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Intravaginal delivery of reverse micellar epigallocatechin loaded in κ -carrageenan and HPMC K100M-based gel

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ABSTRACT: A gel with mucoadhesive properties and a controlled release profile is a suitable dosage form for reverse micellar EGCG delivery. In this study, κ -Carrageenan and HPMC K100M were used as the gel components at a weight ratio of 1:1.5, respectively, for loading native and reverse micellar EGCG. The characteristics of the gel were determined based on pH, swelling index, disintegration time, hardness, and entrapment efficiency. The *in vitro* EGCG release rate was further determined for EGCG levels. Moreover, *in vivo* cervical penetration studies of rhodamine-labeled EGCG gels in mice at two and six hours after intravaginal administrations were conducted. The results showed that the pH and hardness characteristics of the gels for each formula did not differ significantly, while the gel-loaded reverse micellar EGCG had a higher swelling index than that of native EGCG gels. In addition, the rate of release and cervical penetration of rhodamine-labeled reverse micellar EGCG loaded in gels was higher than those of rhodamine-labeled native EGCG gels. Therefore, it can be concluded that loading reverse micelles EGCG into gels prepared with κ -Carrageenan and HPMC K100M successfully controlled the release rate and improved cervical penetration, thereby enabling its potential use in cervical cancer treatment.

KEYWORDS: Epigallocatechin gallate; cancer; hydroxypropyl methylcellulose; κ -carrageenan; reverse micelle; cervical penetration

1. INTRODUCTION

Cervical cancer has a high prevalence in women. World Health Organization states that cervical cancer is the fourth most common cancer in women. In 2018, 570,000 women were diagnosed with cervical cancer worldwide, and about 311,000 women died from the disease(1).It is reported that the persistent infection with carcinogenic human papillomavirus (HPV) types is the leading cause in triggering the development of cervical cancer (2). HPV16 and HPV18 were the most prevalent HPV subtypes among cervical cancer patients, followed by HPV58, HPV53, and HPV33 HPV16 and HPV18 were the most pervasive HPV subtypes among cervical cancer patients(3). Risk factors of developing cervical cancer are low socioeconomic status, smoking, marrying before age 18 years, young age at the first coitus, multiple sexual partners, multiple sexual partners of a spouse, and multiple childbirths (4).Management of cervical cancer is primarily by surgery, radiation therapy, or chemotherapy(5). However, chemotherapy can cause several serious side effects(6), encouraging researchers to look for alternative therapies, including natural products. One natural active ingredient that has been widely reported for cancer treatment is Epigallocatechin gallate (EGCG).

EGCG represents the most effective anticancer polyphenol among the ten contained in green tea (7) and has been shown to have anti-proliferative, anti-metastatic, and pro-apoptotic properties (8).It has been reported to be capable of inhibiting HeLa cell viability, inducing HeLa cell apoptosis, and reducing matrix metalloproteinase-9 (MMP-9) in HeLa cells (9). However, EGCG has low lipophilicity, is easily degraded at high temperatures, and demonstrates low tissue permeability (10).Moreover, it lacks high bioavailability for

Rosita N, Hariyadi DM, Amelia CC, Mamurrie AM, Miatmoko A. Intravaginal delivery of reverse micellar EGCG loaded in κ -Carrageenan and HPMC-based gel. J Res Pharm.2022; 26 (4): 849-858

oral preparations because it can be degraded by the metabolism of digestive bacteria (11). It has previously been reported that the concentration of EGCG decreases at 50°C during storage periods (12). However, although degraded at pH 6.8, it remains stable at pH 4.5 (13). Intravaginal administration at a pH level of 3.5-4.5 with cervical tissue as the target renders gels appropriate as delivery carriers of EGCG in the treatment of cervical cancer.

Gels have certain advantages in the delivery of drugs that protect against first-pass metabolism and enzymatic degradation in the digestive tracts (14). Moreover, the use of mucoadhesive polymers in intravaginal gels can increase the contact time of therapeutic drugs with the tissue mucosa, thus prolonging the therapeutic onset time (15). The most commonly used mucoadhesive polymers include Hydroxy Propyl Methyl Cellulose (HPMC) and κ -Carrageenan (16). HPMC is non-toxic, stable within a wide pH range, and a non-irritating polymer (17). In the previous studies of vaginal tablets prepared with HPMC as the polymer matrix, it has been shown that almost 100% of drugs can be released within 40–150 hours (18,19).

Meanwhile, κ -Carrageenan is a family of gel-forming polysaccharides extracted from certain red seaweed species (20). Gel made from κ -Carrageenan possesses strong and rigid physical properties. Potassium ions have been identified as the optimum crosslinking agents for κ -Carrageenan gels. κ -Carrageenan, as a mucoadhesive polymer of vaginal tablets, successfully releases 100% of Acyclovir within 48 hours (21).

It is known that drug release from the carrier matrix constitutes the initial stage before its penetration into tissues. This process is influenced by several factors, namely, the amount of drug, polymer types, other pharmaceutical excipients, physical or chemical interactions between components, and manufacturing methods (22). The drug release profile can be defined by the mechanism, kinetics, and rate of release. The drug release mechanisms in polymer-based delivery carriers include controlled diffusion, osmotic-controlled, and swelling drug release (22). After the dissolution of the drug, tissue permeability, which is represented by the Log P value, will become an important factor determining concentration in target organs or tissues (23). EGCG has a log P value of 1.1 at pH 4.0 (24). However, optimal lipophilicity for topical drug penetration is indicated by a log P value of between 2 and 3 (25). Modification of EGCG into reverse micelle with the addition of Tween 80 and Span 80 as a surfactant combined with a Hydrophilic-Lipophilic Balance (HLB) value of 6 at pH 5.0 \pm 0.5 was proven to increase the log P value of EGCG to 2.1 (24). This increased lipophilicity of EGCG has also been shown to increase the penetration of EGCG into the skin and also increase its cellular uptake, as indicated by its high cytotoxicity against HeLa cells (24).

The presence of surfactants as components of EGCG reverse micelles at very low concentrations, combined with the polymer, allows aggregate formation (13,26). The aggregates will be formed spontaneously and are reversible at levels above the Critical Aggregation Concentration (CAC) (27). The systems containing polyelectrolytes and non-ionic surfactants have lower CAC. However, interactions will still occur compared to their Critical Micelle Concentration (CMC), albeit in very weak states (13). Consequently, possible physical interactions result from the combination of anionic polymers, including κ -Carrageenan and non-ionic polymers such as HPMC with non-ionic surfactants, i.e., Tween 80 and Span 80 (26).

In this study, the effect of reverse micellar EGCG formation on the characteristics of gels prepared with κ -Carrageenan and HPMC K100M, as well as the release profiles of EGCG and cervical penetration, were investigated. These evaluations proved beneficial for determining the potential use of gels for cervical delivery of reverse micellar EGCG.

2. RESULTS

2.1. Physical characteristics of gel loading reverse micellar EGCG

The physical characteristics of the gels are shown in Table 1. It can be seen that the pH of gel loading native EGCG (EGCG-Gel) was not significantly different from the gel loading reverse micellar EGCG (RM-EGCG-Gel) ($P > 0.05$), being 4.24 and 4.23, respectively. Moreover, the gel hardness of both formulas was approximately 49.75 N, which was higher than the hardness value of a suppository ranging between 17.7–24.5 N (28,29). However, these gels were produced in good firmness resulting in easy intravaginal insertion. The swelling index evaluation revealed a significant difference between the two formulas in that EGCG-Gel had a larger swelling index than that of RM-EGCG-Gel. The use of surfactants for RM-EGCG-Gel can probably form aggregates with polymers from the gel matrix (13), thereby reducing the wettability, which prevents water from causing the polymer to swell. Crucially, such polymer swelling may affect stability and the drug release profile.

Table 1. The physical characteristic of gel loading EGCG prepared with κ -Carrageenan and HPMC K100M. The data represent the mean \pm SD (* $P < 0.05$).

Parameter	EGCG-Gel	RM-EGCG-Gel
pH (n=3)	4.24 \pm 0.01	4.23 \pm 0.06
Hardness (N, n=10)	49.76 \pm 0.11	49.74 \pm 0.04
Swelling Index (n=3)	2.95 \pm 0.54	1.46 \pm 0.19 [*]

2.2. Profiles of EGCG released from gels

The release study showed that approximately 35% of EGCG was released from the gels during the first two hours, as shown in Figure 1. This burst of drug release was probably due to the presence of EGCG on the gel surface and was beneficial in inducing an immediate therapeutic effect. Moreover, the release occurred continuously and was maintained at a slower rate.

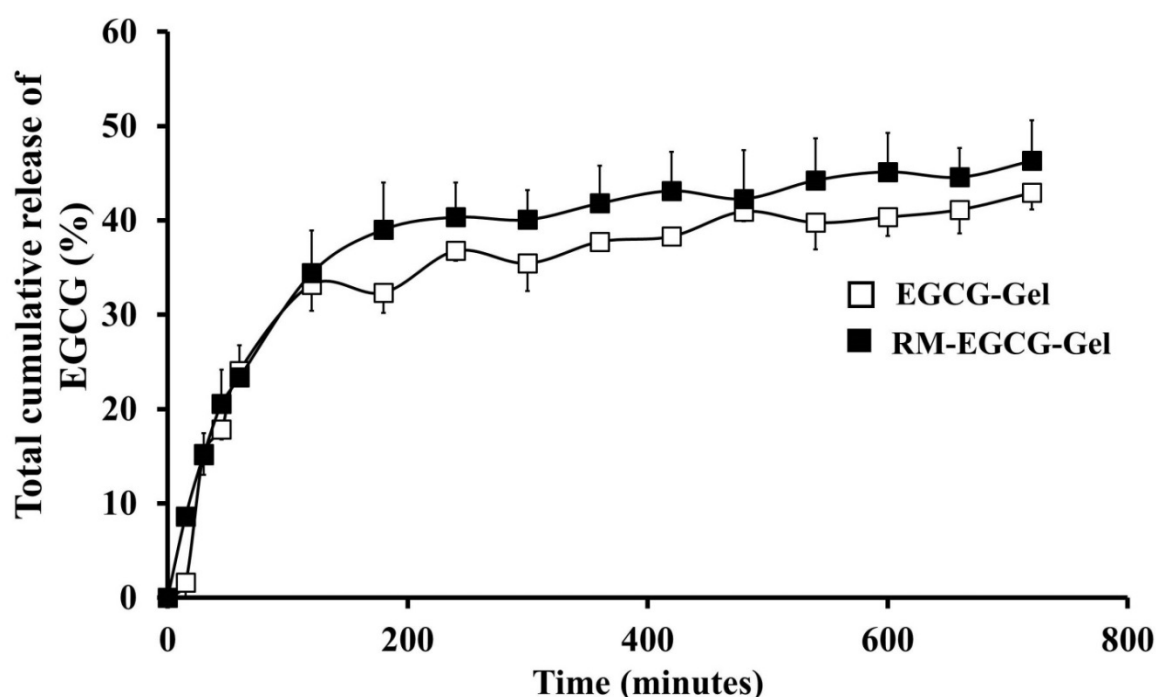


Figure 1. Release Profiles of EGCG from EGCG-Gel and RM-EGCG-Gel prepared with κ -Carrageenan and HPMC K100M. The data represent the mean \pm SD (n=3).

This data was subjected to a further calculation for release kinetics modeling. As can be seen from Table 2, the release kinetics of EGCG from the EGCG-Gel fitted the Higuchi model. In contrast, the EGCG released from RM-EGCG-Gel is based on Korsmeyer-Peppas release kinetics. The Higuchi mechanism requires water absorption for drugs to dissolve and diffuse throughout the media. This is in accordance with the increasing index value of EGCG-Gel, which is greater than that of RM-EGCG-Gel.

Table 2. Release kinetic models and their coefficient correlation to match EGCG release profiles from gels

Release Kinetic Model	Coefficient correlation(R ²)	
	EGCG-Gel	RM-EGCG-Gel
Zero Order	0.684	0.701
First Order	0.449	0.582
Korsmeyer-Peppas	0.758 (n=0.54)	0.898 (n=0.38)
Higuchi	0.824	0.849
Hixson-Crowell	0.495	0.617

There was a significant difference in the rate of release or EGCG flux between these two formulas, while the release rate of EGCG gels was obtained from the slope value of each corresponding release kinetics regression. The use of surfactants in reverse micellar EGCG gels (RM-EGCG-Gels) increased the rate of EGCG release from gels prepared with a combination of HPMC K100M polymer and κ -Carrageenan, as shown in Figure 2.

The Korsmeyer-Peppas mechanism determines that drug release does not involve the water absorption process, thus producing a higher RM-EGCG-Gel release rate than EGCG-gels.

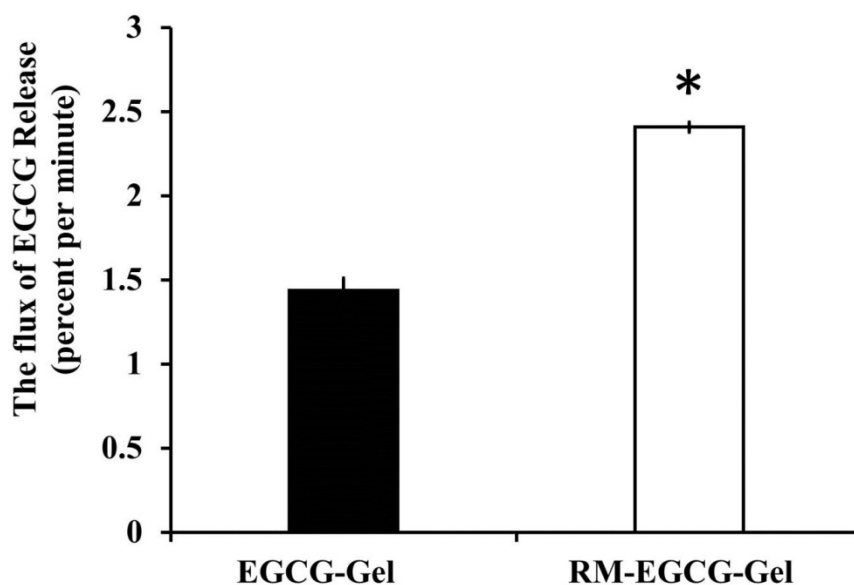


Figure 2. The flux of EGCG release from gels prepared with κ -Carrageenan and HPMC K100M. The data represent the mean \pm SD (n=3, *P<0.05).

2.3. In vivo penetration study of gels loading EGCG

The fluorescence intensities of Rhodamine-Labelled EGCG gels at two and six hours of intravaginal administration are shown in Figure 3. There was no Rhodamine or low intensities of the dye observed in the surfactant-blank control group, while the use of surfactants improved its cervical penetration, especially after six hours of administration.

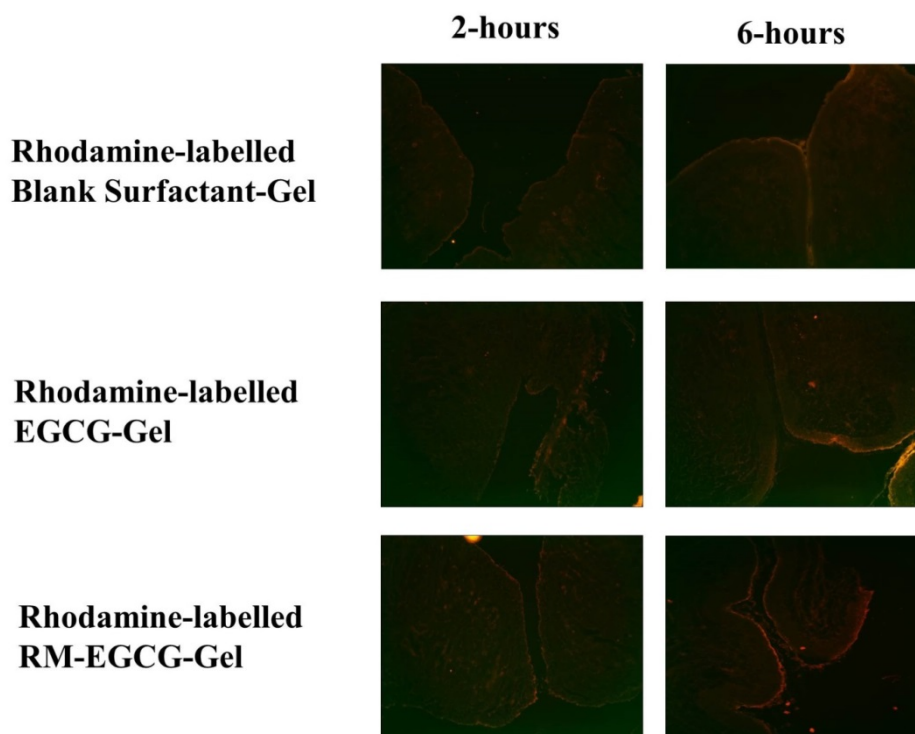


Figure 3. The penetration of Rhodamine-Labelled gel loading native and reverse micellar EGCG in the cervical tissue observed using a fluorescence microscope at two and six hours after intravaginal administration.

Figure 4 shows that the penetration of Rhodamine-labelled EGCG gels after two hours was to a depth of $161.92 \pm 12.30 \mu\text{m}$, while after six hours, it reached a depth of $184.67 \pm 52.98 \mu\text{m}$ tissue penetration, indicating that the longer the contact time of the gels, the deeper the penetration of EGCG. However, both results are based on weak-moderate fluorescence intensity. On the other hand, after two hours, the gel loading reverse micellar EGCG reached a tissue depth of $173.38 \pm 30.79 \mu\text{m}$ with moderate intensity. Then, six hours after administration, the penetration depth was $210.29 \pm 24.11 \mu\text{m}$ with a moderate fluorescence intensity stronger than that of the native EGCG gels.

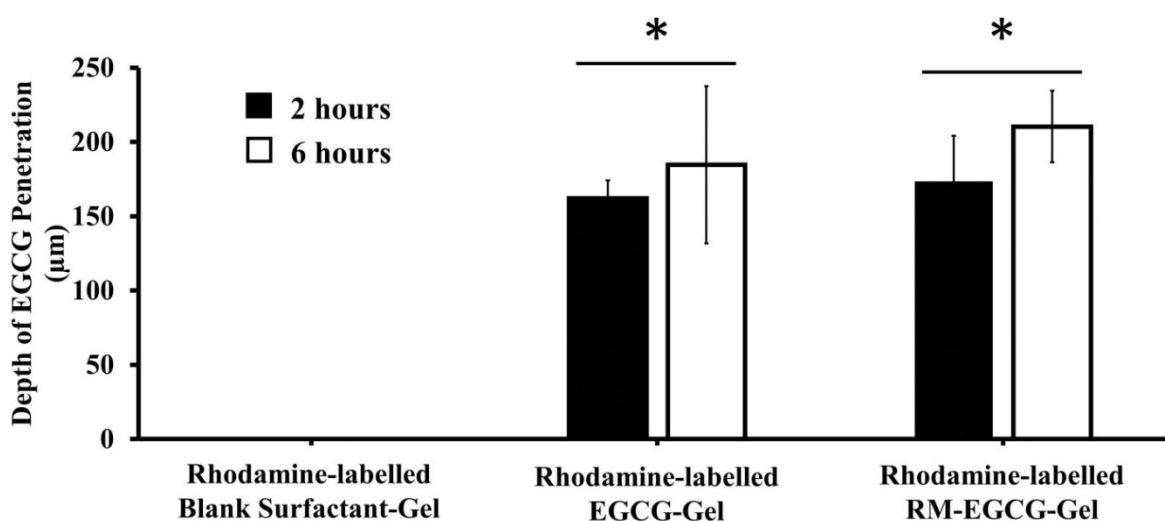


Figure 4. The depth of EGCG penetration was observed at two and six hours after intravaginal administration of gels. The data represent the mean \pm SD ($n=4$, $*P < 0.05$ compared to blank surfactant gel).

3. DISCUSSION

The presence of surfactants in reverse micellar EGCG produces a lower swelling index of the gel prepared with κ -Carrageenan and HPMC K100M (1:1.5) polymers than that of native EGCG. The interaction between polymer-surfactants probably affects the swelling characteristics of polymer-based gels (28). HPMC is a non-ionic polymer, while κ -Carrageenan is an anionic polyelectrolyte polymer (29–31). On the other hand, Tween 80 and Span 80 are non-ionic surfactants (26). It has been reported that interactions may still occur in the systems containing polyelectrolytes, anionic and/or non-ionic polymers with non-ionic surfactants even though their intensity is very low (13,26). These interactions may form aggregates, thereby reducing water wettability and causing the polymer to expand. However, there was no difference between these two formulas in terms of gel hardness.

According to the drug release profiles, during the first two hours, there was an initial burst of EGCG to a level of 35%. The rapid release at this incipient stage may have been due to the intrinsic characteristics of κ -Carrageenan and HPMCK100M related to the water uptake and degree of polymer swelling (32). Nevertheless, the presence of surfactants in reverse micellar EGCG affected the release rate. The existence of this burst produced an immediate therapeutic effect. Furthermore, the release occurred continuously with sustained effects. It can be seen from the contents of Table 2 that the kinetics of the EGCG release from EGCG-Gel matched those of Higuchi release, while the RM-EGCG-gel drug release kinetics fitted the Korsmeyer-Peppas model. Within the Higuchi kinetic model, the release of the drug is based on a diffusion process following Fick's law (33). This mechanism usually occurs in a tablet matrix containing water-soluble drugs with drug release following Higuchi's model, which requires water uptake in the early stages (33–35). It correlates well with the higher swelling index value of EGCG-Gel rather than that of RM-EGCG-Gel.

The release rate analysis indicated that RM-EGCG-Gel has an EGCG release rate greater than that of EGCG-Gel. It appears that the formation of aggregates between surfactants present in the RM-EGCG-Gel had no effect on the EGCG release process. In this case, the release process was more influenced by the polymer-dependent-release mechanism. The rate of drug release of a nanocarrier was also affected by other factors, such as polymer and other excipients, physical or chemical interactions between components, and manufacturing methods(22). The type and number of monomer units of polymers remarkably determined the release of the drug from the dosage form (36).

The penetration study showed that application time significantly affects the depth of EGCG penetration. The rhodamine-labeled EGCG observed during the sixth hour after application produced higher intensity in cervical tissue, which experienced deeper EGCG penetration than those tissues observed two hours after topical administration. In addition, EGCG penetration of the RM-EGCG-Gel indicated greater penetration than that of the EGCG-Gel. The presence of surfactants in reverse micellar EGCG produced an enhancing effect on EGCG penetration. This could also have been caused by an increased EGCG partition coefficient. Physical modification of EGCG with the addition of surfactant Tween 80 and Span 80 produced more lipophilic EGCG by increasing the coefficient partition of EGCG from 1.89 to 2.1 (24). It has been established that the optimal Log P value for tissue penetration is between 2 and 3 (23,24). The addition of Tween 80 and Span 80 surfactants to reverse micellar EGCG systems can also increase membrane fluidity which is affected by the fatty acid chain of surfactants (37). Unsaturated fatty acids of Tween 80 and Span 80 have a double bond that can cause the membrane structure to stretch and render, making it easier for EGCG to penetrate the membrane (37).

In this research, the intensity of rhodamine indicated that EGCG could penetrate as far as the deep layer of cervical tissue. However, the concentration of EGCG required in the target tissue to support anticancer therapy should be further analyzed.

4. CONCLUSION

The characteristics of the gel-loaded reverse micellar EGCG were not significantly different from those in the original EGCG. However, the use of surfactants in these gels produced a higher swelling index than that of native EGCG. Moreover, surfactant Tween 80 and Span 80 in reverse micellar EGCG increased the release flux and cervical penetration of EGCG from κ -Carrageenan and HPMC K100M-based gels, indicating the future application of EGCG for therapeutical purposes.

5. MATERIALS AND METHODS

5.1. Materials

EGCG with a purity grade of 98% was obtained from Xi'an Rhongsheng Biotechnology Co., Ltd. (Shaanxi, China). HPMC K100M is a product of Aldrich (USA). κ -Carrageenan was purchased from Danisco-Cultor (Copenhagen, Denmark). Tween 80 is a product of KOA Corporation (Tokyo, Japan). Span 80 was acquired from Nanhang Industrial Co. Ltd. (Zhejiang, China). Other chemicals and reagents used represented available non-technical grades.

5.2. Preparation of EGCG Gels

Gels were prepared using the components in Table 3. κ -Carrageenan and HPMC K100M were initially dispersed in citrate buffer pH 4.2 and mixed after complete swelling had occurred. The EGCG solution in citrate buffer pH 4.2 ± 0.3 was subsequently added to the polymer dispersion, followed by 0.025 M KCl solution as the crosslinking agent and stirred until homogeneous. At that point, the gels were put into molds and stored at 4°C for 24 hours.

Table 3. Formulation of gels loading native and reverse micellar EGCG

Components	Function	EGCG-Gel		RM-EGCG-Gel	
		%	mg	%	mg
Native EGCG	Active substance	3	90	-	-
EGCG reverse micelle	Active substance	-	-	Equivalent to 3% EGCG	
Tween 80	Surfactant	-	-	0.01	0.3
Span 80	Surfactant	-	-	0.05	1.5
κ -Carrageenan	Polymer	7.5	225	7.5	225
HPMC K100M	Polymer	5	150	5	150
0.025 M KCl solution	Crosslinking agent		1 mL		1 mL
Citrate Buffer pH 4.2	Solvent		Up to 3 grams		Up to 3 grams

In preparing gel loading reverse micellar EGCG, the modified reverse micelle EGCG was first prepared in the manner previously described (24) using Tween 80 and Span 80 HLB 6 in citrate buffer at pH 4.2. The gels were then prepared to employ the same method as gel loading native EGCG.

5.3. Characterization of gel loading EGCG

For preparing the gel, the gel mass was shaped into a mold and stored at a cool temperature for 24 hours, and then the hard gel was left at room temperature for a while and measured for further evaluation. The pH was initially evaluated by dissolving the gels in warm water until all matrix components had melted and dispersed. At that point, the pH was measured at 37 ± 0.5 °C using a pH meter (38). Moreover, a gel hardness evaluation was carried out on ten gels using an Erweka® hardness tester at a temperature of 25 ± 0.5 °C (38–40). The swelling index was further evaluated by weighing gels individually (W_0). Firstly, the beaker glass, funnel, and filter paper were weighed before approximately 50 mL citrate buffer pH 4.2 was added and the weight of the buffer solution was calculated. A gel was then introduced into the expanding medium. After six hours, it was transferred to another container, and the final buffer solution was weighed. The weight of the buffer solution absorbed by the gel was obtained, giving the final gel weight (W_t). The swelling index was subsequently calculated using the following formula (41)

$$\text{Swelling Index} = \frac{W_t - W_0}{W_0}$$

where W_t : weight of gel at time t , and W_0 : initial weight of the gel

5.4. Evaluation of EGCG released from gels

The EGCG released from gels was examined for gels equivalent to 270 mg of EGCG using 100 mL citrate buffer pH 4.2 as the release media at 37°C with continuous stirring at 100 rpm. Each sample of approximately 5 mL was extracted with a syringe at several determined points up to 720 minutes prior to filtering through paper with a pore size of 0.45 μm . The same volume of citrate buffer pH 4.2 was then added to the release medium to replace the extracted volume of samples. The EGCG level in the sample was determined using a UV-Vis Spectrophotometer at a maximum wavelength of 271 nm prior to the EGCG release profile being analyzed. To determine the release kinetics of native EGCG and reverse micellar EGCG from HPMC and κ -Carrageenan-based gels, the release profile was further plotted and analyzed for zero-order, first-order, Korsmeyer-Peppas, Higuchi, and Hixson-Crowell kinetics models.

5.5. *In vivo* cervical penetration study of EGCG gels

The protocol of this experimental study was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia, through ethical clearance letter No. 2.KE.095.06.22021. Twenty-four female mice were divided into six groups of four subjects that had been acclimatized for a week prior to initiation of the study.

The samples were prepared for the *in vivo* penetration study by adding about 2.56 mg Rhodamine-B to the mixtures of surfactants and gel bases as presented in Table 2 for preparing Rhodamine-labelled Blank Surfactant, EGCG- and RM-EGCG Gels.

The subjects were anesthetized with ketamine at a dose of 70 mg/kg body weight and administered intraperitoneally. The sample was subsequently applied at an amount equivalent to 0.234 mg EGCG intravaginally using a syringe connected to a catheter. After two and six hours of application, the subjects were sacrificed using cervical dislocation. Cervical tissue sections were then sliced to a thickness of 5 μm at -20°C using a cryo-microtome (Leica Cryocut, Germany) before being observed using a fluorescence microscope (FSX100 Olympus, Japan).

Data analysis was performed using a fluorescence intensity scoring system based on sample penetration after two and six hours of intravaginal administration. The fluorescence intensity scores were (-) for no fluorescence intensity, (+) for weak fluorescence intensity, (++) for moderate fluorescence intensity, and (+++) for strong fluorescence intensity.

5.6. Statistical analysis

In this study, the data represent the mean \pm standard deviation (SD). The data was analyzed statistically using Independent Samples t-Test with IBM SPSS Statistics v.22 software. The P -value less than 0.05 expressed the significant difference between samples.

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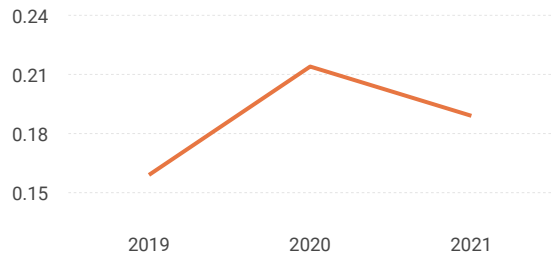
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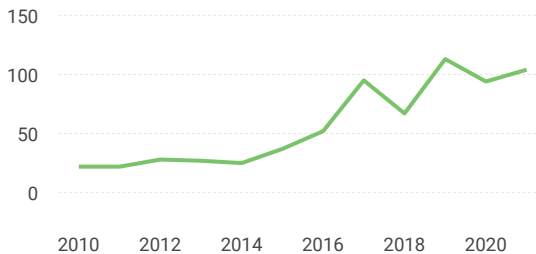
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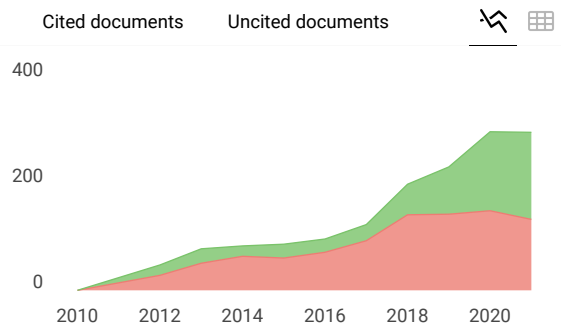
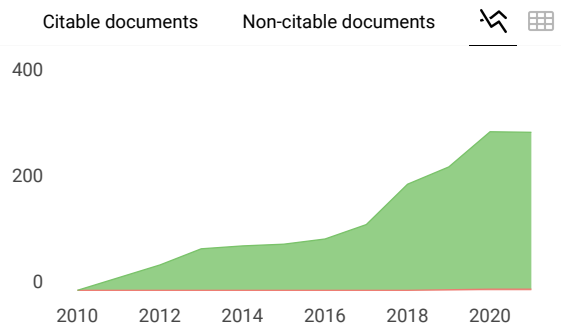
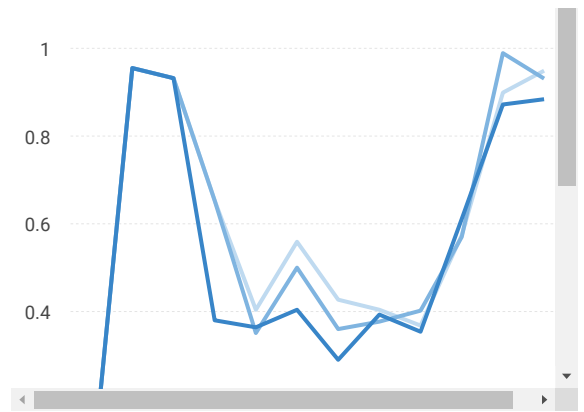
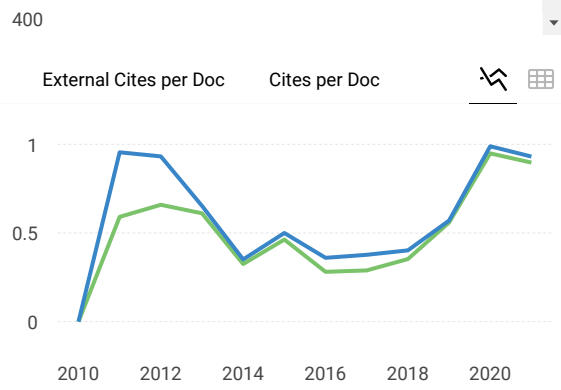


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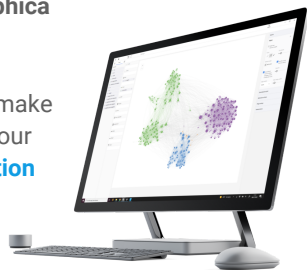
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