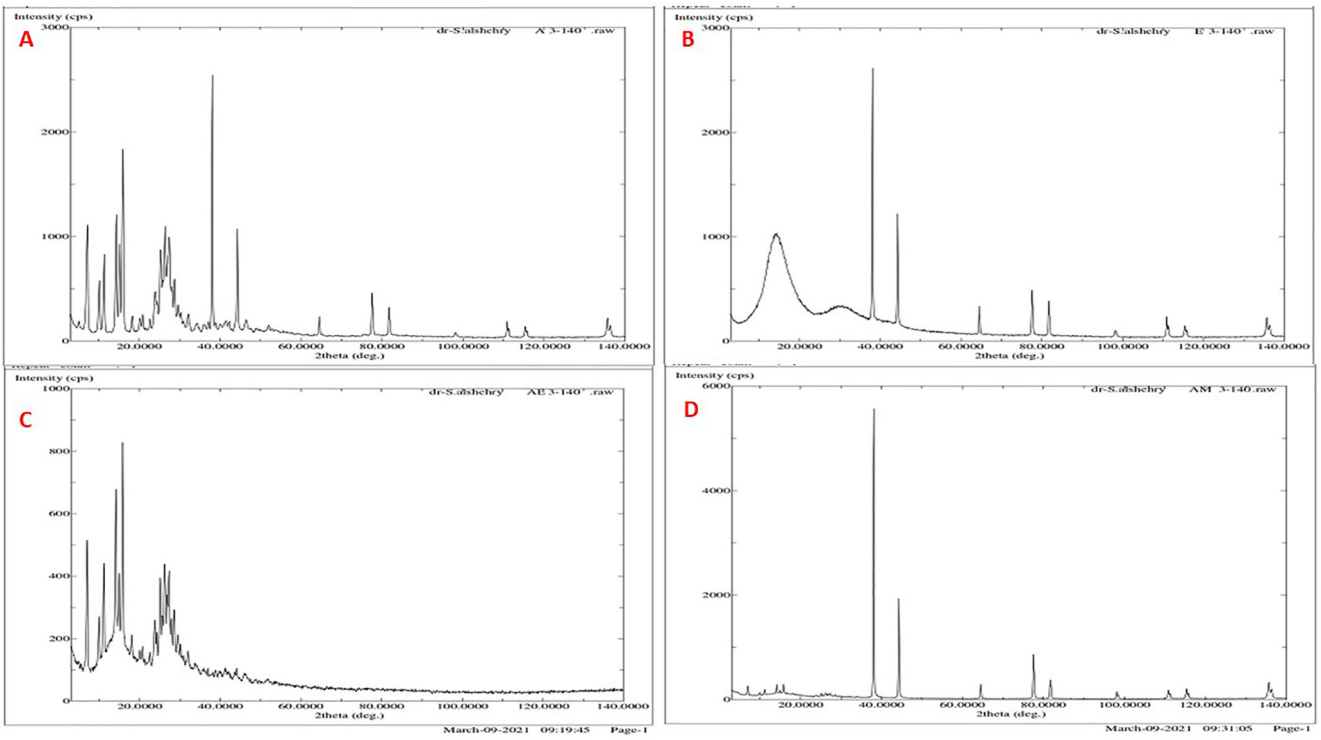

Fig. 5. X-ray graphs of APG (A), EGT (B), APG-EGT Physical mixture (C), Microsponge (D).

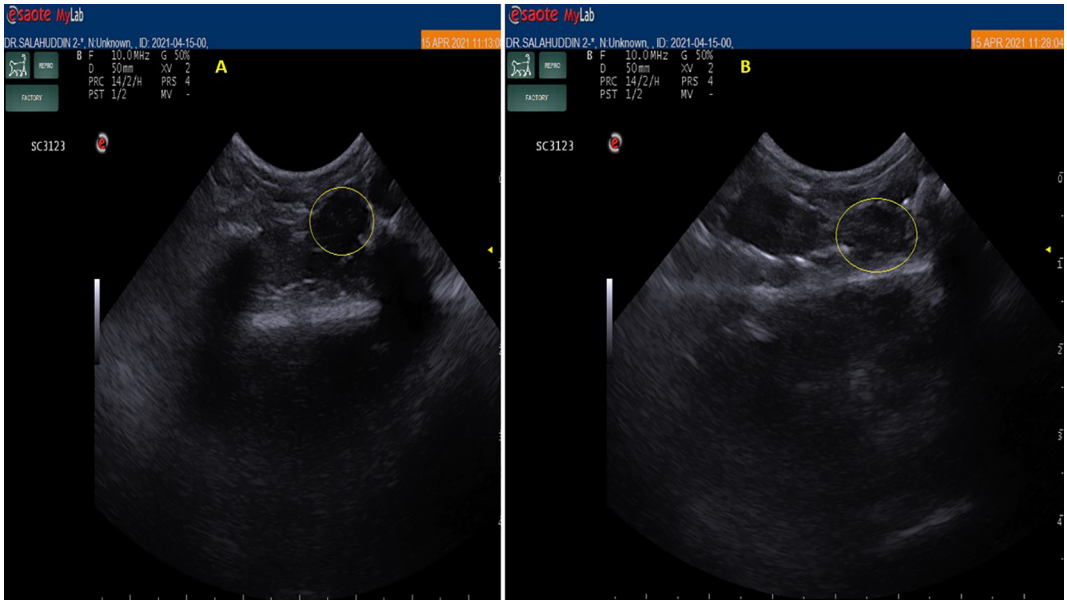

Fig. 6. In vivo assessment of gastro-retention.

the low pH of gastric juice (Tursi et al., 2014; Erah et al., 1997). In contrast, the drug metronidazole has less influenced by low pH, making it a popular choice for treating *H. pylori* (Erah et al., 1997, Ribaldone et al., 2017). However, resistance to this treatment in *H. pylori* has been noted. Because of the restricted options of efficient therapeutics and the widespread use of some bacteriostatic antibiotics in the common population has led to selection pressure on *H. pylori*. As a result, having exceptional adaptation ability this species has undergone mutational changes and has displayed fast advancement of primary antibiotic resistance (Thung I et al, 2016, Kasahun et al, 2020, Nishizawa et al, 2014). In *H. pylori*, resistance to macrolide has been attributed to the point mutations in domain V of 23S rRNA for the peptidyl transferase region (Versalovic et al., 1996) and to the efflux pump system (Bina et al., 2000). On the other hand, the antimicrobial action of fluoroquinolones is due to the inhibition of enzyme DNA gyrase (Ohemeng et al., 1993). But point mutations in the Quinolones Resistance Determining Region (QRDR) of *gyrA* in *H. pylori* inhibit the binding of fluoroquinolones and DNA gyrase, bestowing antibiotic resistance (Nishizawa et al., 2009). Moreover, *H. pylori* lacks

Table 3
In-vitro anti *H. Pylori* activity of APG and its Microsponge.

Test samples	MIC (µg/mL)	Duration of Action (at 2 × MIC) Hours
Apigenin	8	36
Apigenin gastroretentive microsponge (F-3)	16	72

topoisomerase IV genes which are considered to be crucial target as mechanism of action of fluoroquinolone in other bacteria (Nishizawa and Suzuki 2014). Whereas point mutations in the genes like *frxA, frxB, rdxA, fur, pbp1, 16 s rRNA,* and *rpoB* are accountable for resistance to antibiotics like metronidazole, amoxicillin, tetracycline and rifabutin (Nishizawa and Suzuki 2014, Gerrits and de Zoete 2002, Nishizawa et al., 2011).

Therefore, it is vital to look for different therapeutic options that can contend with the problem of *H. pylori*'s treatment resistance. Novel strategies must be adapted such as exploitation of the established therapeutic ability of phytocompounds. Considering this, we tested the antibacterial properties of the natural chemical apigenin and its microsponge formulation using *H. pylori* ATCC43504, a familiar reference strain that is resistant to metronidazole. Although, it has been found that this strain is sensitive to other active pharmaceutical ingredients such as amoxicillin and clarithromycin (González et al., 2019). According to Table 3, apigenin, a phytochemical of the flavone class, and apigenin-microsponge showed MICs of 8 and 16 µg/mL, respectively against *H. pylori* ATCC43504. In additional research, MICs for apigenin and other flavones against drug-resistant/susceptible *H. pylori* strains were shown to be between 4 and 32 g/mL (González et al., 2021; Chung et al., 2001). According to the research stated above, apigenin alone or in a microsponge formulation may be able to prevent *H. pylori* strain growth at reduced concentrations than other flavones as well as metronidazole. The apigenin-microsponge formulation was found to have a longer duration of effect (up to 72 h) than apigenin (36 h). We found that the improved microsponge formulation of apigenin has in vitro antibacterial action that is comparable to that of regular apigenin against *the H. pylori* - strain. It might be postulated that the inclusion of other biologically inert substances such as eudragit in developed formulations results in a reduction in the active concentration of apigenin and therefore is the cause of increased MIC of microsponge formulation of apigenin by two-fold in comparison with pure apigenin. These compounds could cause a one-half reduction of active compound quantity in the improved formulation of microsponges, i.e., apigenin, as already stated in Table 1. Consequently, there is a twice increase in MIC of the developed microsponge. Nonetheless, a quiet susceptibility to apigenin in the improved formulation was noticed in *the H. pylori* test strain for a prolonged duration with significantly lower apigenin concentration to inhibit bacterial growth.

Similar to other phytocompounds, the antimicrobial activity of apigenin is appeared to be multifactorial by showing multiple molecular targets in the pathogen (Gonzalez et al., 2021. Besides showing antioxidant effects, other well-known molecular targets of apigenin in *H. pylori* include certain enzymes (Wu et al., 2008; Zhang et al., 2008), and secretion systems (Yeon et al., 2019). Apigenin being lipophilic flavonoids could penetrate the lipid bilayer membrane causing disruption of the cell membrane by disarranging and disorientation of lipid molecules (Gorniak et al., 2019) in *H. pylori* and thereby resulting in a bactericidal effect. Another bactericidal and noticeable mechanism of action of apigenin is reported to be binding with HsrA in *H. pylori*, thereby inhibiting the essential functions needed for survival like cell division, response to oxidative stress, virulence potential and overall, a homeostatic regulator of various metabolic functions depending upon the nutrients availability (Gonzalez et al., 2019, Gorniak et al., 2019). An added advantage of exploiting flavonoids including apigenin as antimicrobial agent against *H. pylori* infections could be their ability to interact synergistically with anti-*H. pylori* primary antibiotics such as amoxicillin, clarithromycin, and metronidazole (Gonzalez et al., 2021, Gonzalez et al., 2019) and thereby reverting antibiotic-resistant phenotypes. Moreover, by possessing multiple sites of action and bactericidal effects, this compound as a therapeutic drug would not allow *H. pylori* to develop resistance and would slow down the development and propagation of newer resistance mechanisms.

As shown by prior research (Jafar et al., 2021), the MICs of apigenin and APG-developed formulation against metronidazole-resistant *H. pylori* ATCC43504 in our investigation are much lower than those of metronidazole against *H. pylori* strains. Our research thus supports the idea that apigenin and apigenin-loaded microsponge can successfully target different *H. pylori* strains including sensitive and resistant strains. The current study further demonstrated that apigenin microsponge exhibits potent and prolonged antibacterial action against *H. pylori*. As a result, it may represent a viable alternative medication with improved bioavailability that can effectively combat the treatment of peptic ulcers brought on by *H. pylori*.

4. Conclusion

With improved physicochemical properties, such as good production yield, high drug content, and excellent drug entrapment efficiency, in addition to achieving slow in-vitro drug release and prolonged retention in the albino rat stomach, apigenin gastroretentive microsponge was successfully developed. Additionally, antimicrobial experiments against the *H. pylori* bacteria indicated that the improved microsponge formulation had far longer and better antibacterial action than apigenin alone. As a result, the APG gastro-retentive microsponge formulation has the potential to be an effective new method for both eradicating the *H. pylori* infection and delivering apigenin to the stomach region. To get many beneficial results, additional pharmacokinetic investigations, histological examinations, and therapeutic evaluations of F-3 gastroretentive microsponge are needed.

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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