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by Noorma Rosita

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RESEARCH ARTICLE

Characteristics, Stability and Activity of Epigallocatechin Gallate (EGCG)-Chitosan Microspheres: Effect of Polymer Concentration

Noorma Rosita, Yuyun Nailufa, Dewi Melani Hariyadi
Pharmaceutics Department, Faculty Pharmacy, Universitas Airlangga, Surabaya, Indonesia
*Corresponding Author E-mail: **itanr@yahoo.com**

ABSTRACT:
Epigallocatechin gallate (EGCG) is a natural product compound which has known to have anticancer activity. However, its bioavailability was low of 0.1% limited by poor stability. This study was aimed to produce microspheres as drug delivery system to improve the stability of drug. EGCG-Chitosan microspheres were formed by ionotropic gelation-aerosolization technique and were produced from chitosan and sodium tripolyphosphate. This study evaluated effect of 1, 2 and 3% of chitosan concentration on physical characteristics, stability and activity of EGCG-Chitosan microspheres. Physical characteristics were evaluated in terms of particle size, morphology, moisture content, entrapment efficiency, drug loading, yield, swelling index, physical stability and activity. Stability was evaluated by measuring size, entrapment efficiency and drug loading at 25ºC and 50ºC temperature for storage period of 7, 14, 21 and 30 days. Activity test was evaluated with MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay using HeLa cells. Particle sizes of formula were 2.29, 2.68, and 3.11 µm respectively with entrapment efficiency of 33.94, 52.27, 62.14% and drug loading of 23.25, 29.36, and 32.24 % correspondingly. Morphology was spherical with smooth surface. Yields were 78.25, 79.36, and 79.77% respectively. No significant differences between all formulas indicated that microspheres were stable during storage. Activity results showed that formula with chitosan 3% was the most active as anti-cervical cancer showing by IC_{50} was 83.58 µg/mL. EGCG-chitosan microspheres demonstrated potential as drug delivery system and as anti-cervical cancer.

KEYWORDS: Chitosan, EGCG, Microspheres, Characteristics, Stability, Activity.

INTRODUCTION:

Cervical cancer is a malignancy that occurs in cervical (cervix) which is the lowest part of the uterus that protrudes into the peak of the rut of the vagina. Cervical cancer are normally caused by infected of HPV (Human Papillomavirus Human)[1]. The development of cancer drugs currently leads to the development of drugs from natural products because it is believed to be safer and do not cause adverse effects on normal cells[2]. One of the compounds of natural product that have been researched as effective as chemotherapy in cancer was epigallocatechin gallate (EGCG)[3]. Epigallocatechin gallate (EGCG) is a compound of natural product that are known to have anti-cancer activity, EGCG can inhibit the proliferation and growth of cervical cancer[4]. However, bioavailability of EGCG were categorized as low of 0.1% due to the low stability of EGCG[5]. To

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improve stability of EGCG, microspheres are one alternative of selected drug delivery system.

Microspheres can act as drug delivery systems by entrapping drug molecules in their interior structures, adsorbing drug molecules on the surface or covalently bonded. Microspheres provide advantages such as increasing solubilization of hydrophobic drug active agents, improving bioavailability, improving pharmacokinetics of drugs, protecting drug from physical, chemical or biological degradation[6].

Common methods used in the manufacture of microspheres include spray drying (atomization), ionotropic gelation, and supercritical fluid precipitation, single and double emulsification[7].

The ionotropic gelation method with aerosolization techniques involving two-phase aqueous mixture in generating second-phase ionic interactions has the advantage of encapsulating the drug to protect from the environment, the process is easy, fast and relatively inexpensive[7,8]. The preferred method is ionotropic gelation with aerosolization technique because this can be done at room temperature considering EGCG is not stable at high temperature. In this study the polymer used is chitosan which suitable with stability pH of EGCG. Chitosan is soluble in pH <6, biodegradable, biocompatible, nontoxic and capable of regulating drug release[9]. Chitosan can also precipitate with the addition of polyanion solution as crosslinking and forming the gel at low pH[10].

The polyanion solution which can be used as crosslinking in chitosan polymer is tripolyphosphate (TPP). TPP is a non-toxic polyanion that can interact with the electrostatic forces between NH^{3+} from chitosan and $-P_3O_{10}^{5-}$. The bond intensity between NH^{3+} and $-P_3O_{10}^{5-}$ will affect the physicochemical characteristic such as chemical physics interaction, matrix density, morphological structure, particle size and drug entrapment ability which will influence the drug release, bioavailability and activity[11-13].

Physical characteristics of chitosan microspheres with ionotropic gelation method are influenced by several factors such as amount of drugs, concentration of drug and chitosan, pH of chitosan solution, chitosan solution temperature, acetic acid concentration and stirring speed[7]. In this research we will study the effect of chitosan polymer concentration on physical characteristic and stability of EGCG-Chitosan microspheres.

Cytotoxic activity of EGCG-Chitosan microspheres was measured using MTT assay. The aim of this study is to evaluate the cytotoxicity of EGCG-Chitosan microspheres against cervical cancer (HeLa) cell line *in vitro*.

MTT assay showed that EGCG treatment resulted in a time- and concentration- dependent inhibition of cell proliferation, and 50% inhibition concentration (IC50) at 24 h appeared to be 27.3μM for CaSki, and 47.9μM for HeLa cells[14].

MATERIALS AND METHODS:

Materials:

EGCG with 98% purity from Xi'an, Shaanxi, China; Chitosan (20 cps) with deacetylation degree 95,2% from CV. Bio Chitosan Indonesia; (sodium tripoliphosphate p.a.; sodium citrate p.a; citric acid p.a; and acetic acid p.a.) from Bratachem.

Methods:

Experimental design for optimization:

Microspheres were made by ionotropic gelation method with aerosolization technique with different chitosan concentrations of 1%, 2% and 3%. EGCG 3% as topical intravaginal dosage dose was dissolved into chitosan solution. Ratio of Sodium tripolyphosphate and chitosan were: Na TPP 10:1. Sodium TPP solution was sprayed using a spray nozzle with a pressure 40 psi into EGCG-Chitosan solution at distance 8 cm and continued stirring for 3 hours at 1000rpm followed by centrifugation at 3000rpm for 10 min and the sediment was washed twice with demineralized water and dried with a freeze dryer at -80°C for 30 hours. The freeze dried microspheres were then evaluated for physical characteristics, stability and activity tests. Ratio of Sodium tripolyphosphate and chitosan were: Na TPP 10:1. Formula can be seen in Table 1.

Table 1 EGCG-Chitosan Microspheres Formula:

Material	Function	Formula (% w/v)		
		F1	F2	F3
EGCG	Active Agent	3	3	3
Chitosan	Polymer	1	2	3
Na TPP	Crosslinker	0.1	0.2	0.3

Chitosan in 2% acetic solution

EGCG-chitosan microspheres characteristics:

Evaluation of physical characteristics of EGCG-Chitosan microspheres include:

Spectroscopy FTIR:

This evaluation was performed to identify the materials used and to identify whether or not the interaction was formed due to the interaction between chitosan and TPP. Measurements were done using FTIR from Agilent Cary 630 FTIR Spectrometer with ATR sampling technology and PLS DA method from Agilent Technologies, Inc.

Morphology and Particle size Determination:
To observe morphology of microspheres, Scanning Electron Microscopy (SEM) CarlZeiss type EVO MA 10, Germany was used and to measure the particle size used Novel OptiLab, LLC.

Swelling index:
The swelling test was performed by weighing the microspheres (W_0) using an analytical scale and placed separately on the pH 4.0 citrate buffer and incubated at 37±1°C. At intervals of 15 minutes, 30 minutes, 1 hour, 2 hours, and 4 hours, the microspheres were removed from the cup and the excess water was removed using filter paper, the expanded microsphere was weighed again (Wt) and the swelling index was calculated using the following formula:

$$\text{Swelling Index} = (Wt\text{-}W0)/W0 \times 100\%$$

Yield:
Yield is a determined recovery factor by comparing the total weight of the microspheres obtained to the weight of the microspheres-formed. Thoroughly microspheres were weighed and chitosan, sodium tripolyphosphate and EGCG as microsphere-forming materials were recorded.

The dry microsphere were weighed and were recorded as the total weight of the microspheres. The yield was then put into below equation[15]:

$$\text{Yield} = \frac{\text{(Weight of Dry Microspheres)}}{\text{(Weight of Drug and Polymer)}} \times 100\%$$

Entrapment efficiency and Drug Loading:
Entrapment efficiency was measured using spectrophotometer because EGCG has chromophore and conjugated group[16]. The EGCG entrapment efficiency was calculated from the determination of EGCG content in microspheres by using below equation[15]:

$$\text{Entrapment Efficiency(\%)} = \frac{\text{Weight of drug in the Microspheres}}{\text{Weight of drug used in Formulation}} \times 100\%$$

While to know the percentage of EGCG content or drug loading each formula, below equation was used (Zhang et al., 2013):

$$\text{Drug Loading (\%)} = \frac{\text{Weight of drug in the Microspheres}}{\text{Weight of Microspheres}} \times 100\%$$

Physical Stability Test:
Stability evaluation were performed at 25ºC and 50°C for 30 days and observed on organoleptic, entrapment efficiency and drug loading compared to initial day (Day 0).

Observations were made after 7, 14, 21 and 30 days of storage. Physical stability evaluation of microspheres was done at cool temperature 4ºC ±1ºC, at room temperature 25ºC ± 1ºC and at high temperature 50ºC for 1 month.

Activity test:
Cell and cell culture conditions:
Human cervical cancer cell lines with HeLa obtained from CCRC were cultured in RPMI 1640 and were supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37ºC in humidified atmosphere of 5% CO_2.

Microspheres were dissolved in DMSO (dimethylsulfoxide) and were stored at -20ºC before used. In all experiment control cultures were made of medium, DMSO and cells.

MTT cell viability assay:
The effect of microsphere on cell proliferation was examined by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. HeLa cells (10^4 per well) were seeded in 96-well plates and allowed to grow 24 h. Then microspheres or DMSO (vehicle control) was added at specified concentrations. Each treatment was in triplicates. After 24 h, 20μL of MTT solution (5mg/mL) was added to each well and was incubated for 4 h at 37°C. The MTT formazan crystal was then dissolved in DMSO, and the absorbance was measured by ELISA reader at a wavelength of 495nm.

Data analysis:
Physical characteristics data were analyzed using statistical method of one-way ANNOVA with 95% (α = 0.05) degree of confidence to know whether there was significant difference between formulas while the stability data was analyzed using Factorial Design ANOVA method with degree of confidence of 95% α = 0.05).

RESULTS AND DISCUSSION:

Interaction of drug and polymer:
From the result of FTIR evaluation of EGCG and all microspheres formulas, same absorption peaks were observed, this indicated the presence of EGCG on the microspheres confirming that EGCG was stable and did not degrade during formulation process using ionotropic gelation using aerosolization technique. For result of FTIR evaluation of microspheres, a confirmed peak at wavelength 3348.256nm were shown shifts at wavelength 3345.946nm and looked more gentle peak, this can be interpreted the microspheres were formed after crosslinking bond interaction occurred between chitosan polymer and TPP cross linker encapsulated EGCG.

Particle Size:

The average of particle size of formula F1, F2 and F3 with 1, 2 and 3% chitosan concentrations were 2.29, 2.68, and 3.11µm respectively. From the results of statistical tests one-way ANNOVA obtained sig value. 0.000 < 0.05 it showed that chitosan concentration had significant effect on particle size, the higher the chitosan concentration the larger the particle size. This was in accordance with research which stated the higher polymer concentration caused larger particles and greater entrapment efficiency.

Particles larger than 1 micron cannot diffuse through the mucus layer. However, for micron-sized particles it can interact with the mucus layer when it contains a mucoadhesive polymer. Chitosan is one of mucoadhesive polymer therefore EGCG-Chitosan microspheres can be used for topical therapy intravaginal preparations for cervical cancer because in the vagina there was cervico vaginal mucus that can help penetration of EGCG-Chitosan microspheres.

According to statistical analysis of entrapment efficiency and drug loading with one way ANNOVA with 95% confidence degree (α = 0,05) the obtained sig value of entrapment efficiency was 0.000 while at drug loading was 0,005. The sig value of drug loading, entrapment efficiency <0.05 then there is a significant difference in drug loading and entrapment efficiency, meaning that chitosan concentration had a significant effect on drug loading and entrapment efficiency (Table 2).

Table 2 Yield, drug loading, entrapment efficiency, swelling index, moisture content and particle size of microspheres formulas

Formulation	Yield (%±SD)	Drug Loading (%±SD)	Entrapmet Efficiency (%±SD)	Swelling Index (%±SD)	Moisture Content (%±SD)	Particle Size (µm±SD)
F1	78.25±5.61	23.25±0.54	33.94±1.73	0.33±0.24	1.44±0.16	2.29±0.03
F2	79.36±1.60	29.36±3.44	52.27±6.52	0.69±0.27	1.55±0.28	2.68±0.06
F3	79.77±5.67	32.24±0.74	62.14±1.22	0.85±0.21	1.68±0.21	3.11±0.02

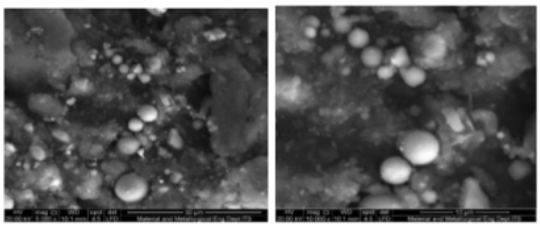
From the table, it shown the yield percentage obtained of F1 was 78.25%, F2 was 79.36% and F3 was 79.77%. Yield is a value that describes the effectiveness of the method of manufacture used. High percentage values can be interpreted as encapsulation methods were effective and efficient. The entrapment efficiency F1, F2 and F3 were 33.94%, 52.27% and F3 62.14% respectively. For drug loading of all formulas were 23.35%, 29.36% and F3 32.24% respectively. This described that the greater concentration of chitosan, greater entrapment efficiency and drug loading were resulted. The higher the chitosan concentration, the higher the viscosity and the greater entrapment of drug[17].

In addition to increasing the viscosity, to improve the entrapment efficiency and drug loading can be done by enhancing more effectively formation of encapsulated particles by adding surfactants. Surfactants can improve entrapment efficiency and decrease particle size[18,19].

From the statistical evaluation, sig value of yield, moisture content and swelling index on three formulas were > 0.05, with yield sig value 0.921, moisture content sig value. = 0.487 and swelling index 0.089 therefore in conclusion the three formulas did not significantly different in moisture content and swelling index. This means chitosan concentration did not give significant effect on moisture content and swelling index. The swelling test of this index is to understand profile drug release. Drug release system in the body is divided into 3 ie swelling system, floating system and bio adhesive system. To obtain the desired drug release rate can be done by modifying the drug delivery system by regulating solubility, drug penetration with biological fluid or by adjusting the rate of drug diffusion[20]. The rate of drug release also depends on the process of water migration into the matrix and the diffusion of the drug from the expanding matrix[21].

Morphology of microspheres:

From the result of entrapment efficiency evaluation it was seen that the formula F3 had the highest entrapment efficiency. Therefore, this physical characteristics will then continue to observe morphology of microspheres by using Scanning Electron Microscopy (SEM) and the spherical microspheres of particles were appear as smooth surface microspheres of F3 (Figure 1 A and Figure 1 B).



A B

Fig 1.Microspheres morphology of F3 microspheres formula using Scanning Electron Microscopy (SEM) at magnification 5000x (A) 10000x (B)

Stability test results can be seen in (Figure 2-5). According to statistical analysis in term of stability test using ANNOVA Factorial Design method, EGCG stability evaluation at 25ºC and 50ºC temperature during storage period, 7 days, 14 days, 21 days and 30 days, it was obtained sig value 0.000 < 0.05, it means that the temperature and duration of the storage period give a

significant effect on EGCG concentration. In the EGCG stability test at 50ºC, it was shown that EGCG concentration decreased with increasing days of storage (Figure 2). Stability test at extreme 50°C showed the reduced of EGCG, but not in room temperature. This was because EGCG is one compound that were auto oxidative and easily degraded at high temperature[22].

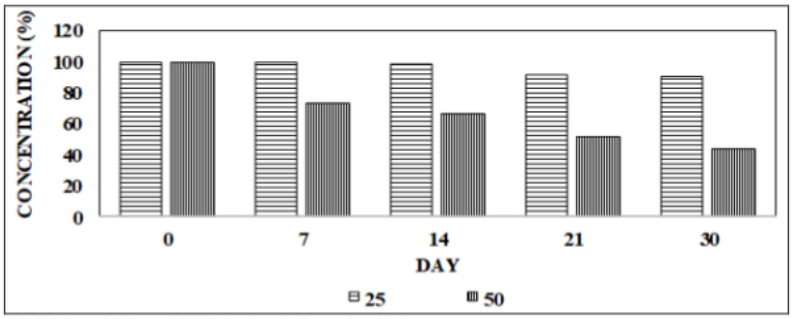
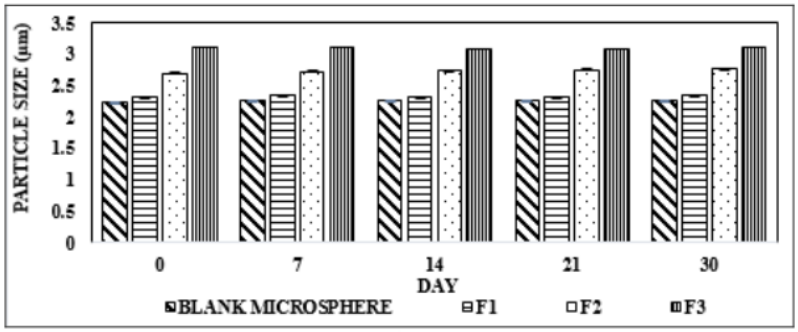


Fig 2. Stability test of EGCG at 25ºC and 50ºC Period 0, 7, 14, 21, 30 days



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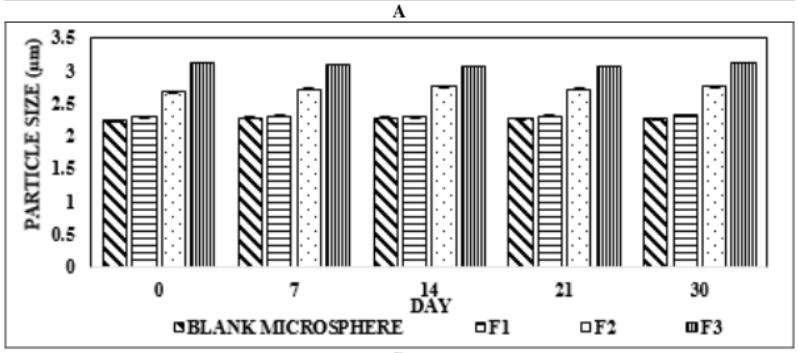
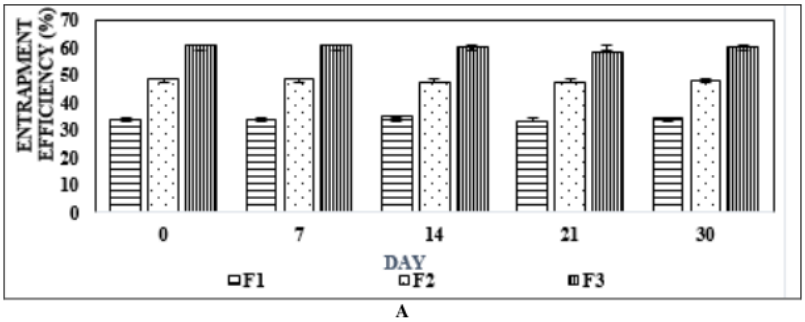


B

Fig 3. Stability test of Particle size EGCG-Chitosan microspheres at Period 0, 7, 14, 21, 30 days at 25ºC (A) and 50ºC (B)



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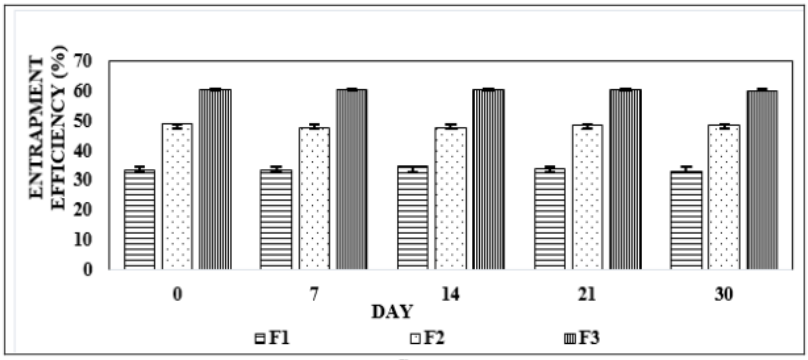
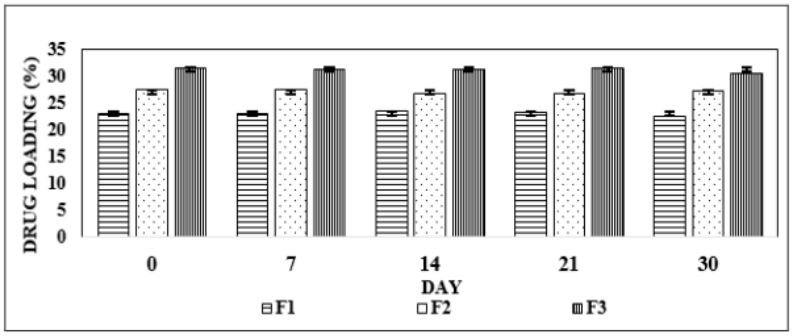


B

Fig 4. Stability test in terms of Entrapment efficiency of EGCG-Chitosan microspheres at Period 0, 7, 14, 21, 30 days at 25ºC (A) and 50ºC (B)



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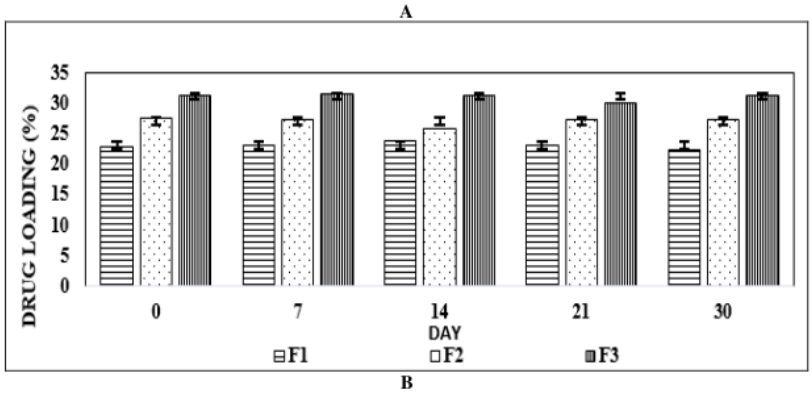

B

Fig 5.Stability test of Drug loading of EGCG-Chitosan microspheres at Period 0, 7, 14, 21, 30 days at 25ºC (A) and 50ºC (B)

From statistic test result using ANNOVA Factorial Design method on particle size stability evaluation at 25ºC and 50ºC and during storage period at day 7, 14, 21, 30, it was obtained sig value of F1, F2, F3 was 0.979, 0.437 and 0.065. On the entrapment efficiency sig value of F1 was 0.928, F2 was 0.562, and F3 was 0.285. For drug loading, sig value of F1, F2 and F3 were 0.417, 0.456, and 0.284 respectively. Because the values of all sig were > 0.05, it means that the temperature and duration of the storage period did not give a significant effect on the particle size, entrapment efficiency and drug loading. It can be concluded microspheres drug delivery system in this study proved able to maintain stability of EGCG. Result of physical stability test in this EGCG-Chitosan microspheres had been in accordance with the results of stability evaluation conducted by Dashora at room temperature 25ºC ± 1ºC and at high temperatures 50ºC for 1 month. They obtained results that microspheres remain stable either at room temperature or high temperatures[23].

For activity test, according statistical analysis using one way ANNOVA method, it was obtained that sig value was 0.000<0.05. This means that the chitosan

concentration gave a significant effect on activity of microspheres as anti-cervical cancer. Formula 3 had the highest anti cervical cancer with MTT assay. The anti-cancer activity of a compound can be seen in IC_{50} values. According Kamuhabwa et al., extract had to assess IC_{50} = 100μg/ml to be categorized having anti-proliferation potency[24]. In the formulas 1,2 and 3, IC_{50} values was respectively of 95.51, 86.44 and 83.58 μg/mL.

CONCLUSIONS:

The ionotropic gelation method using aerosolization technique for encapsulation of EGCG produced successfully EGCG-chitosan microspheres with particle size between 2.29 - 3.11µm, entrapment efficiency of 33.9 - 62.14% and drug loading of 23.25 - 32.24%. Further research to improve entrapment efficiency and drug loading were recommended. Formula F3 with the highest concentration of chitosan polymer was the optimized formula with entrapment efficiency and drug loading. Formula 3 with chitosan 3% produced IC_{50} 83.58μg/ml. This means that this EGCG-Chitosan microspheres drug delivery system was potential for cancer treatment.

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CONFLICT OF INTEREST:

The authors declared no conflict of interest.

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