Cytotoxic effects of the synthesized *Citrus aurantium* peels extract nanoparticles against MDA-MB-231 breast cancer cells

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Abstract. Currently, nanoparticle is at fastest pace due to the enhanced implication in drug delivery system. The synthesis of chitosan nanoparticles through ionic gelation methods is becoming increasingly relevant due to the low cost involved and environment-friendly approach. In this study, cytotoxic effect of chitosan-*Citrus aurantium* peels extract nanoparticles (NPs-CA) against MDA-MB-231 breast cancer cells was investigated. The initial confirmations of NPs-CA were using Particles size analysis (PSA), which showed the size of NPs-CA. The Zeta potential was measured to check the stability of NPs-CA. The cytotoxic effect was carried out in MDA-MB-231 breast cancer cells under MTT assay. Based on PSA analysis, the size of F1 and F2 were ranging between 372 – 4472 nm and 94 – 373 nm, respectively. The zeta potential mean value of F2 NPs-CA 34.9 mV, it can be concluded that the particles show less chances for agglomeration. On the various test concentration, the F2 NPs-CA performed better cytotoxic effects than *Citrus aurantium* peels extract with IC₅₀ value of 83 µg/mL and 320 µg/mL, respectively. These findings pose a positive impact to expand further studies in the development of nanoparticle to cancer therapy.

1. Introduction

Breast cancer (BC) has emerged as one of the most serious diseases of deaths among women worldwide [1]. It is triggered by a variety of factors due to uncontrolled cell division and metastatic [2]. In well-established medical treatment, the use of chemo preventive agent is a strategy to block BC metastatic [3]. Many studies have reported chemo preventive agent use natural product due to the ability of the bioactive compound have multiple targets to kill cancer [4,5].

Thus, the development of potent and effective anticancer drugs is one of the most persuaded goals. One of the potential alternative chemotherapy is plant natural bioactive compound that can be enhance the efficacy and less side effects. In addition, the bioactive compound was reported to stimulated immune system and has some beneficial effect, such as antioxidant. *Citrus aurantium* peels (CA) were reported to contain many secondary metabolite compounds such as hesperidin, hesperetin, naringenin, tangeretin dan nobiletin [6]. These compounds have been proven to have anticancer effect on several type of cancer [7–9]. However, the secondary metabolite compound in CA have insoluble in water...
characterization, rapidly degraded, and low bioavailability. Whereas to get a good activity a compound must have a high bioavailability, good solubility, and good permeability.

Nanotechnology is one of the approaches that can be used to solve this problem. Nanotechnology optimizes the use of secondary metabolite compounds from plant in their transport for efficient absorption, as in chitosan (CS) nanoparticles cross-linked with sodium alginate (AL). The ionic gelation method is the most common methods to produce nanoparticles. The method is simple, low cost, relatively easy to control and environment friendly. Therefore, in this study, the synthesis of chitosan- *Citrus aurantium* extract nanoparticle (NPs-CA) was carried out with the goal of enhancing the physical characteristics and the effect on the highly metastatic breast cancer MDA-MB-231 proliferation.

2. Methods and Material

2.1. Plant Material

*Citrus aurantium* (Rutaceae) peels were collected from Bandungan, Semarang – Central Java, Indonesia. The identity of the plant was confirmed by biologist at Biology Department, Diponegoro University.

2.2. Preparation of Plant Extract

The peels of *Citrus aurantium* was collected, chopped into small pieces and dried under shade. The dried peels (250 g) were re-macerated using ethanol 96% for 3 days at room temperature based on [10] with slight modification. The extracts were evaporated in the under reduce pressure in a rotary vacuum evaporator at 50°C to result the crude extracts. The crude extracts were dried in a vacuum freeze dryer and preserved at -20°C for subsequent analysis.

2.3. Synthesis of *Citrus aurantium* peels extract-loaded chitosan nanoparticles

*Citrus aurantium* peels extract (CA)-loaded chitosan nanoparticles (NPs-CA) were prepared using ionic gelation method according to [11] In this method, we use chitosan (CS) cross-linking with sodium alginate (AL) at several concentration (Table 1) to obtain the optimal characteristics of nanoparticles. In brief, AL was dissolved in water with 1% (w/v) tween-80 (pH between 5.0-5.5). Subsequently, CA ethanolic solution was added dropwise into this mixture, sonicated for 15 minutes. Calcium chloride was added and the mixture was vortex for 30 minutes. In addition, CS was dissolved in water with 1% (v/v) acetic acid (pH between 4.5-5.0) and vortex for the next 30 minutes. The resulting mixture was equilibrated overnight and undissolved material removal was achieved by centrifuge at 2500 rpm for 10 minutes [12].

| Table 1. Synthesis formulation of NPs-CA |
|------------------------------------------|
| Concentration of CS (mg/mL) | Concentration of AL (mg/mL) | Concentration of CaCl (mg/mL) |
| F1 | 0.3 | 0.3 | 0.67 |
| F2 | 0.6 | 0.3 | 0.67 |

2.4. Particle size and zeta potential

The particle size and zeta potential of NPs-CA were determined by Laser Particle Sizer Testing LLPA-C10. Measurements were made using aqueous diluted sample with ratio 2:1 and then the instrument also gives the measurement of particle-size distribution in the range between 10-500 nm.

2.5. Cell culture

MDA-MB-231 (ECACC #92020424) was maintained in DMEM high glucose medium (Gibco, USA) enrich with 10% fetal bovine serum (Gibco, USA), 12.5 μg/ml Amphotericin B (Gibco, USA), 150 μg/ml Streptomycin, and 150 IU/ml Penicillin (Gibco,USA). Cells were cultivated at 37°C under 5% CO₂. Culture media were renewed every two to three days, and cells were subculture when confluent of
For assays, only cells with >90% viability, passage number <10, and in the log growth phase were used.

2.6. Cytotoxic assay

The cytotoxic assay was based on a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay according to [5,13] with slight modification. Briefly, density of 5x10⁢³⁵ cells/well were seeded into 96 well-plate and incubated at 37°C under 5% CO₂ for 24 hours. Subsequently, cells were treated in triplicate with CL rhizome (5-200 μg/ml) and PN herbs (5-200 μg/ml) and exposed for 24 hours. Untreated cells were regarded as negative controls. After treatment, cells were treated with 0.5 mg/mL of MTT (Biovision) and incubated further for four hours. MTT formazan were soluble using 100μl DMSO and incubate for 15 minutes. After incubation, the absorbance was measured by ELISA reader (Biorad iMarkTM Microplate Reader) at λ 595 nm.

2.7. Statistical analysis

Values were presented as the mean ± SD. All calculations were carried out using one-way analysis of variance followed by the last significant difference test (Excel 2016 software; Microsoft, Redmond, WA). A P value of <0.05 was considered significant.

3. Results and Discussion

3.1. Optimum synthesis of NPs-CA and its physical characterization

One of the factors that affects the particle size of nanoparticles is the portion of CS to AL in the synthesis formulation. Comparison of the synthesis formulation from F1 and F2 was evaluated one by one to get the optimum synthesis formulation. The results show that F2 was selected to be the optimum formulation based on visual morphology observation (Table 2). The observation based on the presence of sedimentation after centrifuge 2500 rpm for 10 minutes.

Table 2. Optimization of nanoparticles characterization

| Formula | Visual observation            | Particle size (nm) | Zeta potential (mV) |
|---------|--------------------------------|--------------------|---------------------|
| F1      | White precipitate              | 373.369            | 27.7                |
| F2      | Yellow clear (no precipitate)  | 110.163            | 34.9                |

The size of particles is a crucial factor in the synthesis of nanoparticle. The average particle size of F1 and F2 were obtained in 373.369 nm and 110.163 nm (Figure 1A), respectively. This suggested that particle size was influenced by the concentration of CS. The primary factor in the formation of nanoparticles is the tendency of AL chains functional group, particularly carboxylic group that could form complex ion calcium structures. The previous study also reported that the high concentration of CS leads more functional groups to gather around calcium ions than the more CS chains to form calcium cations complexes.

Furthermore, to ensure the stability of the nanoparticles, we also measure the potential zeta value of the formulas. Zeta potential is a description of the surface load of the particles. The possible measurement of zeta potential is a crucial factor in determining the nanoparticles stability. Zeta potential is capable of defining the stability of the colloid system due to aggregation. The zeta potential value of F1 and F2 were 27.7 and 34.9 mV (Figure 1B), respectively. The zeta potential value is about > 30 mV indicated the particles show less chances for agglomeration. Based on this data, it can be concluded that the F2 is the optimum formula based on the physical characterization.
3.2. Cytotoxic activity of NPs-CA on MDA-MD-231 cells

Cytotoxic assay was performed to measure the effect of NPs-CA in inhibiting cancer cell proliferation compared to CA extract. The resulted showed that the NPs-CA dan CA treatment for 24 h significantly decreased the cell viability with dose-dependent manner. Interestingly, the IC50 value of NPs-CA (83 µg/mL) was four-fold lower than CA (320 µg/mL) (Figure 2A). This resulted suggested that the particle size of the compounds that involved in the effect on BC cell growth. In addition, the NPs-CA also cause the morphological change under inverted microscope observation (Figure 2B). The concentration of 50 µg/mL NPs-CA induced cell CL induced cell shrinkage, pyknosis and fragmentation. Cell shrinkage and pyknosis are visible through inverted microscopy during the early process of apoptosis [14,15]. However, in the same concentration the treatment of CA has not shown significant morphological changes. These findings clearly indicated that NPs-CA has more potential reduce cell viability than CA on highly metastatic breast cancer MDA-MB-231 cells due to the involved of particle size.
Figure 2. Cytotoxic activity of NPs-CA and CA on MDA-MB-231 cells. (A) Cells 5×10^3 were seeded for 24 h in 96-well plate, then treated with NPs-CA and CA individually and incubated for 24 h. Cell viability profile expressed mean ± SE of 3 experiments. IC50 obtained from a linear regression calculation of log concentration vs cell viability with p<0.05. (B) The effect of NPs-CA and CA on cell morphology. Visible morphological changes and population of cells in treatment of untreated, NPs-CA 50µg/mL and CA 50µg/mL. Observations of Cell morphology performed using an inverted microscope with a magnification of 100x.

4. Conclusion
Based on this study, it can be concluded that for improved the solubility and efficacy of *Citrus aurantium* extract the optimum synthesis of nanoparticles formulation was chitosan 0.6 mg/mL, sodium alginate 0.3 mg/mL, CaCl\(_2\) 0.67 mg/mL, and extract 20 mg/mL. The cytotoxic effect of nanoparticle *Citrus aurantium* peels extract is more potential than the *Citrus aurantium* peel whole extract using MTT assay. More importantly, our findings suggested that nanoparticle *Citrus aurantium* peels extract potential to be developed as a choice of dosage form for natural cancer therapeutic agent.

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