Ex Vivo and In Vivo Retention Time Evaluation of Fucoidan Isolated from *Macrocystis pyrifera* Through a Thermosensitive Gel System in The Vaginal Route

Anwar Sam[1], *Ardiyah Nurul Fitri* Marzaman[2], *Mukarram* Mudjahid[2], *Tri Puspita* Roska[2], *Andi Dian* Permana[2], *Marianti A*. Manggau[1*]

[1]Department of Biopharmacy, Faculty of Pharmacy, Hasanuddin University, 90245, Indonesia
[2]Department of Pharmaceutical Science and Technology, Faculty of Pharmacy, Hasanuddin University, 90245, Indonesia

Abstract. This study evaluated Fucoidan from *Macrocystis pyrifera* as a potential treatment for cervical cancer. The research aimed to examine Fucoidan's in vivo retention capacities in poloxamer-based in situ gels for vaginal drug delivery systems. Five different thermosensitive gel formulations were developed, each with varying concentrations of Pluronic F127 and F68 polymers. The incorporation of HPMC affected the gelation temperature, viscosity, and bioadhesive strength. The accepted formula, F3, had a bioadhesive value of 5415.93 ± 98.74 dyne/cm2 and could form a gel at physiological temperature. Ex vivo animal models showed that Fucoidan components retained well on vaginal tissue. Only F1, F2, and F3 achieved the media after 8 hours of examination. In vivo evaluation showed F3 had the highest drug concentration retained in the vaginal mucosa of female rats after 8 hours ($24,115 \pm 4,842$ μg), slowly removed after 24 hours ($13,014 \pm 5,596$ μg). In conclusion, increases in the hydrophilic content of formulations led to the retained hydrogel formula, which increased drug release and lowered intravaginal elimination.

1 Introduction

Cervical cancer is the growth of abnormal cells in the lining of the cervix. In 2020, it is well known that about 90% of new cases of cervical cancer and deaths happened in low and middle-income countries with a total of 604.000 new cases and 342,000 patients' deaths worldwide [1]. Cervical cancer was the second most common cancer in women in Indonesia when observed in 2018, with 348.809 new cases and 207.210 deaths, which is almost 60% of the new cases [2]. Deaths from cervical cancer are expected to keep going up and are expected to reach 12 million by 2030 if things aren't done right. It is thought that there are 180,000 new cases of cervical cancer in Indonesia every year and that 75% of those who get it die in the first year [3]. This death is mostly linked to the fact that most newly diagnosed patients are already in an advanced stage and due to a lack of early detection and as well as

---

* Corresponding author: mariantimanggau@unhas.ac.id

early treatment of cervical precancerous lesions [3, 4]. Therefore, it is crucial to expand the treatment of algorithms to prevent or even cure this disease.

Researchers have been studying Fucoidan as a complex polysaccharide with bioactive capabilities, found naturally in several different types of brown algae (*Phaeophyceae*). Fucoidan is potential to treat several diseases that include immunomodulatory, anti-inflammatory, anticancer, and could perform antioxidant effects because of the structure and its chemical composition [5, 6]. Fucoidan is a sulfate polysaccharide found in the cell wall that is composed of several different kinds of monosaccharides. To enter cells, Fucoidan relies on its low molecular weight polysaccharide content, which has a high absorption efficiency [7]. The majority composition of Fucoidan is α-L-fucose and sulfate, however, galactose, mannose, xylose, uronic acid, and an acetyl group are also the additional monosaccharide components [8].

In a study conducted using mice as experimental animals, giving Fucoidan at repeated doses (10 mg/kgBW), was able to show an antitumor effect by inhibiting tumor growth by 33% and antimetastatic activity by 29%. Several supporting studies with administration of Fucoidan via the intraperitoneal injection route and/or administration of Fucoidan through food, gavage, subcutaneous injection, and intravenous injection have also been widely studied [9-13]. However, the complex heterogeneity of polysaccharides has an influence on the conditions for collecting and processing data, as well as the lack of analysis using pure products that have been standardized. This study attempted to validate the Fucoidan analysis method by examining the polysaccharide groups using the phenol-sulfuric acid method.

In the process of treating cervical cancer, the selection of the route of administration and the type of preparation is a very important factor. Several studies have shown that the vaginal route is the most suitable route for delivering drugs directly to targets in cervical cancer. This route has various advantages, such as large surface area, rich blood supply, high permeability, increased drug bioavailability, first-pass metabolism, minimized side effects that occur, and how to use it easily when compared to injections that require the assistance of health workers [14].

There many examples of vaginal dosage form available, and one of those types is gel. However, this form gives a feeling of discomfort during the application process which has an impact on patient adherence to therapy. To increase patient comfort and obtain sustainable drug release, preparations are formulated in the form of in situ gels [15].

Thus, the development of preparations using delivery systems on the vaginal route continues to be developed. The thermosensitive gel was chosen in this research due to its ability to form gel when applied to the vaginal mucosa. To measure the susceptible criteria of formulation, it is important to carry out in vitro and in vivo tests to confirm the physico-chemical characterization of the preparation. Based on the explanation above, this study aims to design Fucoidan in the form of a thermosensitive gel preparation to increase the bioavailability of Fucoidan in vaginal tissue. This preparation is then subjected as an initial study for the development of effective preparations in the treatment of cervical cancer.

2 Materials and method

2.1 Materials

The materials used in this study included deionized water, acetic acid, hydrochloric acid (HCl), lactic acid, DMDM Hidantoin, Fucoidan, glucose, glycerin, potassium hydroxide (KOH), calcium hydroxide (Ca(OH)2), methanol, sodium chloride (NaCl), Pluronic F127, Pluronic F68, and HPMC.

2.2 Preparation of artificial vaginal fluid

Artificial vaginal fluid was prepared by weighing 5 grams of glucose, 3.51 grams of sodium chloride (NaCl), 3 grams of lactic acid, 1.4 grams of potassium hydroxide (KOH), 1 gram of acetic acid (CH3COOH), 0.4 grams of urea, 0.222 grams of calcium hydroxide CaH_2O_2, and 0.16 grams of glycerol. The ingredients that have been weighed are then dissolved in Erlenmeyer. Then, the pH was measured and adjusted until it reached pH 4.2 using 0.1 N HCl, then added up to 1 L using distilled water [16, 17].

2.3 Fucoidan analysis

2.3.1 Preparation of fucoidan stock solution

A total of 5 mg of Fucoidan was weighed and put into a 5 mL volumetric flask. After that, it was dissolved to the mark using purified water (1000 µg/mL).

2.3.2 Determination of Phenol-Sulfuric Acid Concentration for fucoidan analysis in Polysaccharides

This method is carried out to determine the appropriate concentration to obtain the highest absorbance that can be read on a UV-Vis Spectrophotometer [18, 19]. The test was carried out three times using several comparisons of Fucoidan concentration, 5% phenol and sulfuric acid as shown in Table 1 and Table 2.

After obtaining the desired colour change (orange-gold), the absorption was measured using a UV-Vis spectrophotometer to determine the maximum wavelength that would be used for the next analysis.

2.3.3 Determination of the maximum wavelength of fucoidan in water solvent

500 µL of stock fucodain solution was sampled and then diluted using distilled water up to 1000 µL (concentration 500 µg/mL). After that, the absorption of the solution was measured using a UV-Vis spectrophotometer at a wavelength of 200-400 nm. The maximum wavelength is determined based on the highest absorption in the sample [18][19].

2.3.4 Determination of fucoidan maximum wavelength in Artificial Vaginal Fluid (AVF)

Fucoidan stock solution with a concentration of 1000 µg/mL was then diluted to a final concentration of 100 µg/mL using artificial vaginal fluid. Fucoidan solution was added with 5% phenol:sulfuric acid (1:8). After that, the absorption was measured using a UVV is spectrophotometer at a wavelength of 400-800 nm. The maximum wavelength is determined from the absorption in the sample

2.3.5. Determination of the maximum wavelength of fucoidan in vaginal tissues

Fucoidan stock solution with a concentration of 7,500 µg/mL was sampled as much as 10 µL and was added to the vaginal tissue preparation as much as 90 mg. Then 200 µL of tris buffer was added. The preparation was then sonicated for 15 minutes, then vortexed for 5 minutes and centrifuged for 15 minutes. Furthermore, 100 µL of the supernatant was sampled, then 5% phenol:sulfuric acid (1:8) was added. After that, the absorption was measured using a

UV-Vis spectrophotometer at a wavelength of 400-800 nm. The maximum wavelength is determined from the absorption in the sample [18-20].

Table 1. Variation of fucoidan concentrations with the addition of 5% phenol and sulfuric acid visible measurements.

No	Fucoidan in Water Solvent (µg/mL)	Phenol 5% (µL)	Sulfuric acid (µL)
1	500	50	450
2	250	50	450
3	125	50	450
4	62.5	50	450
5	31.25	50	450

Table 2. Variation of fucoidan concentrations with 5 the addition of 5% phenol and sulfuric acid visible measurements.

No	Fucoidan in Water Solvent (µg/mL)	Phenol 5% (µL)	Sulfuric acid (µL)
1	100	100	800
2	50	100	800
3	25	100	800
4	12.5	100	800
5	6,25	100	800

2.3.6 Determination of the maximum wavelength of fucoidan in water

The stock Fucoidan solution was diluted to a concentration of 100, 50, 25, 12.5, 6.25 µg/mL using distilled water, respectively. Each solution concentration was then measured for its absorption at a predetermined maximum wavelength using a UV-Vis spectrophotometer. The relationship between absorption and concentration is then plotted in the form of a curve [21].

2.3.7 Determination of the maximum wavelength of fucoidan in Artificial Vaginal Fluid (AVF)

Fucoidan stock solution with a concentration of 1000 ppm, then diluted using artificial vaginal fluids to reach concentrations of 100, 50, 25, 12.5, and 6.25 ug/mL. Next, each Fucoidan solution was added with 5% phenol:sulfuric acid (1:8). The absorbance of each solution concentration was measured using a UV-Vis spectrophotometer with a predetermined maximum wavelength, then a standard curve was created with the relationship between absorption and concentration then plotted in the form of a curve [18, 20]

2.3.8 Determination of the maximum wavelength of fucoidan in vaginal tissue

Fucoidan solution with a concentration of 30,000 µg/mL, 15,000 µg/mL, 7,500 µg/mL, 3,750 µg/mL, 1,875 µg/mL. Each sampled as much as 10 µL was added to the tissue slurry as much as 90 mg. Then 200 µL of tris buffer was added. Sonicated in a sonicator for 15 minutes, then vortexed for 5 minutes and centrifuged for 15 minutes. 100 µL of the supernatant was sampled, then 5% phenol:sulfuric acid (1:8) was added. Each solution concentration was then measured for its absorption at a predetermined maximum wavelength using a UV-Vis

spectrophotometer. The relationship between absorption and concentration is then plotted in the form of a [18-20].

2.4 Fucoidan Gel Thermosensitive (FTG) preparation

The thermosensitive-mucoadhesive gel formula was prepared by a modified cold method. Pluronic® F127 and F68 were added to cold distilled water (4°C) while continuously stirring using a magnetic stirrer. The mixture is then left in the refrigerator until a clear mixture is obtained. HPMC was then added to the mixture, then allowed to stand for 1 x 24 hours in the refrigerator. The polymer mixture and DMDM hydantoin were then added and stirred until homogeneous [22, 23]. The thermosensitive-mucoadhesive gel formula can be seen in Table 3.

Table 3. Fucoidan thermosensitive-mucoadhesive gel formula.

Ingredients	Composition (%b/b)				
	F1	F2	F3	F4	F5
Fucoidan	3	3	3	3	3
Pluronic F-127	12	14	16	18	20
Pluronic F-68	4	4.5	5	5.5	6
HMPC	1	1	1	1	1
Glycerin	10	10	10	10	10
DMDM Hidantoin	0.1	0.1	0.1	0.1	0.1
Aquadest	Up to 100%				

2.5 Characterization of Fucoidan Thermosensitive Gels (FTG)

2.5.1 Organoleptic

Organoleptic testing was carried out by directly observing the color, texture, and homogeneity of the gel.

2.5.2 Gelation temperature measurement

Gelation temperature measurements were carried out using a modified method in which a thermosensitive gel was sampled and placed in a closed test tube and then placed in a water bath at 20°C which was increased to 65°C. The test tube was observed visually every 1°C temperature increase. The gelation temperature recorded was the temperature at which the gel did not move when the tube was inverted to 90° [24].

2.5.3 pH measurement

The pH of the thermosensitive gel was measured using a pH-meter at room temperature by inserting the tip of the electrode into the gel and the results were recorded after 2 minutes of observation [24].

2.5.4 Spreadability

The in situ thermosensitive gel formulation was weighed as much as 500 mg and placed on a glass plate and covered with a glass plate with a weight of 100-100 grams, then, a load weighing 500 g was then placed on top of the formula. After 5 minutes of adding the load, the diameter of the gel formed was measured with a caliper [23].

2.5.5 Extrudability

The gel formulation was placed in collapsible tubes and sealed as much as 15 grams for each formula. The weight of the tubes was measured at the beginning. The tubes were placed between two glass slides and were clamped. As many as 500 g weight was put on the top of the slides and then the cap was opened. The amount of gel extruded was collected and was calculated. The accepted criteria if the percentage of extruded gel achieves>90% which is classified as excellent, >80% are classified as Good and >70% classified as Fair [25].

$$\% \text{ Extrudability} = \frac{total\ mass\ of\ extruded\ gels}{total\ mass\ of\ gels\ inserted\ in\ the\ tube} \times 100 \qquad (1)$$

2.5.6 Bioadhesive strength evaluation

The mucoadhesive strength of Fucoidan thermosensitive gel was measured using a modified weighing method at physiological body temperature (37°C). The porcine vaginal mucosa that had been rinsed using artificial vaginal fluid was used as a membrane attached to the upper and lower vials. After that, 1 g of each FTG formula was taken and glued between the vaginal mucosa which was attached to the left arm of the scales. Meanwhile, on the right arm of the scales a weight of 1 gram is placed with the addition of certain amount of weight in every 30 seconds to measure the total weight needed for the gel to be released from the vaginal mucosa. The addition of the load was stopped when the surfaces of the two vials were separated [22, 26]. The mucoadhesive strength of a Fucoidan thermosensitive gel can be calculated using the following equation:

$$Bioadhesive\ strength\ (dyne/cm^2) = (m.g)/A \qquad (2)$$

m= the load required to release the gel from the vaginal mucosa (g)
g= acceleration due to gravity (980 cm/s^2)
A= exposed mucosal surface area (cm^2)

2.5.7 Viscosity

The viscosity of the thermosensitive gel was measured using a Brookfield viscometer at different temperature conditions using spindle 3 (before forming the gel) and 7 (after forming the gel) and was set at 50 rpm [24].

2.5.8 Rheology

The rheology of the thermosensitive-mucoadhesive gel was determined using a Brookfield viscometer using spindle 7 at speeds of 5, 10, 20, 50, and 100 rpm. Measurements were made at vaginal physiological temperature (37°C). The measurement results are then processed into a graph, so that the type of flow can be determined from the thermosensitive mucoadhesive gel preparation [27].

2.5.9 Ex vivo permeation test

The *Ex Vivo* permeation test was carried out using a Franz diffusion apparatus using the porcine vaginal mucosa as the donor compartment. The vaginal mucosa washed using artificial vaginal fluid, then placed between the donor compartment and the Franz diffusion cell receptor, the artificial vaginal fluid is adjusted to a pH of 4.2 and put into the receptor compartment. The test temperature was set at $37\pm1°C$ while keep stirred using a magnetic stirrer at 100 rpm. After that, 1 gram of each FTG formula was taken and placed in the donor compartment. The sampling was done by taking 1 mL of sample at 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, and 24 hours, then the absorption was measured using a UV-Vis spectrophotometer. The amount of receptor medium sampled was replaced with fresh medium ($\pm 37°C$) equal to the volume sampled in the receptor compartment. The drug concentration in the sample was calculated using the standard curve, then the release rate (flux) of the permeation preparation was calculated [28].

2.5.10 Ex vivo retention test

The *Ex Vivo* permeation test was carried out using a Franz diffusion apparatus, porcine vaginal mucosa, and artificial vaginal fluid at pH 4.2. The temperature is set at $37\pm1°C$. The vaginal mucosa was obtained through a surgical process and rinsed using water, then the porcine vaginal mucosa was stored in a tightly closed container and cooled at -20°C until the next stage of evaluations [29].

The sample was put into the donor compartment and the porcine vaginal mucosa was collected after 24 hours. Next, tris buffer was added in a ratio of 1: 2 (w/w) between the mucosa that had been made into tissue pulp. The Fucoidan extraction process was carried out using a sonicator for 15 minutes, then vortexed for 5 minutes, and centrifuged for 15 minutes. 100 µL of the supernatant was taken, 100 µL of 5% phenol was added, 800 µL of sulfuric acid was added and homogenized. The absorbance is measured using a spectrophotometer. The drug concentration in the sample was calculated using the vaginal tissue standard curve, then the release rate (flux) of the permeation preparation was calculated [28].

2.6 In Vivo Test

2.6.1 Experimental animal setup

The test animal used in this study was an adult female white rat (*Rattus norvegicus*). A place for keeping test animals is prepared, namely cages, husks, places to eat and places to drink. Rats were acclimatized for 7 to 14 days in order to adapt to their new environment. 45 rats were used, 9 rats each for each formula which were divided into 3 groups, namely the 1st, 8th, and 24th hours. The testing procedure in this research studies was reported to the Hasanuddin University Ethics Commission and was approved with ethical approval number 121022092285 and in adherence to the National Institutes of Health guide for the care and use of Laboratory animals [29].

2.6.2 Treatment of experimental animals

The animals were applied with the gel's formula, and evaluated at 1, 8 and 24 hours of monitoring.

2.6.3 Extraction of fucoidan in the blood of experimental animals

Each mouse vaginal tissue sample collected was weighed on an analytical balance. Then tissue slurry is made by adding aquadest with a tissue:aquadest ratio (9:1). Then added the filter solution using tris buffer with a ratio of 100 mg/ 200 µL. The sample was sonicated for 15 minutes, then vortexed for 5 minutes, and centrifuged for 15 minutes [20, 30].

2.6.4 Determination of retained fucoidan content using spectrophotometry Uv-Vis

The determination of retained Fucoidan was measured by taking 100 µL of the supernatant and added with 100 µL of 5% phenol, 800 µL of sulfuric acid and homogenized and then the absorbance was measured using a Uv-vis spectrophotometer [20, 31].

2.7 Data collection and data analysis

The research data obtained will then be tabulated and analyzed using a statistical approach using the IBM SPSS Statistics application. *Ex vivo* evaluation of pH, viscosity, gelation temperature, mucoadhesive strength, permeation, and retention was analyzed using the One Way Anova method. In addition, the data obtained is also processed into graphical form using the GraphPad application.

3 Results and discussion

3.1 Determination of maximum wavelength of fucoidan with various fucoidan concentration.

In this study, the maximum wavelength of Fucoidan was measured using phenol-sulphuric acid to detect the presence of polysaccharides. The concentrated sulfuric acid is capable of converting all polysaccharides, disaccharides, and oligosaccharides into monosaccharides during this process. These pentoses (5-carbon substances) are then dehydrated to furfural, while the hexoses (6-carbon substances) are dehydrated to hydroxymethyl furfural. When the compounds react with phenol, they produce an orange-gold color which lasts a long time, and the procedure's precision is within +-2% under ideal conditions [32]. It was found that, using the ratio of 1:8 between phenol and sulphuric acid with Fucoidan concentration starting at 100 ppm, 50 ppm, 25 ppm, 12,5 ppm and 6,25 ppm, showed a good curve pattern (Figure 1). Furthermore, this calibration curve used for all evaluations which involved absorbance analysis.
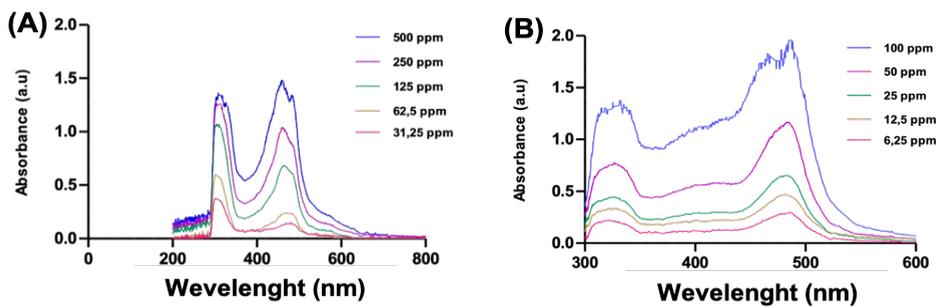
3.2 Fucoidan thermosensitive gel formulation

In this study, Fucoidan was formulated into a thermosensitive gel dosage form as a vaginal delivery system to increase patient comfort when applying the gel and increase the concentration of Fucoidan retained in the vagina. The thermosensitive gel was made by using a combination of two types of Pluronic® namely Pluronic F127 and F68 as thermosensitive polymers. The addition of 0.5% w/w HPMC as a viscosity increaser will also affect the bioadhesive ability of the preparation on the vaginal mucosa. The Fucoidan thermosensitive gel formulation was made into 5 types of formulas with different concentration variations between Pluronic®, namely Pluronic F127 and F68.

3.3 Evaluation of fucoidan thermosensitive gel formulation

3.3.1 Organoleptic test

Fucoidan thermosensitive gel preparations that have been formulated can be seen Figure 2. The organoleptic test results showed that all formulas of Fucoidan thermosensitive gel (FTG) preparations were white, odorless, formed liquid at cold temperature and homogeneous. In addition, the results of the organoleptic test also showed that the addition of HPMC showed a viscosity increment at F5 when evaluated in room temperature which could be due to the ability of HPMC to increase the viscosity of the preparation [33].
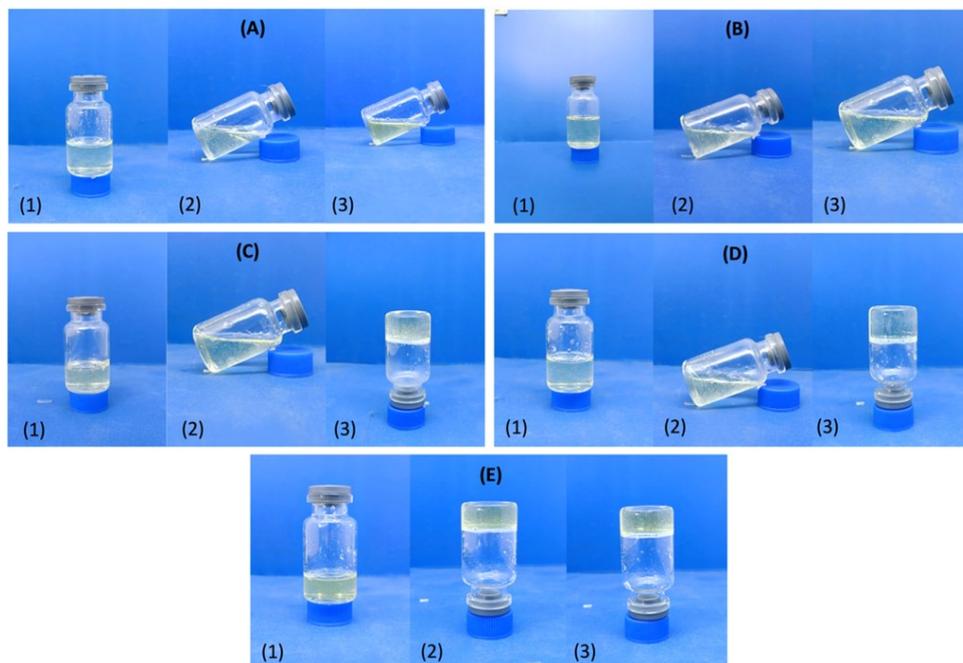

Fig. 1. Maximum wavelength of Fucoidan using phenolic sulphuric acid methods at a ratio of (4:5) with concentration of Fucoidan at 500 ppm, 250 ppm, 125 ppm, 62,5 ppm and 31,25 ppm (A) Maximum wavelength of Fucoidan using phenolic sulphuric acid methods at a ratio of (1:8) with concentration of Fucoidan at 100 ppm, 50 ppm, 25 ppm, 12,5 ppm and 6,25 ppm (B).

3.3.2 Measurement of gelation temperature, extrudability, pH, and bioadhesivity

Determining the gelation temperature was done using the test tube inversion method. Gelation temperature is a very important parameter to be tested in thermosensitive gel formulations because the gelation temperature of the preparation must match the temperature at which the gel is applied. Normal vaginal temperature ranges from 36-37°C, so the test is done at that temperature. The results of gelation temperature tests between F1 – F5 respectively were 77.33 ± 1.15°C, 46.53 ± 1.5°C, 36.16 ± 0.28°C, 33.66 ± 1.15°C, and 27.5 ± 0.5°C. Statistical calculation based on the data obtained, it shows a significant difference between F1 to F2, F3, F4 & F5. Moreover, F2 also showed significant difference with the other formulas with a *p* value <0.05.

However, the gelation temperature on F1 and F2 does not meet the criteria for a good thermosensitive gel, because gelation occurs above the physiological temperature of the vagina. However, the gelation temperature F3 and F4 fulfilled the criteria because they experienced *in-situ* gelation at normal vaginal temperature and formed as liquid at room temperature (±25°C), while F5 had formed a gel at room temperature and also did not meet the criteria.

Pluronic® is known to be a non-ionic tri-block copolymer containing a hydrophilic side (polyethylene oxide (PEO) and a hydrophobic core) (polypropylene oxide (PPO)). The difference between each type of Pluronic® is by the ratio of PEO and PPO, where Pluronic® F127 has a higher PPO ratio than Pluronic® F68. The hydrophobic part (PPO) is associated with a decrease in gelation temperature while the hydrophilic part (PEO) associated with an increase in gelation temperature, therefore changing the PEO/PPO ratio will cause a change in the gelation temperature of the formula [34].

Fig. 2. Visual appearance at cold temperatures of 4°C (1) room temperature 25°C (2), and vaginal physiological temperature 37°C (3). Thermosensitive gel with a concentration of Pluronic F127 12% and F68 4% (A); Thermosensitive gel with a concentration of Pluronic F127 14% and F68 4.5% (B); Thermosensitive gel with a concentration of Pluronic F127 16% and F68 5% (C); Thermosensitive gel with a concentration of Pluronic F127 18% and F68 5.5% (D); Thermosensitive gel with a concentration of Pluronic F127 20% and F68 6% (E).

3.3.3   pH measurement

pH testing was carried out using a pH meter. A diagram of the results obtained in the pH test can be seen in Figure 3C. The pH of the thermosensitive gel preparation is an important parameter to evaluate because the discrepancy between the pH of the preparation and the pH of the vagina can cause irritation or an uncomfortable burning feeling when the gel is applied. A normal vagina has a pH that is in the range of 3.8 – 5.0 (Y.-P. Lin et al., 2021). The pH values of F1 – F5 respectively were 4.11 ± 0.07, 4.15 ± 0.07, 4.64 ± 0.07, 4.62 ± 0.02, and 4.89 ± 0.01. The results obtained indicated that the pH of the five preparations corresponded to the normal vaginal pH. Based on the results of statistical analysis, it was found that the pH value did not differ significantly ($p < 0.05$) between all formulas.

3.3.4 Spreadability

Spreadability is a key indicator of how comfortable a topical dosage form is when applied to the skin [35]. The gel should have adequate coverage and meet standards for optimal topical administration. The spreading power of the semi-solid formulation was measured based on the average diameter of the spreading circle. The larger the diameter, the greater the spreading power that will occur, so that more surface area will be covered by the gel [35]. The Diagram of spreadbility test results (mean ± SD, n=3) can be seen in Figure 4B.

The spreadability values from F1 – F5 respectively were 143.94 ± 0.74, 130.79 ± 1.12, 75.01 ± 0.65, 55.46 ± 1.35, and 38.4 ± 0.46. Spreadability on F1 & F2 showed the greatest value because they were still in liquid form when tested at 37°C. Meanwhile, F3 shows preparations that match the desired characteristics where the good spreadability values are in the range of 50-70 mm. Statistically, based on the data obtained, each formula was significantly different from one another because the significance value of each formula combination was at $p < 0.05$.
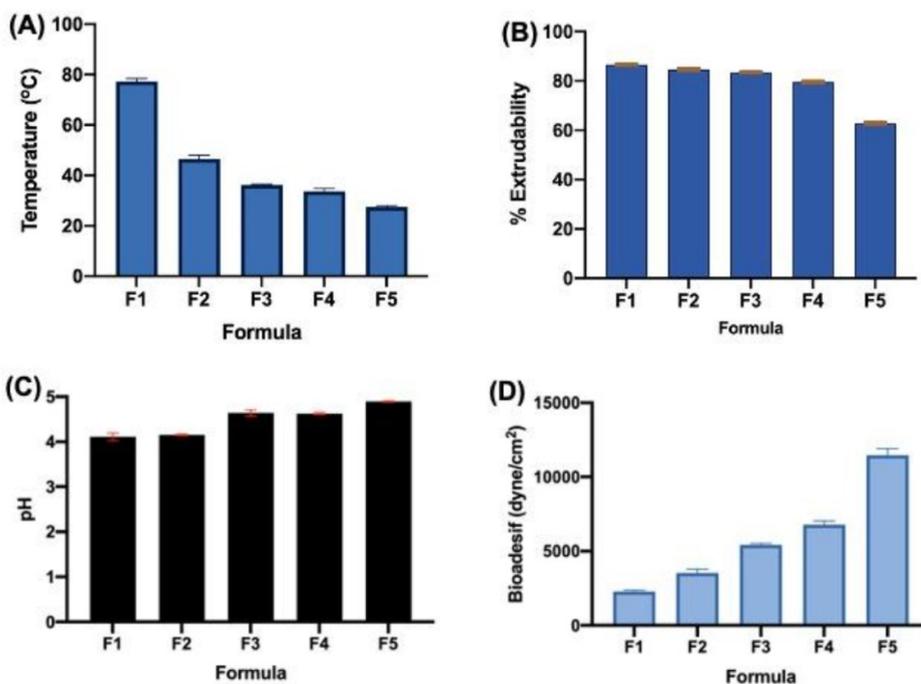

Fig. 3. Fucoidan thermosensitive gel evaluation of gelation temperature (A), Extrudablity (B), pH value (C), and gels bioadhesivity (D) (mean ± SD, n=3).

3.3.5 Extrudability

The extrudibility test was carried out with the aim of seeing the extrusion of the Fucoidan thermosensitive gel from the tube. Good gel consistency is needed for the gel application process in releasing the gel outside of the tube. The extrudability of the Fucoidan gel formula was shown in Figure 3B. From the results obtained, it can be seen that the extruded percentage values of F1 to F5 were 86.54 ± 0.20%, 84.63 ± 0.34%, 83.49 ± 0.15%, 79.50 ± 0.33% dan 62.77 ± 0.32%, respectively. From the results obtained, it can be seen that F1 to

F3 achieved the good criteria with a percentage value above 80%, F4 is included in the fairly good category with a percentage value above 70%, and F5 is included in the poor category with an extrusion percentage value of less than 70% [31]. This shows that the concentration of pluronic F127 and F68 can affect the viscosity, so that the higher the pluronic concentration, the lower the percentage of extrusion.

Table 4. The results of the Fucoidan thermosensitive gel viscosity test (Mean ± SD (n=3)).

Formula	Viscosity (cPs)		
	4°C	**25°C**	**37°C**
F1	76.66 ± 5.77	510 ± 26.45	1053.33 ± 30.55
F2	112.5 ± 6.61	610 ± 43.58	1066.66 ± 35.11
F3	165 ± 5.0	690 ± 26.45	37733.33 ± 832.66
F4	214.16 ± 20.96	744 ± 46.13	43466.66 ± 1616.58
F5	393.33 ± 20.81	1086.66 ± 58.59	51466.66 ± 923.76

3.3.6 *Bioadhesive strength on the vaginal mucosa*

Mucoadhesive strength was measured using a modified weighing method at vaginal physiological temperature (37°C). The results of measuring the mucoadhesive strength of Fucoidan thermosensitive gels can be seen in Figure 3D.

Measurement of mucoadhesive strength needs to be done to determine the ability of the Fucoidan thermosensitive gel to adhere to the vaginal mucosa. Vaginal gel preparations must have good mucoadhesive strength because it can affect the contact time of the gel with the vaginal mucosa. Based on the test results, the mucoadhesive strength of Fucoidan F1 – F5 thermosensitive gels were 2280.39 ± 98.74 dyne/cm^2, 3534.61 ± 261.25 dyne/cm^2, 5415.93 ± 98.74 dyne/cm^2, 6784.17 ± 261.25 dyne/cm^2, and 11458.99 ± 452.502 dyne/cm^2 . The addition of HPMC in the formulation was able to increase the bioadhesive strength of the preparation because it was known that the use of Pluronic® F127 alone as a polymer was not able to provide sufficiently good mucoadhesive properties [36]. Therefore, incorporation of polymers with the addition of materials such as HPMC was highly desirable to extend the dosing period of the dosage form applied to the vaginal mucosa which will directly contribute to the enhancement of the effect and localization of the drug. In the absence of HPMC, the hydroxyl contact between Pluronic® F127/F68 and the mucosal layer is not as strong as when the polymer is combined with HPMC to form a thermosensitive gel. It is well known that, HPMC as a cellulose derivative, has a very good mucoadhesive abilities [37]. The hydroxyl contact between Pluronic® F127/F68 and the mucosal layer was not as strong when the polymer is combined with HPMC to form a thermosensitive gel.

3.3.7 *Viscosity evaluation*

Viscosity testing using a Brookfield viscometer with spindle No.3 at cold temperature (4°C) and room temperature (25°C) and spindle No.3 at physiological body temperature (37°C) for F1 and F2, while for F3, F4 and F5 used spindle No.7. A diagram of the results of the Fucoidan thermosensitive gel viscosity test can be seen in Figure 4.

Evaluation with three different temperatures were carried out to proved the nature of the thermosensitive gel which has a low viscosity below the body's physiological temperature in order to facilitate the application of the gel to the vagina when the preparation is in a liquid state. The viscosity of the preparation was greater at 37°C to estimate the ability of the

preparation to form a gel when applied which can increase the localization time of the drug in the vagina [20].

The optimal viscosity of thermosensitive gel preparations before experiencing gelation is 5 – 1,000 cPs (Ahmed & Goli, 2018). The results of the viscosity test in table 4 show that at 4°C the viscosity of the Fucoidan thermosensitive gel is in the range of 76 – 393 cPs and at 25°C it is in the range of 510 – 1086 cPs, so that the viscosity of the Fucoidan thermosensitive gel at 4°C and 25 °C has met the criteria for thermosensitive gel preparation. After performing statistical analysis, it was found that the viscosity between all formulas at 4°C and 25°C was significantly different ($p < 0.05$). Meanwhile, the results of testing the viscosity of Fucoidan thermosensitive gels at 37°C were in the range of 1053 - 51466 cPs, where the optimal viscosity for thermosensitive gel preparations after experiencing gelation was 50 - 50,000 cPs (Ahmed & Goli, 2018). This shows that the viscosity of the Fucoidan thermosensitive gel at 37°C also meets the requirements of a desirable gel properties. Based on the results of observations at physiological body temperature, it was found that the viscosity between all the thermosensitive gel formulas showed a statistically significant difference ($p < 0.05$). This was influenced using a higher polymer concentration ratio between pluronic F127 and F68, and the addition of HPMC as a viscosity increasing agent also contributed to increasing the viscosity of the preparation.
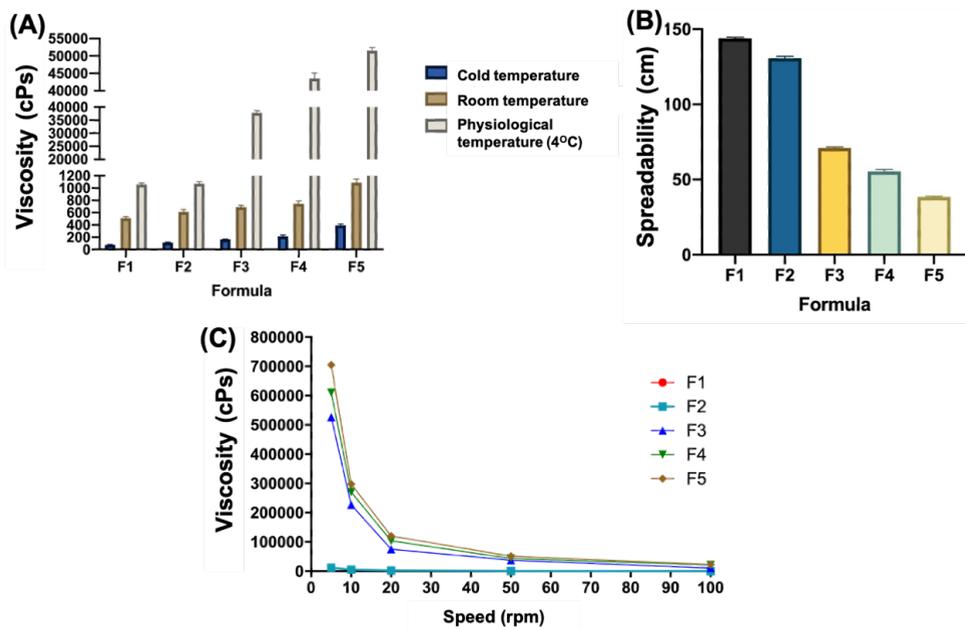

Fig. 4. Diagram of Viscosity test result (A), Spreadability (B), and Rheology (C) (mean ± SD, n=3).

3.3.8 Rheology

Viscosity and rheology are interrelated and play an important role in the process of delivering active substances to the target by influencing the dispersion ability, adhesion, and drug penetration [38]. The results of the rheological measurements of the CAP-MP's thermosensitive gels can be seen in Figure 4C. Based on the results of the rheological measurements that have been carried out, it was found that F3, F4 and F5 of the Fucoidan thermosensitive gel had a pseudoplastic flow type. Pseudoplastic flows are known to be flows

that have a viscosity that continues to decrease as the rate of shear increases [31]. Based on these results, it can be concluded that the Fucoidan thermosensitive gel will spread more easily (decreased viscosity) when a higher shear speed was applied.
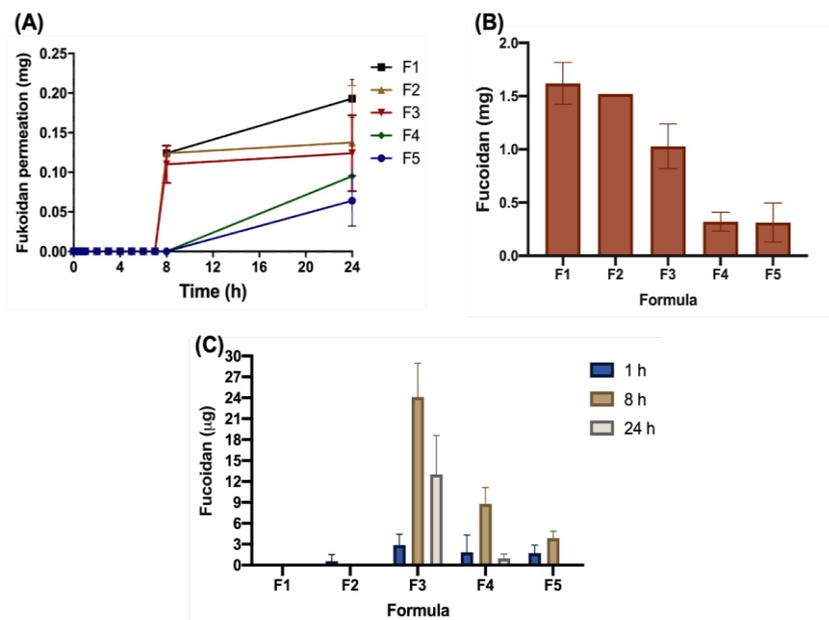

Fig. 5. Permeation test *ex vivo* (A), Retention test *ex vivo* (B), Permeation test *in vivo* (C) (mean ± SD, n=3).

3.3.9 Ex vivo permeation test

The permeation test was carried out ex vivo using a Franz diffusion cell. The results of the Fucoidan thermosensitive gel permeation test can be seen in Figure 5.

The ability of a drug to permeate across a system or membrane describes the concentration of drug that can enter the systemic pathway. This ability can be assessed based on the drug levels that managed to pass through the porcine vaginal mucosa used in the Franz diffusion cell. UV-Vis spectrophotometer is a tool used to analyze drug levels that are able to pass through the porcine vaginal mucosa and penetrate up to the systemic. In this study, the diffusion medium used was artificial vaginal fluid. Furthermore, the Fucoidan standard curve in artificial vaginal fluid as a medium in the permeation test is used to analyze the dissolved Fucoidan content in the medium. The maximum wavelength of Fucoidan in artificial vaginal fluid solution obtained from analysis using a UV-Vis spectrophotometer is 472.8 nm. Fucoidan standard curve in artificial vaginal fluid as a medium in a permeation test was made to analyze the dissolved Fucoidan content in the medium.

The test results in table 5 showed that at after 8 hours there were already permeated Fucoidan in F1, F2, and F3 with the obtained measurement results are 0.134 ± 0.0006, $0.133 \pm 0, 0006$ and 0.117 ± 0.027 respectively. The results of the F4 and F5 evaluation after 8 hours did not show any permeated Fucoidan passing through the vaginal mucosal tissue.

The time required by the permeated compound to start appearing in the receptor compartment is called the *lag time*. The results of the lag time calculation (table 6) can be seen that the higher the viscosity of the Fucoidan thermosensitive gel, the longer the time

required for the preparation to appear in the receptor compartment. The permeation rate (flux) per unit time can be seen in table 7, which is the amount of active substance transported per unit time per unit area. The statistical results showed a significance different between F4 and F5 compared to the other formulas, because at higher concentration of Pluronic which in line with the higher of viscosity, the drug component was retained in the skin surface and not being permeated to the receptor compartment.

Table 5. Permeation test results of Fucoidan thermosensitive gel Mean ± SD (n=3).

Time (h)	Amount of permeated Fucoidan (mg)				
	F1	**F2**	**F3**	**F4**	**F5**
0.25	0	0	0	0	0
0.5	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	0	0	0
4	0	0	0	0	0
5	0	0	0	0	0
6	0	0	0	0	0
7	0	0	0	0	0
8	0.134±0.0006	0.133±0	0.117±0.027	0	0
24	0.218±0.028	0.154±0.083	0.137±0.056	0.1±0.0013	0.063±0.037

Table 6. Determination of lag time.

Formula	*Lag time* (h)
F1	7,00047
F2	7,00086
F3	7,03077
F4	9,99884
F5	9,99927

The drug concentration in the donor compartment is calculated from the amount of drug transported assuming that the reduced drug in the donor compartment is transferred to the receptor compartment and the amount of drug in the membrane is neglected. The results of the calculation of the permeation rate showed that the higher the viscosity of the Fucoidan thermosensitive gel, the less permeated the amount of Fucoidan. Regarding the hydrogel, which showed a higher level of stickiness compared to the liquid control solution, there is a possibility of increased residue on the skin surface, which means that the drug or chemical compound remains on the skin and cannot be absorbed properly. In a study by Binder et al. in 2019, showed that both HEC- and HPMC-containing hydrogels showed a decrease in penetration depth as the gel viscosity increased. Increased gel viscosity may inhibit the model drug's diffusion from the formulation into the skin, ultimately leading to limited total penetration depth. In conclusion, the viscosity of the vehicle appears to influence drug distribution within the skin [39]. Therefore, F1 & F2 showed a high drug permeation after 8 and 24 hours, but both formulation did not meet the criteria of *in-situ* hydrogel. However, the F3 was chosen as it shows a good drug permeation compared to those F4 and F5.

Table 7. Calculation of flux data.

Formula	Time (h)	*Flux* ($\mu g/cm^2.h$)
F1	8	4,750
	24	4,103
F2	8	4,736
	24	3,340
F3	8	4,156
	24	2,955
F4	24	1,181
F5	24	0,752

3.3.10 Ex vivo retention test

The retention test was carried out after the permeation test using a franz diffusion cell. The results of the Fucoidan thermosensitive gel retention test at 24 hours can be seen in Figure 5.

Retention test was conducted to determine the amount of Fucoidan retained in vaginal tissue after application. This is important to know because cervical cancer target cells are found in the cervix in the vagina so it is hoped that the amount of retained Fucoidan can have an anticancer effect.

The test results showed that F1 had far more levels of Fucoidan deposited in vaginal tissue, followed successively F2, F3, F4, and F5 which were 1.052 ± 0.127 mg, 0.889 ± 0.179 mg, 0.668 ± 0.136 mg, 0.207 ± 0.057 mg, and 0.102 ± 0.039 mg. The results of the retention test also showed that the higher the viscosity, the lower the amount of Fucoidan deposited in vaginal tissue.

However, F1 and F2 did not show good physical characteristics, because when applied and exposed to physiological body temperature, they were not able to experience gelation, which would reduce patient comfort during application. F3 and F4 have met the characteristics of a good thermosensitive gel in which they are liquid at room temperature and will experience gelation when exposed to physiological body temperature, while F5 has experienced gelation temperature even though it is still at room temperature.

Table 8. Results of Fucoidan thermosensitive gel retention test using experimental animals (in vivo) Mean $\pm$ SD (n=3).

Time (h)	Amount of permeated Fucoidan (μg)				
	F1	**F2**	**F3**	**F4**	**F5**
1	0	0.0570 ± 0.93	$2.879 \pm 1,562$	$1,831 \pm 2,489$	$1,700 \pm 1,173$
8	0	0	$24,115 \pm 4,842$	$8,792 \pm 2,330$	$3,855 \pm 1,009$
24	0	0	$13,014 \pm 5,596$	$0,956 \pm 0,627$	0

3.4 In vivo retention studies

The purpose of this method is to determine the amount of Fucoidan retained in vaginal tissue in experimental animals after application. The experimental animals used were adult female rats of the Wistar strain which were first acclimatized to adjust to the new environment for 7 days. In this study, drug retention profiles in the vaginal tissues of experimental animals were

carried out by comparing each formula with three-time intervals, namely at 1 hour, 8 hours, and 24 hours. Figure 5C shows the results obtained from the amount of Fucoidan deposited in the vaginal tissue of experimental animals. Table 8 shows the amount of Fucoidan deposited in the vaginal tissue of experimental animals.

The test results showed that in F1 there was no Fucoidan deposited in the tissue either after 1 hour, 8 hour, or even 24 hour. This is due to the rapid physiological clearance caused by mucus secretion and turnover inhibiting effective vaginal retention because the F1 and F2 formula are still in liquid form even though they have been exposed to physiological body temperature. The clearance within the vaginal lumen is strong enough that the residence time of the formulation is usually too short for the active ingredients to carry out their therapeutic role, resulting in insufficient dosing and/or duration of action (D'Cruz & Uckun, 2014). After 1 hour of application, F3 showed retained capacity in the experimental animal vaginal tissue, with a value of 2,879 ± 1.562 **μ**g, after 8 hour it was 24.115 ± 4.842 **μ**g, and at the 24 hour it was 13.014 ± 5.596 **μ**g. Compared to F4, the amount of Fucoidan retained in the vaginal tissue after 1, 8 and 24 hour was 1.831 ± 2.489 **μ**g, 8.792 ± 2.330 **μ**g and 0.956 ± 0.627 **μ**g, respectively. Compared to F5, at the first observation (1 h) Fucoidan was retained in the experimental animal tissues was 1,700 ± 1,173 **μ**g, after 8 hour it was 3,855 ± 1,009 **μ**g, and there was no Fucoidan found in the tissue after 24 hour.

From the results of *in vivo* experimental retention test using adult female rats, it was found that F3 had the highest amount of Fucoidan deposited into the vaginal tissue, while the results of the F5 test after 24 hour showed no Fucoidan are retained in the tissue, this was influenced by the viscosity of the thermosensitive gel which is too large thereby reducing the ability of the active ingredient to penetrate into the tissue.

4 Conclusion

Based on the results that have been obtained it can be concluded that the use of various concentrations of pluronic®F127 and F68 polymers can affect the Fucoidan thermosensitive gels properties such as organoleptic, gelation temperature, spreadability, extrudability, pH, rheology, and viscosity. The increase in the concentration of pluronic®F127 and F68 was inversely proportional to the gelation temperature, rheology and spreadability and directly proportional to the viscosity, pH and bioadhesive. The different viscosity of each formula caused by variations in pluronic®F127 and F68 concentrations can affect the Fucoidan release profile which can be seen in the amount of Fucoidan permeated *ex vivo* and the amount of Fucoidan retained *ex vivo* and *in vivo*. It is well accepted that F3 is an optimal formula with the use of pluronic®F127 and F68 concentrations at 16% and 5% respectively, which contributes to the good physical characteristics and good Fucoidan release profiles when evaluated *ex vivo* and *in vivo*.

References

1. H. Sung, J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, F. A Cancer Journal for Clinicians, **71**, 3, 209–249 (2021)
2. Word Health Statistics, Monitoring health for SDG's (2019)
3. K.A. Kundrod, C.A. Smith, B. Hunt, R.A. Schwarz, K. Schmeler, R. Richards-Kortum, Expert Review of Molecular Diagnostics, **19**, 8, 695–714 (2019)
4. L. Denny, M. Quinn, R. Sankaranarayanan, Vaccine, **24**, 3 (2006)

5. H. Cui, Z. Wang, J. Liu, Y. Wang, Z. Wang, J. Fu, Z. Wan, R. Li, Q. Li, J. Helen Fitton, Y. Liu, M. Zhang, Aquaculture Nutrition, **26**, 1, 47–59 (2020)

6. J.H. Fitton, D.N. Stringer, A.Y. Park, S.S. Karpiniec, Marine Drugs, **17**, 10 (2019)

7. T. Zuo, X. Li, Y. Chang, G. Duan, L. Yu, R. Zheng, C. Xue, Q. Tang, Food and Function, **6**, 2, 415–422 (2015)

8. A. Synytsya, W.J. Kim, S.M Kim, R. Pohl, F. Kvasnička, J. Čopíková, Y. il Park, Carbohydrate Polymers, **81**, 1, 41–48 (2010)

9. A.F. Hifney, M.A. Fawzy, K.M. Abdel-Gawad, M. Gomaa, Food Hydrocolloids, **54**, A, 77-88 (2016)

10. K. Azuma, T. Ishihara, H. Nakamoto, T. Amaha, T. Osaki, T. Tsuka, T. Imagawa, S. Minami, O. Takashima, S. Ifuku, M. Morimoto, H. Saimoto, H. Kawamoto, Y. Okamoto, Marine Drugs, **10**, 12, 2337–2348 (2012)

11. Y. Lin, X. Qi, H. Liu, K. Xue, S. Xu, Z. Tian, Cancer Cell International, **20**, 1, 154, (2020)

12. P.V. Turner, T. Brabb, C. Pekow, M.A. Vasbinder, Journal of the American Association for Laboratory Animal Science, JAALAS, **50**, 5, 600–613 (2011)

13. W. Zhang, H.B. Park, D. Yadav, J. Hwang, E.K. An, H.Y. Eom, S.J. Kim, M. Kwak, P.C.W. Lee, J.O. Jin, International Journal of Biological Macromolecules, **174**, 477–484 (2021)

14. C. K. Sahoo, P. Kumar Nayak, D.K. Sarangi, T.K. Sahoo, American Journal of Advanced Drug Delivery, **1,** 1 , 43–45 (2013)

15. A. Majeed, N.A. Khan, Journal of Drug Delivery and Therapeutics, **9**, 1, 337–347 (2019).

16. J. das Neves, C.M.R. Rocha, M.P. Gonçalves, R.L. Carrier, M. Amiji, M.F. Bahia, B. Sarmento, Molecular Pharmaceutics, **9**, 11, 3347–3356 (2012)

17. D.H. Owen, D.F.H. Katz, Contraception, **59**, 2, 91–95 (1999)

18. M. Kondo, R. Mulianda, M. Matamura, T. Shibata, T. Mishima, A. Jayanegara, N. Isono, Animal Science Journal, **92**, 1 (2021)

19. M. Rasouli, A. Ostovar-Ravari, H. Shokri-Afra, (2016)

20. C. K. Enggi, H.T. Isa, S. Sulistiawati, K.A.R. Ardika, S. Wijaya, R.M. Asri, S.A. Mardikasari, R.F. Donnelly, A.D. Permana, International Journal of Pharmaceutics, **609** (2021)

21. A. Noyes, R. Godavarti, N. Titchener-Hooker, J. Coffman, T. Mukhopadhyay, Vaccine, **32**, 24, 2819–2828 (2014)

22. U.C. Galgatte, A.B. Kumbhar, P.D. Chaudhari, Drug Delivery, **21,** 1, 62–73. (2014)

23. A. D. Permana, R.N. Utami, A.J. Courtenay, M.A. Manggau, R.F. Donnelly, L. Rahman, Journal of Photochemistry and Photobiology B: Biology, **205** (2020).

24. A. Nurul Fitri Marzaman, Sartini, M. Mudjahid, T. Puspita Roska, A. Sam, A.D. Permana, International Journal of Pharmaceutics, **628**, 122323 (2022).

25. S. Das, P.K. Haldar, G. Pramanik, International Journal of PharmTech Research CODEN, **3**, 1 (2011)

26. S. Manna, U. Lakshmi, M. Racharla, P. Sinha, L. Kanthal, S. Kumar, Journal of Applied Pharmaceutical Science, **6**, 8, 022–029 (2016)

27. O. Solar, S. Gunasekaran, Journal of Food Engineering, **99**, 3, 338–343 (2010)

28. A.D. Permana, A.J. Paredes, F.V. Zanutto, M.N. Amir, I. Ismail, M.A. Bahar, Sumarheni, S.D. Palma, R.F. Donnelly, ACS Applied Materials and Interfaces, **13**, 32, 38745–38760 (2021)

29. J.C. Schwarz, E. Pagitsch, C. Valenta, European Journal of Pharmaceutical Sciences, **50**, 5, 595–600 (2013)

30. NIH Guidance grants and contracts (1978)

31. A. D. Permana, E. Utomo, M.R. Pratama, M.N. Amir, Q.K. Anjani, S.A. Mardikasari, S. Sumarheni, A. Himawan, A. Arjuna, U. Usmanengsi, R.F. Donnelly, ACS Applied Materials and Interfaces, **13**, 15, 18128–18141 (2011)

32. S.S. Nielsen, Phenol-Sulfuric Acid Method for Total Carbohydrates. in Food Analysis Laboratory Manual. Food Science (Springer, Boston, 2010)

33. Rowe, R.C., Sheskey, P.J. and Quinn, M.E. Handbook of Pharmaceutical Excipients. 6th Edition, Pharmaceutical Press, 506-509 (2009)

34. A. Khattab, S. Marzok, M. Ibrahim, Journal of Drug Delivery Science and Technology, **53**, 101134 (2019)

35. M. Bercea, M. Constantin, I.A. Plugariu, M. Oana Daraba, D. Luminita Ichim, Journal of Molecular Liquids, **362** (2022).

36. I.S. Kurniawansyah, T. Rusdiana, I. Sopyan, H. Ramoko, H.A. Wahab, A. Subarnas, Heliyon, **6**, 11 (2020)

37. A. Chowhan, T.K. Giri, International Journal of Biological Macromolecules, **150**, 559–572 (2020)

38. M.T. Islam, N. Rodríguez-Hornedo, S. Ciotti, C. Ackermann, Pharmaceutical Research, **21**, 7, 1192–1199 (2004)

39. L. Binder, J. Mazál, R. Petz, V. Klang, C. Valenta, Skin Research and Technology, **25**, 5, 725–734 (2019)