

Synthesis, Characterization, and Cytotoxicity Evaluation of Gallic Acid Nanoparticles Towards Breast T47D Cancer Cells

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ABSTRACT

Introduction: Gallic acid is a naturally polyphenolic acid which shows cytotoxicity against several cancer cells, as well as it displays chemo-preventive activity which is attributed to its strong apoptosis-inducing and antioxidant effects. Thus, gallic acid has become an attractive substance to be further developed due to its strong cytotoxic activity. This study aimed to synthesize gallic acid nanoparticle coating with alginate-chitosan, and evaluate its cytotoxicity against breast T47D cancer cells. **Methods:** Gallic acid nanoparticle was synthesized using ionic gelation method. The yield, size and morphology of the nanoparticles were determined by UV-Vis Spectroscopy, Transmission electron microscopy (TEM) and Fourier Transform Infrared (FTIR) spectroscopy. Cytotoxicity evaluation of gallic acid nanoparticle towards breast T47D cancer cell is carried out by MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) assay. **Results:** Spherical nanoparticles of gallic acid with the size of 100-200 nm has been successfully synthesized in 96% of yield. Compared to gallic acid (IC₅₀: 20.86 µg/mL) and alginate-chitosan nanoparticle (IC₅₀: 38.46 µg/mL), gallic acid coating with alginate-chitosan nanoparticles demonstrated higher cytotoxicity towards breast T47D cancer cells with IC₅₀ value of 9.03µg/mL. **Conclusion:** Our results clearly confirmed that gallic acid nanoparticles coating with alginate-chitosan showed a strong cytotoxicity towards breast T47D cancer cells, which is potential to be developed as a candidate for new anti-breast cancer agent.

Key words: Synthesis; Gallic acid; Nanoparticle; Cytotoxicity; T47D cells.

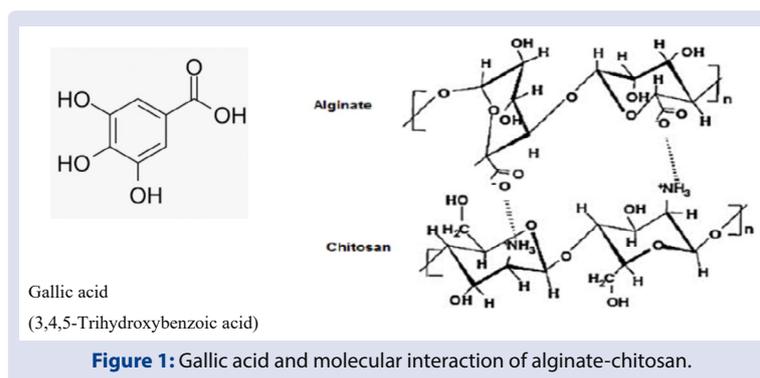
INTRODUCTION

3,4,5-trihydroxybenzoic acid, also known as gallic acid (Figure 1), is a polyphenolic compound found in plants and fruits such as mangoes, grapes, raspberries, etc. Gallic acid is known to have anticancer, antimicrobial, and antiviral properties.¹⁻⁴ A research by Wang *et al.* in 2014 showed that gallic acid has anticancer effects towards MCF-7 breast cancer cells by inhibiting the cancer cells proliferation and inducing apoptosis. Gallic acid works by activating the Fas/FasL apoptotic pathway. In addition, gallic acid also induces apoptosis through mitochondrial pathway. Gallic acid is hydrophilic, causing it difficult to penetrate into the wall of cancer cells.⁵ Preparation of gallic acid in form of nanoparticles is believed to increase the hydrophobicity. Therefore, this study aimed to synthesize nanoparticle of gallic acid coating with alginate-chitosan (Figure 1), and evaluate its cytotoxicity toward breast T47D cancer cells. Gallic acid coated alginate-chitosan nanoparticle is expected to be able to diffuse easily through the cancer cell membrane, that may lead to the increasing in its absorption and bioavailability, as well as the improvement of its anticancer activity.

Nanoparticles are particles between 1-1000 nanometre in size. In the field of Pharmacy, nanoparticles have two meanings/interpretations, namely the nanometre-sized drug compounds or nanocrystal, and drug compounds that are

encapsulated in nanometre-sized carrier system termed as nanocarriers.⁶ There are several types of nanocarriers, such as nanotubes, liposomes, solid lipid nanoparticles, polymeric nanoparticles, etc.⁷ The carrier polymer can be chitosan, which is a polysaccharide derived from chitin deacetylation process. Chitosan can be utilized in mucoadhesive drug delivery systems because its positively charged polymer chains can interact electrostatically with the negatively charged mucose.⁸ In 2018, Adhikari and Yadav reported that chitosan and its derivatives had anticancer activity through cellular enzymatic, antiangiogenic and apoptotic pathways.⁹ Whereas alginate, is a polysaccharide polymer that can be obtained from brown algae with α-L-guluronic acid and β-D-mannuronic acid as the constituent monomers. Alginate is easily dissolved and degraded under normal physiological conditions, making it suitable to be used as systemic drug delivery.¹⁰ Chitosan-alginate nanoparticles can be prepared, for example, by using the ionic gelation method. This method has some advantages such as simple preparation process with mild conditions without using hazardous or toxic organic solvents and without using high temperature heating process that may cause the decomposition of active compounds, so that this method is suitable to be used for the preparation of nanoparticles that contain thermolabile active compounds.¹¹ Nanoparticles have several advantages and disadvantages. The advantages of nanoparticles include: the capability to overcome physiological barrier in the body

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that is caused by the drug delivery system which is influenced by the particle size; increase the solubility of compounds that are poorly water-soluble so that it increases the bioavailability, active compounds stability, efficiency of drug distribution and allows better penetration in tumors with pores ranging 100-1000 nm in diameter. However, the disadvantages of nanoparticles are that they are easily aggregated so that it is difficult in handling and storage; small in size and not suitable for drugs that require large dosage; due to the nanometre size, able to penetrate undesirable parts such as nuclear envelope and cause genetic damage or mutations.⁸ Nanoparticles characterization to determine the physicochemical properties, size, shape, and particles distribution can be done by using some instruments, namely Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), Nuclear Magnetic Resonance (NMR), Fourier-Transform Infrared Spectroscopy (FTIR), Dynamic Light Scattering (DLS), Static Light Scattering (SLS), etc.¹²

Research about synthesis of gallic acid and its derivatives nanoparticles is still very limited. In 2010, Moreno-Alvarez *et al.* reported that preparation of gold-gallic acid nanoparticles has antibacterial activity.¹³ Li and Niu (2015) successfully synthesized silver-gallic acid nanoparticles that have high antimicrobial and low cytotoxicity activities towards normal cells.¹⁴ Radoman *et al.* (2015) reported the synthesis and characterization of TiO₂ nanoparticles modified with octyl gallate¹⁵, whilst the latest research by Cordova *et al.* in 2017 showed that solid lipid-octyl gallate nanoparticles can improve the antimetastatic activity in mice model of lung cancer.¹⁶ Synthesis and characterization of gallic acid nanoparticles with ionic gelation methods, as well as study to determine the its *in-vitro* anticancer activity has never been reported. This serves as the novelty aspect of this research.

MATERIAL

Chemicals

Gallic acid, alginate, chitosan, and doxorubicin were purchased from Sigma-Aldrich Chemical Company.

Syntheses of the nanoparticles were conducted in dried glasswares. All chemical solvents in pro analysis grade were purchased from chemical distributor of Brataco Indonesia. Syntheses of the nanoparticles and its cytotoxicity evaluation were carried out in Departement of Medical Chemistry, Faculty of Medicine, University of Indonesia. UV-Vis and FTIR analysis of the synthesized nanoparticles were conducted in Drug Development Research Cluster, Indonesia Medical Education and Research Institute (IMERI), Faculty of Medicine, University of Indonesia. TEM analysis of the nanoparticles were performed by JEOL TEM 1010, 80 KV (magnification of 40,000x) in Laboratory of TEM and Histology, Ejkman Institute, Jakarta, Indonesia.

Breast T47D cancer cell lines

Breast T47D cancer cell is the cell culture collection of the Medical Chemistry Department, Faculty of Medicine, University of Indonesia.

METHODS

Synthesis procedure of gallic acid nanoparticles with ionic gelation method

100 mg of CaCl₂ is dissolved in 50 mL distilled water, then 50 mg of gallic acid is added, stir it until the solution is homogenized (Solution 1). Amount of 200 mg of Sodium alginate is dissolved in 25 mL distilled water. The pH is adjusted until 5.1 with 0.01 M HCl (Solution 2). Subsequently, the solution 1 is dropped into Solution 2 by using a syringe. Then, stir at the speed of 1400 rpm for 24 hours (Solution A). Amount of 100 mg of chitosan is dissolved in 25 mL of 1% (v/v) of glacial acetic acid. The pH is adjusted until 5.5 with 1N NaOH, then added 0.31 g of Tween 80, and stir it for 24 hours at 60°C (Solution B). Solution B is then dropped into Solution A by using syringe whilst being stirred at the speed of 1300 rpm for an hour. The mixture is then centrifuged at 3000 rpm for 10 minutes until the nanoparticles are obtained in the form of pellets. The synthesized nanoparticle were freeze dried prior to use for analysis. Alginate-chitosan nanoparticles were prepared by using the same procedure with the synthesis of gallic acid nanoparticles, but without the addition of gallic acid.

Procedure of *in vitro* cytotoxicity determination of the nanoparticles by MTT assay

Breast T47D cells are seeded in RPMI 1640 (Gibco, USA) culture medium, which has been supplemented with 10% fetal bovine serum (Gibco, USA). Then, it is incubated at 37°C in a humidified atmosphere of 4% CO₂. The cell viability is determined by 0.1% trypan blue method. The test sample (nanoparticle) is diluted to reach the final concentration are 51.2; 25.6; 12.8; 6.4; 3.2; 1.6; 0.8; 0.4 µg/mL. Diluted samples were added to the target cells, and incubated for 48 hours. Amount of 100 µl of 5 mg/mL of MTT phosphate-buffered saline (PBS) was then added into the target cells of breast T47D in well plate, and the mixtures were reincubated for 4 hours. The mixtures were then centrifuged, the medium is separated. DMSO in amount of 200 µl is added to each well to dissolve the blue purple-colored sediments. The absorbance is measured at 590 nm on a microplate reader model 550 (Bio-Rad, USA). The inhibition (in %) was calculated by using the formula below:

$$\% \text{inhibition} = 1 - \left(\frac{\text{Absorbance of treatment group}}{\text{Absorbance of control group}} \right) \times 100\%$$

Cytotoxicities of the nanoparticles are expressed by median inhibitory concentration (IC₅₀) value. The results will be compared with free-gallic acid (gallic acid not in form of nanoparticle) and doxorubicin as a positive control.

RESULTS AND DISCUSSION

In this work, chitosan polymer is used as nanocarrier. Chitosan is non-toxic, biocompatible, and biodegradable, but it is very fragile. Therefore, it requires alginate as a cross-linker to make it more stable. It has been reported that biopolymeric alginate-chitosan nanoparticle is effective and stable as an anticancer drug delivery^{17,18}, in which, inspired us to perform the synthesis of gallic acid nanoparticles coating with alginate-chitosan biopolymer.

Analysis of % yield of the synthesized gallic acid nanoparticles by UV-vis spectrophotometry

Liquid (filtrate) obtained from centrifugation of the mixture of the synthesized nanoparticles was analyzed by UV-Vis spectroscopy at 690 nm to determine the concentration of free-gallic acid (gallic acid which did not convert into nanoparticles). Absorbance data of gallic acid standar solution are displayed in Table 1. Calibration curve of linear regression with the equation: $y = 0.005x - 0.0062$, is obtained by plotting absorbance (mean value from three replication) of gallic acid in Y axis with the concentration of gallic acid (ppm) in X axis (Figure 2). The concentration of free-gallic acid ($x = 20.4867$ ppm) is generated by substituting Y in linear line equation of $Y=0,005x-0.0062$ with mean absorbance of filtrate (0.0962). The initial concentration of gallic acid is 500 ppm, thus concentration of gallic acid converted into nanoparticle is 479.5133 ppm (500 ppm-20.4867 ppm). So that, % yield of synthesized gallic acid nanoparticle is $(479.5133/500) \times 100\% = 96\%$.

TEM analysis of gallic acid nanoparticles

TEM (Transmission electron microscopy) and SEM (Scanning electron microscopy) can help to identify the shape and size of small particles and nanoparticles. Compared to SEM, TEM has higher resolution. TEM could resolve object near the atomic level, as close as 1 nm. Besides that, SEM's magnifying power is up to 50,000 times, whereas TEM's magnifying power is up to 2 million times.¹⁹ Therefore, in this work, identification of morphology and size of the synthesized nanoparticle

using TEM.

TEM analysis of the nanoparticles (Figure 3) were conducted in Laboratory of TEM and Histology, Ejkman Institute, Jakarta, Indonesia, by using JEOL TEM 1010, 80 KV, with magnification of 40,000x. As shown, gallic acid nanoparticles (Figure 3a) have a spherical morphology with the size of 100-200 nm. Whereas alginate-chitosan nanoparticles (Figure 3b) also have a spherical shape with slightly smaller nanosize.

FTIR analysis of gallic acid nanoparticles

FTIR is used to confirm the molecular interaction between gallic acid and chitosan-alginate nanocarrier. FTIR analysis of the nanoparticles are displayed in Figure 4a-c. FTIR spectrum of gallic acid (4a) showed sharp band of hydroxyl (-OH) stretching aromatic at 3200-3500 cm^{-1} , absorption of carbonyl (-C=O) group at 1702 cm^{-1} , and absorption of aromatic carbon at around 1500-1600 cm^{-1} . Whereas, FTIR spectrum of alginate-chitosan nanoparticle (4b) showed N-H stretching vibration of amine group overlapped with carbonyl (-C=O) group of alginate at around 1500-1600 cm^{-1} , as well as a broad hydroxyl (-OH) stretching and amine group (-N-H) band at 3200-3600 cm^{-1} , which indicated the electrostatic interaction and hydrogen bonding between alginate and chitosan. Moreover, a very broad absorption band at 3000-3700 cm^{-1} in FTIR spectrum of gallic acid nanoparticle (4c) has confirmed the presence of hydrogen bonding between gallic acid and chitosan-alginate nanocarrier.

Cytotoxicity of the nanoparticles towards breast T47D cancer cells

Studies on synthesis of gallic acid nanoparticle are limited. Daduang *et al.* in 2015 reported the synthesis of gold nanoparticles conjugated with gallic acid (GNPs-GA), and found that GNPs-GA inhibited cervical cancer cells less effective than gallic acid, but it was not toxic against normal vero cells, which indicated that GNPs-GA could be an alternative for cervical cancer treatment with less side effects to the normal cell.²⁰ Another researcher, Hu *et al.* (2015) reported that nanoparticles of gallic

Table 1: UV-vis absorbance data of gallic acid.

Conc. standard solution of Gallic acid (ppm)	Absorbance				Free-Gallic acid in filtrate (ppm)
	1	2	3	Mean	
0	0.0001	0.0001	0.0001	0.0001	20.4867
2	0.0090	0.0090	0.0089	0.0090	
4	0.0142	0.0143	0.0141	0.0142	
8	0.0302	0.0301	0.0300	0.0301	
16	0.0556	0.0556	0.0557	0.0556	
32	0.1626	0.1626	0.1626	0.1626	
Liquid (Filtrate)	0.0963	0.0962	0.0962	0.0962	

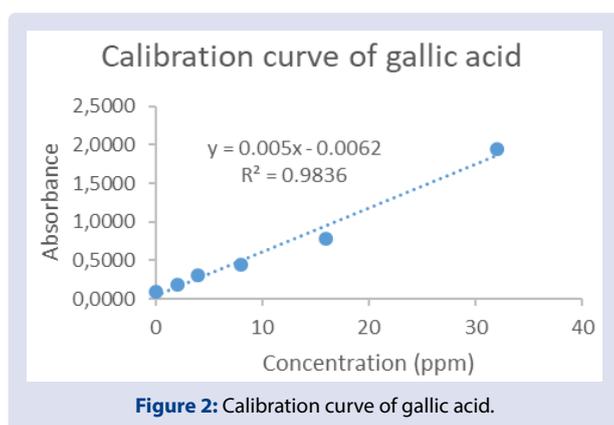


Figure 2: Calibration curve of gallic acid.

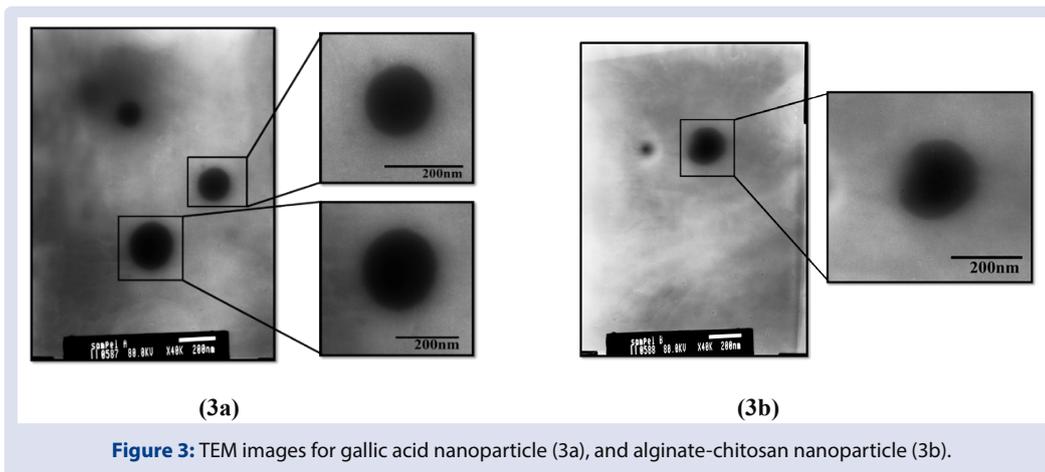


Figure 3: TEM images for gallic acid nanoparticle (3a), and alginate-chitosan nanoparticle (3b).

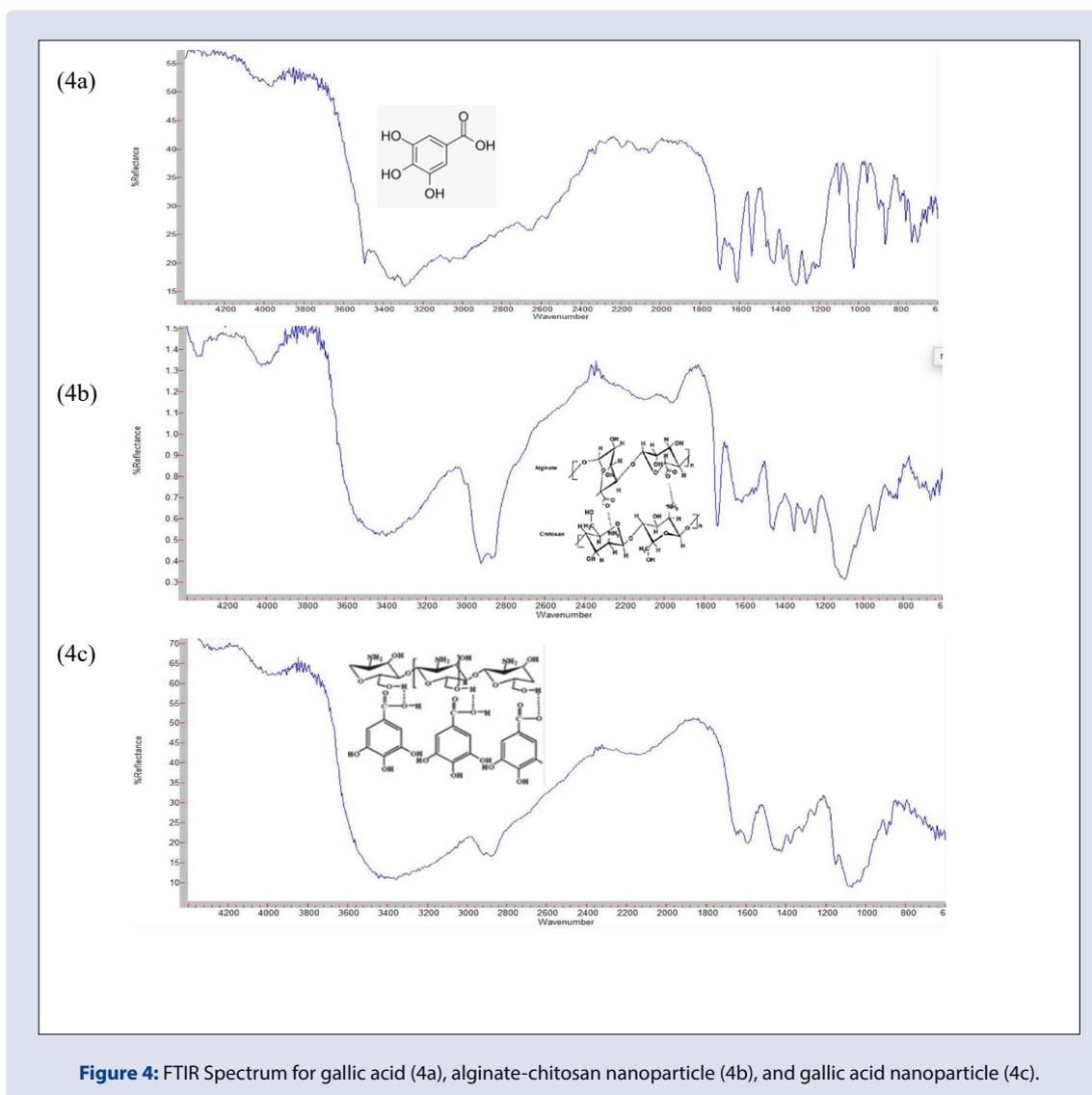


Figure 4: FTIR Spectrum for gallic acid (4a), alginate-chitosan nanoparticle (4b), and gallic acid nanoparticle (4c).

Table 2: Cytotoxicity of the nanoparticles, free-gallic acid and doxorubicin towards breast T47D cancer cells.

Tested sample	IC ₅₀ (µg/mL)*
Doxorubicin (positive control)	0.10 ± 0.06
Gallic acid	20.86 ± 3.5
Alginate-chitosan nanoparticle	38.46 ± 4.2
Gallic acid nanoparticle	9.03 ± 2.1

*IC₅₀ is the 50% half maximal inhibitory activity in µg/mL, expressed in mean value (n=3) ±SD (standard deviation)

acid (GA) grafted chitosan (CS) and caseinophosphopeptides (GA-g-CS-CPP). This GA-g-CS-CPP nanoparticles exhibited high antioxidant effect and cytotoxicity against Caco-2 colon cancer cells.²¹ More recent, Saleh *et al.* (2019) evaluated anticancer activity of iron oxide-carboxy methyl chitosan (CMC) nanoparticle conjugating with gallic acid (Iron oxide-CMC-GA), and the result showed that the nanoparticles could higher induce the apoptosis in A549 cancer cells compared with WI-38 normal cells.²² Furthermore, Wang *et al.* (2016) investigated anticancer effect of chitosan-coated alginate nanoparticles, and found that chitosan-alginate nanoparticles had cytotoxic effect on Hep2 cells, and could be a promising vehicle for anticancer drug delivery system.¹⁸

Related to previous works, in this current research we examined *in vitro* anticancer activity of gallic acid coating with alginate-chitosan nanoparticles against breast T47D cells. Table 2 summarizes cytotoxicities of gallic acid coating with alginate-chitosan nanoparticle, alginate-chitosan nanoparticle, gallic acid compound (not in form of nanoparticle), and doxorubicin. As displayed in Table 2, doxorubicin as a positive control has the lowest IC₅₀ value, exhibited the strongest cytotoxicity on T47D cells. Compared to gallic acid (IC₅₀: 20.86 µg/mL) and alginate-chitosan nanoparticle (IC₅₀: 38.46 µg/mL), gallic acid coating with alginate-chitosan nanoparticles exhibited greater cytotoxicity on breast T47D cells (IC₅₀: 9.03 µg/mL). This result suggesting that compared to gallic acid compound, gallic acid nanoparticles coating with alginate-chitosan was successfully improved its cytotoxicity against breast T47D cells, due to the increasing in its hydrophobicity. Thus, it should be further developed as a promising candidate for treatment of breast cancer.

CONCLUSION

Gallic acid nanoparticle coating with alginate-chitosan has been successfully synthesized with ionic gelation method in 96% of yield. Gallic acid nanoparticle exhibited a strong cytotoxicity towards breast T47D cancer cells with IC₅₀ value of 9.03 µg/mL, which is potential to be developed as a candidate for new anti-breast cancer agent.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

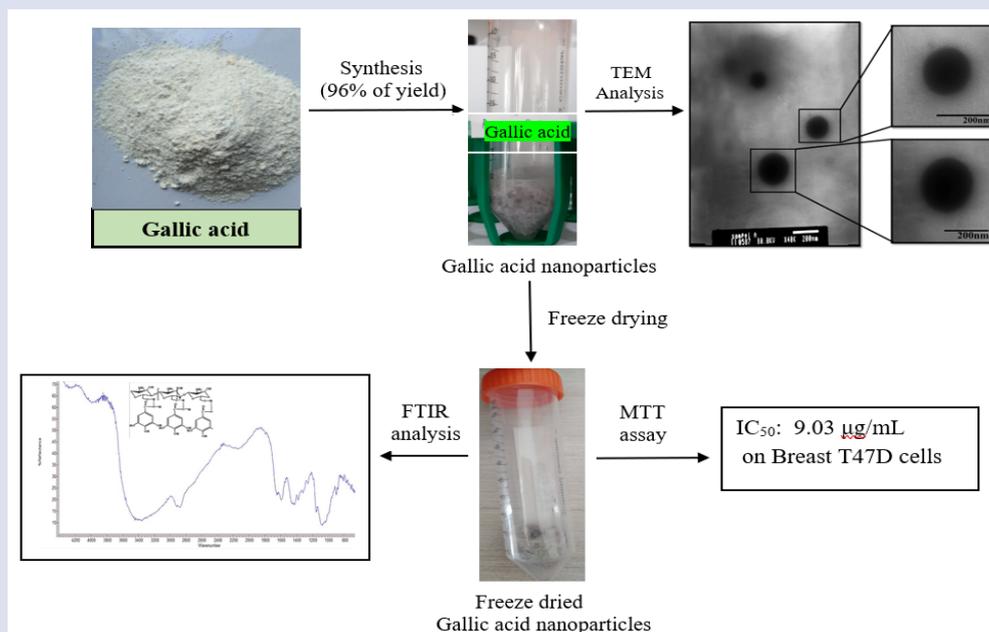
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide); **IC₅₀:** median Inhibitory Concentration; **µg/mL:** microgram/milliliter; **g:** gram; **mL:** milliliter; **N:** Normality; **DMSO:** Dimethyl

sulfoxide; **UV-Vis:** Ultra violet-Visible, **FTIR:** Fourier Transform Infrared; **TEM:** Transmission Electron Microscopy; **RPMI:** Rosewell Park Memorial Institute; **h:** hour; **PBS:** Phosphate-Buffered Saline; **°C:** degree Celsius; **µL:** microliter; **CO₂:** Carbon dioxide; **USA:** United States of America.

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GRAPHICAL ABSTRACT



SUMMARY

Gallic acid has become an attractive substance to be further developed due to its strong anticancer activity. In this work, we succeeded in synthesizing spherical gallic acid nanoparticles coating with alginate-chitosan at the size of 100-200 nm in 96% of yield by ionic gelation method. FTIR analysis has confirmed hydrogen bonding between gallic acid and chitosan-alginate nanocarrier. Compared to gallic acid and alginate-chitosan nanoparticle, gallic acid nanoparticle demonstrated higher cytotoxicity towards breast T47D cancer cells with IC₅₀ value of 9.03 µg/mL.

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Vincent Kharisma Wangsaputra: Third-year medical student in Faculty of Medicine, Universitas Indonesia. Despite technological advancement in treatment and sophisticated therapeutic strategies, neoplasm still remains as the focus of medical problem causing mortality and morbidity. Many researches have been conducted in order to figure out substances that have high potential of anticancer or cytotoxic activity, especially in combating cell resistance. My personal interest of research includes the utilization and incorporation of Indonesian natural compound to be scientifically applied in a way that it can yield efficient antitumorigenic properties towards the cancer cells.



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