Formulation of nanoparticles ribosome inactivating proteins from *Mirabilis jalapa* L. (RIP MJ) conjugated AntiEpCAM antibody using low chain chitosan-pectin and cytotoxic activity against breast cancer cell line

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Abstract: Ribosome Inactivating Proteins (RIPs) isolated from *Mirabilis jalapa* L. (MJ protein) leaves showed high cytotoxic effect on malignant. Chitosan nanoparticles have frequently been used in protein delivery applications. The aim of this study was to develop targeted drug delivery system of RIP MJ for breast cancer therapy with chitosan nanoparticles conjugated antiEpCAM antibody. RIP MJ nanoparticles were prepared using low viscous chitosan and pectin using polyelectrolit complex method, followed by conjugation process with antiEpCAM antibody. Characterization of this formula was then carried out for its entrapment efficiency, particles size, zeta potential, morphology using transmission electron microscope (TEM) and cytotoxic assay against T47D and Vero cell line. The optimal concentration of MJ protein; low viscous chitosan; pectin for preparing AntiEpCAM conjugated of RIP MJ nanoparticles was 0.1%; 0.01%;1% (m/v) respectively and showed satisfactory formula with the average particle size of 376.8±105.2nm, polydispersity index (PI) 0.401, zeta potential 43,71 mV, high entrapment efficiency 98,97±0,12%. Transmission electron microscope (TEM) imaging showed a spherical and homogenous structure for nanoparticles. The in vitro cytotoxicity analysis showed that RIP MJ nanoparticle had more cytotoxic effect compared to unformulated RIP against T47D cell-lines. AntiEpCAM conjugated RIP MJ nanoparticles however, increased cytotoxic effect of RIPs on Vero cell-lines not for T47D cell-lines. Chitosan-Pectin nanoparticles suitable for delivering protein to target cancer cells.

Keywords: *Mirabilis jalapa* L. Ribosome Inactivating Proteins, AntiEpCAM, chitosan, pectin, nanoparticles, T47D, Vero cell line

INTRODUCTION

Ribosome-inactivating proteins (RIPs) were plant's enzyme that could depurinate ribosomal RNA (rRNA) causing for irreversible blocking of protein synthesis process (Stirpe and Battelli, 2006). RIPs isolated from *Mirabilis jalapa* L. plant, indicated to have the potential for breaking the supercoiled DNA and inducing the cell death through the induction of apoptosis and necrosis in cancer cell line (Sismindari *et al*., 1998; Ikawati *et al*, 2003; Ikawati *et al*, 2006). One of RIPs, MJ30, a purified RIP from *M jalapa* using CM-Sepharose CL-6B has a cytotoxic activity on breast cancer cell line (T47D), cervical cancer cell line (HeLa) and blood cancer NSI (myeloma) (Sismindari *et al*., 2010).

The potency of using polymer nanoparticles as drug carriers has been developed with many different conductive materials. Nanoparticles are colloidal structures with the sizes around 10-1000nm (Sahoo and Labhasetwar, 2003). Nanoparticles can be formulated using biodegradable polymers in which the therapeutic agent will be trapped, absorbed and incorporated chemically (Sterzynska *et al*., 2004).

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Chitosan and pectin are the type of polymer that can be used in the manufacture of biocompatible nanoparticles because their properties, biodegradables and muchoadesive (Morris *et al*., 2010; Rashidova *et al*., 2004). Chitosan is a cationic polysaccharide derived from the deacetylation of chitin. Chitosan widely used in drug delivery nanoparticles since it has a good stability, low toxicity, simple on preparation and can almost be used for all route of drug administration (Tiyaboonchai, 2003). Pectin is an anionic polysaccharide with the main composition of poly-D-galacturonic acid. Pectin has natural biocompatible and biodegradable, and has been used on targeted drug delivery or others biomedical applications (Rashidove *et al*., 2004).

EpCAM is a marker for epithelial cells in which a variety of normal cellular function is derived. EpCAM was over expressed on tumor at the proliferation stage and growth of malignancy (Brown *et al*, 2008), especially on colon, breast, prostate and bladder cancer (Premsukh *et al*, 2011). The addition of anti-EpCAM antibody in drug delivery systems hopefully can be possible direct delivery of drugs to the target cells without damaging normal cells. The purpose of this study was to formulate RIP nanoparticle using combination of pectin and chitosan short chain. The prepared nanoparticle was further

conjugated with antibody anti-Ep-CAM for targeted drug delivery systems against breast cancer cell-line, T47D.

MATERIALS AND METHODS

Materials

Fresh leaves of *Mirabilis jalapa* L. (red flower cultivar) were collected from Yogyakarta, Indonesia. A voucher specimen was deposited in the Pharmaceutical Chemistry Laboratory, Faculty of Pharmacy Universit as Gadjah Mada, Yogyakarta, Indonesia.

T47D, Vero cell-lines and pUC18 were obtained from laboratory stock of Life Science Laboratory, LPPT Universitas Gadjah Mada, Yogyakarta. All other reagents used were analytical grade and commercially available.

Methods

Protein extraction and determination of protein concentration

Extraction of protein from the leaves of *M.jalapa* L (RIP MJ) was carried out as described previously (Sismindari *et al.*, 1998). The extract was precipitated using aceton followed by centrifugation at 2.800g for 30 minutes. The precipitated protein was dissolved in 5mM phosphate buffer pH 6, 5 and centrifuged at 8.500g. The protein fraction was then freeze dried and stored at 4^{o}C. Protein concentration was determined using Bradford protein assay (BioRad) as total protein.

Cleavage of supercoil DNA by the protein extract

The presence of RIP activity in the RIP MJ protein, was determined by their capability to cleave supercoiled double stranded DNA, as previously described (Sismindari *et al.,* 1998). Briefly, 1 µg of supercoiled double stranded plasmid DNA (pUC18) was incubated with various concentration of the protein (0.05; 0.1; 0.2; and 0.5mg /mL) to a final volume of 20µl in 50mM Tris-HCl, 10mM $MgCl_2$, 100mM NaCl, pH 8.0, at 30^{o}C for 1 hour. At the end of the reaction, 10µl of loading buffer were added. Electrophoresis was carried out at 0.8% agarose gel in 0.5 x TBE (tris-borate) buffer. DNA fragments were visualized by staining the gel with ethidium bromide.

Preparation of nanoparticles

Nanoparticles was formed by mixing RIP MJ at various concentrations (0.05; 0.1; and 0.2%) with 0.01% chitosan followed by addition of 1% pectin with volume ratio of 1:1 between chitosan and pectin. The concentration of chitosan and pectin in this formula was chosen based on previous optimization of nanoparticle formula done using bovine serum albumin (BSA) as model protein.

Entrapment efficiency

The entrapment efficiency was determined by calculating the total concentration of RIP MJ added and free RIP MJ following separating from RIP MJ nanoparticles using centrifugation at 8.500g for 30 minutes. The free RIP MJ was on the aqueous layer. The amount of the free protein was measured by Bradford protein assay.

The entrapment efficiency of protein nanoparticles was calculated by following equation 1:

$$EE\% = \frac{\text{Total amount Protein} - \text{Free protein}}{\text{Total amount protein}} \times 100\% \quad (1)$$

Conjugation of nanoparticles with antibody anti-EpCAM

10µL solution of 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) 1mg/mL was mixed with 10mL of antibody antiEpCAM solution 1mg/mL and then added 80µL MOPS buffer 20mm pH 6.0. Mixed and incubated mixture for 15 min at room temperature. Then added 1µL mixture to 1mL nanoparticles and dialyzed overnight at 4°C.

Characterization of RIP-nanoparticles

The size of nanoparticles was measured using Particle Size Analyzer (Beckman Coulter). The particle size distribution is reported as a poly-dispersity index (PI). The morphology of nanoparticles was analyzed by Transmission Electron Microscopy (TEM), by placing the samples on a carbon coated copper grid coated using Auto Carbon Coated (JOEL JEC-560, Japan). Nanoparticle was allowed to dry and placed in a holder and further analysis at an acceleration voltage of 120kV with 60000 x magnification.

Cytotoxicity studies

One hundred mL of the exponentially growing cells (5 x 10^4 cells/mL) were seeded in 96-well plate with a serial dilution of nanoparticle formulas. The media without cells was used as a control media, while the phosphate buffer pH 6.5 was used as control for the protein fraction. After 24 h incubation, the number of viable cells was ascertained with MTT reaction (3-(4,5-dimethyltiazol-2-il)-2,5-diphenyl tetrazolium bromide), and measured at λ_{550}nm followed by IC_{50} calculation.

RESULTS

Isolation and double stranded cleavage activity of RIPMJ

Isolated RIP MJ protein was measured by Bradford protein assay. Total protein obtained from 320gram of fresh samples was 125mg (about 0.04%). The RIP activity of RIP MJ was demonstrated by the cleaving activity on super coiled double strand DNA. The result indicated that at 0.2µg and 0.4 µg RIP MJ was able to cleave double stranded pUC18 into nick-circular form. The activity was increased at 2µg protein, as indicated by the degraded DNA (fig. 1). This result supported previous results (Sismindari *et al*, 1998), so that it indicated that isolated

MJ protein contained RIPs. This MJ protein was then ready for nanoparticle preparation.

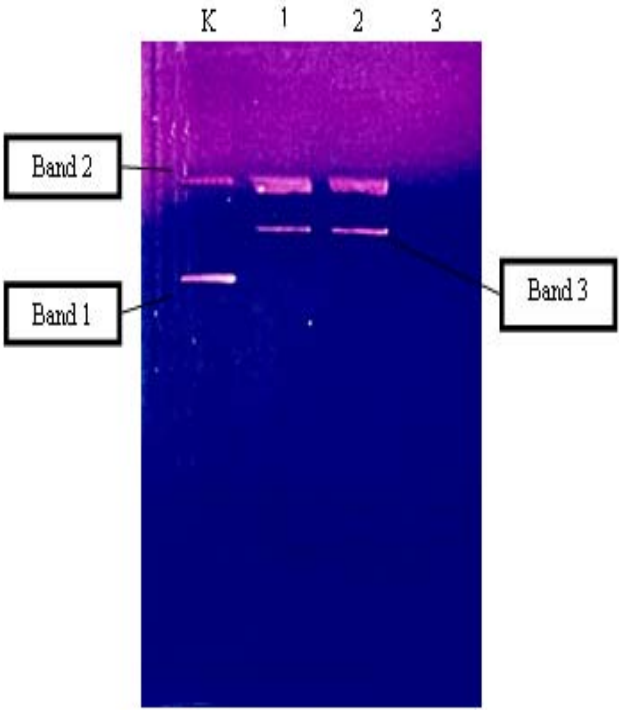

Fig. 1: DNA supercoiled cleavage activity of MJ protein using pUC18. Protein amount 0,2µg (1) and 0,4µg (2) shown cleavage DNA supercoil to nick-circular and linear. Protein amount 2µg (3) DNA was degraded. K: Control untreated pUC18; Band 1: supercoiled form; band 2: nick-circular form; band 3: linear form.

Entrapment efficiency

Purification of unbound protein and nanoparticle-RIP performed by centrifugation, next the unbound protein present in the supernatant was analyzed by Bradford protein assay. Result is shown in table 1 and RIP at concentration of 0.05mg/mL, the percent EE value of more than 100%. It means that all the proteins that are formulated may have been tied or entrapped into nanoparticles. RIP at concentration of 0.2mg/mL, given EE value smaller than concentration of 0.1mg/mL.

Characterization of nanoparticles-RIP

Measurement of nanoparticle complex was done by Particle Size Analyzer (PSA) TM Delsa Nano (Beckman Coulter). Measurements were taken at temperature conditions of 25^{o}C; refractive index of 1.3332; cP viscosity of 0.8858; and scattering intensity of 8777 cps in aqueous solvent.

The results shown in fig. 2 indicated that the size of nanoparticles formed in this study had an average size of 376.8 nm with index polydispersity of 0.401.

Measurement of zeta potential of nanoparticles-RIP acquired zeta potential value of +36.05 mV. Morphological characterization of nanoparticle-RIP was also observed before conjugated with antibodies. The resulting RIP MJ nanoparticles have an average spherical shape with different sizes (fig. 3).

Cytotoxic assay of nanoparticles

Cytotoxic assay of RIP nanoparticles showed that both RIP MJ and antiEpCAM conjugated RIP MJ nanoparticles had a higher cytotoxic effects compared to unformulated MJ protein against either T47D cells and Vero cell-lines as indicated by the level of IC_{50} (fig. 4). The lower IC_{50} indicated higher cytotoxicity level.

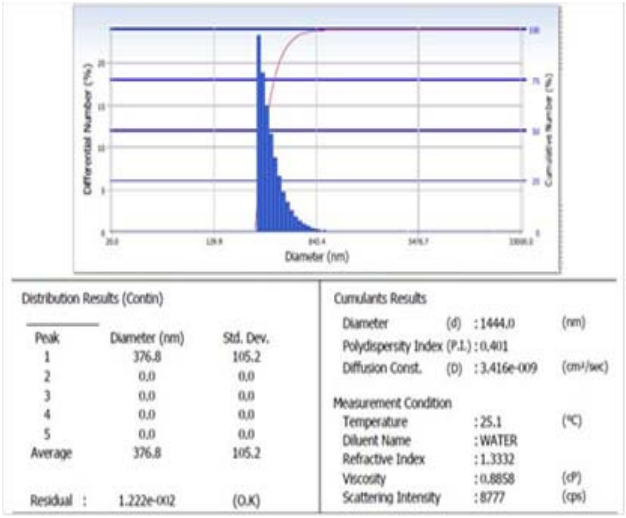

Fig. 2: Particle Size Analyzer (PSA) of RIP MJ nanoparticle. Average size of nanoparticles was 376.8 nm; polydispersity index (PI) 0.401

DISCUSSION

The nanoparticles were formed by complex coacervation technique that relies on the interaction between the negative charge of poly-anions and the positive charge of the cations. Amine (NH_3^+) groups of chitosan will interact with the carboxyl (COO^-) groups of pectin to form polyelectrolyte complexes via hydrogen bonding. The interaction of chitosan and pectin are interesting because they both have a high molecular weight. The structure of these two polysaccharides generate strong intermolecular interactions and rigid polymer chain (Rawat *et al*, 2006).

Protein nanoparticles are formed instantly as pectin, a poly-anion, was added to the chitosan solution and protein. Pectin would form hydrogen bonds with the free amine group owned by chitosan nanoparticles and proteins so that the proteins are formed to be more compact.

Table 1: Formulation Nanoparticles-RIP

Concentration Of RIP (mg/mL)	Chitosan 0.01% Pectin 1%	Entrapment Efficiency (EE%)
0.05	-	100.55 ± 0.22
0.1	-	99.46 ± 0.24
0.2	-	98.85 ± 0.04

Result of EE measurement in table 1 shows that the higher concentration of RIP decreased the EE percentage. Concentration of 0.1mg/mL was considered as the optimal concentration since the EE was decreased at concentration of 0.2 mg/mL while at concentration of 0.05mg/mL, the nanoparticle might still have the ability to entrap more protein. Entrapment efficiency was likely influenced by two mechanisms: interaction of positively charged amine group with a negatively charged carboxyl group and entrapment of protein during the process of complex formation of nanoparticles.

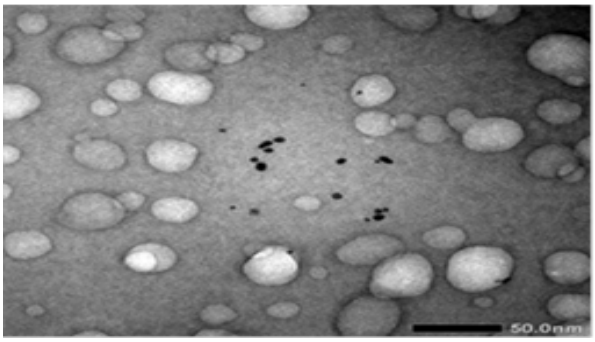

Fig. 3: Morphology of RIP MJ nanoparticle based on measurements using Transmission Electron Microscopy (TEM). Morphology nanoparticles was spherical with different sizes

The particle size and distribution of the particle are important characteristic of the particle since it would determine in vivo distribution, affected drug loading, drug release and the stability of the nanoparticles. The length of the polymer chain and the crosslinking between the polymer will affect the cohesiveness of the matrix and affecting the swelling and drug diffusivity trapped in matrix (Winarti, 2011). The size and shape of the nanoparticles determine the ability of nanoparticles to enter the cell. The smaller particle size would make nanoparticles easier enter into cell. But if the size was too small (<80 nm) it would be easy for nanoparticles to be carried by the flow of blood vessels in tumor tissue without the opportunity to enter into cell. The size of nanoparticles in this study as shown in fig. 2 shown that it wasn't too small for cellular application as it has the average size of 376.8 nm.

PI values generally ranged between 0-1 and the smaller value of PI indicates more uniform particle size distribution. The resulted PI value of nanoparticle was 0.401 (fig. 2) which indicated that the nanoparticles have quite uniform distribution. In addition to indicating the particle size distribution in a sample, the PI can also be used to expect the presence of aggregation in the sample. When aggregation occurs in a sample, the particle size becomes less uniform due to presence of particles that are joined, causing the value of PI to increase (Chabib *et al*, 2012).

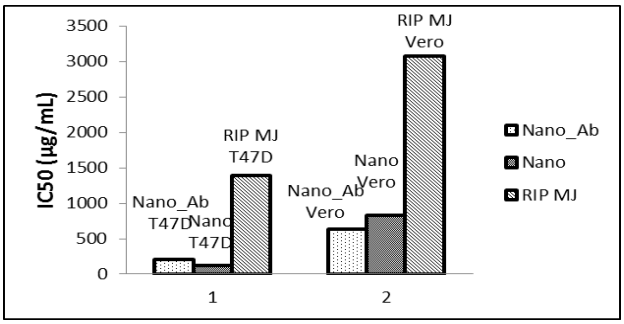

Fig. 4: IC_{50} value of RIP MJ nanoparticles against cancer cells lines. T47D (1) and Vero cells (2) the treatment of antiEpCAM conjugated RIP MJ nanoparticles (blue); RIP MJ nanoparticle - (red); unformulated RIP-MJ (green)

Positive zeta potential value of the nanoparticle indicated that the surface of the nanoparticles had a positive charge. The greater the value of zeta potential charge, the particle will be more stable. This is due to the repulsive force between particles with similar charges so it does not form aggregates in the system. From the morphological observation of the nanoparticle (fig. 3), it appears that some nanoparticles aggregated or piled up to form particles with a larger size. In addition, some other particles were also detected, which may be unbound protein that is not entrapped into nanoparticles.

Conjugation of nanoparticles with antibodies was formed by carbodiimide catalyst. EDAC reacts with carboxylic acid group to form the intermediate O-acylisourea which are highly reactive. These active species then react with nucleophiles such as primary amides to form an amide bond. In this study, EDAC will activated the carboxylic group of antibodies or pectin. Nanoparticles and antibodies have the same carboxyl groups and amines. Therefore, EDAC could activate the carboxyl group possessed by antibodies and also carboxyl groups in surface of nanoparticles derived from pectin. Active carboxyl groups of antibody reacted with the amine group on nanoparticles derived from chitosan. While active,

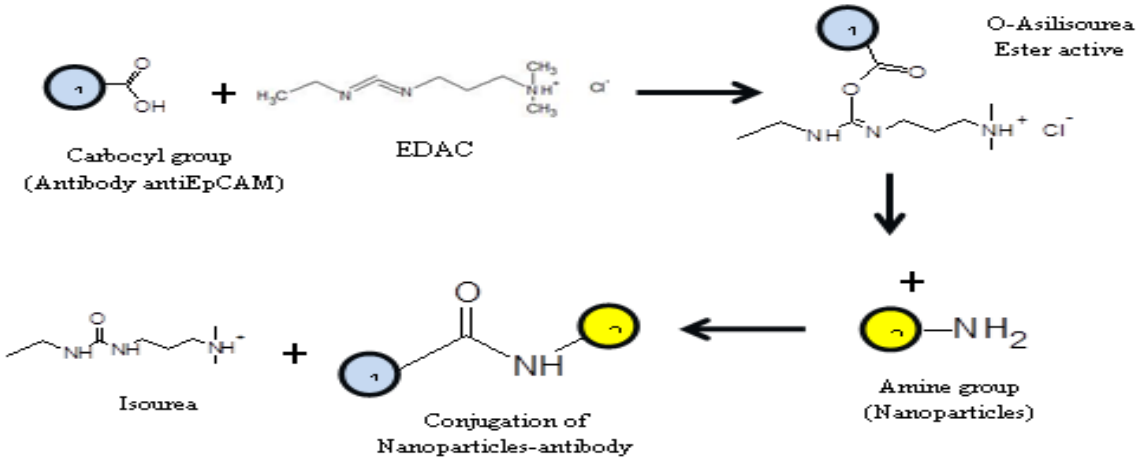

Fig. 5: Mechanism reaction of conjugation of anti-EpCAM antibody and nanoparticles using EDAC.

carboxyl groups on the nanoparticles will bind the amine group of antibodies. Reaction mehanism of nanoparticles conjugation with antiEpCAM antibody is shown in fig. 5.

The result of cytotoxicity analysis showed that nanoparticle formulation increased the cytotoxicity of RIP MJ in both T47D breast cancer and vero cells, with higher cytotoxicity towards T47D compared to vero cells. However, the conjugation of antiEpCAM antibody only increased the cytotocicity of nanoparticles in Vero cells, but not in T47D breast cancer cells. In breast cancer, as many as 90% of 1715 tumor samples showed EpCAM positive. However, the level of expression in lobular carcinoma was significantly higher than the expression of EpCAM on the type of ductal carcinoma or other types of breast cancer. T47D breast cancer cells were the type of ductal carcinoma that EpCAM expression in these cell types was not as big as on other breast cancer cells such as MCF7 or SKBR3. Thus it was possible that EpCAM expression in Vero cells is higher when compared with T47D cells. If this is true, then the nanoparticle-RIP conjugated antiEpCAM will be more specifically targeted at Vero cells than T47D. Another factor that may affect the results of the test are less specific cytotoxic antiEpCAM antibody used in cancer cells T47D. In this study, the type of antibody used is AUA-1. Based on research (Sterzynnska, 2012) by using three kinds of antibody antiEpCAM T47D showed that cells respond positively in immunofluorescence tests using antibody antiEpCAM EBA-1 and 9C4, while the AUA-1 antibody showed a negative response.

In addition to the low specificity of the antibody conjugated to nanoparticle-RIP, the level of nanoparticles uptake into the cell could also affected the result. Nanoparticle size greater than 300 nm will decrease rate of uptake into the cell. The presence of antibodies conjugated on the surface of the nanoparticles would also increases the size of the particles so that adds to the burden of nanoparticles to be taken into cell.

The cytotoxicity test result as shown in fig. 4 indicated that formulated nanoparticle could increase the cytotoxic effect of MJ protein. The increase activity of nanoparticle formula was also detected on Termozolomide (TMZ)-poly lactic-co-glycolic acid (PLGA) nanoparticle formula against C6 glioma cells (Jane *et al*., 2014). Unfortunately, the conjugation of anti-EpCAM had not been able to increase the cytotoxicity. On T47D cell-line, the IC_{50} value of RIP MJ nanoparticle was lower than anti-EpCAM conjugated RIP MJ. While on Vero cells, the anti-EpCAM conjugated RIP MJ appeared to be more cytotoxic compared to RIP MJ nanoparticle. The cytotoxic result indicated that the RIP MJ nanoparticle was more cytotoxic against T47D cell-line compare to that on Vero cells, a normal cell-line.

CONCLUSION

Ribosome Inactivating Protein (RIP) could be encapsulated into nanoparticles of chitosan and cross-linker short chained pectin with an average size of 376.8 nm. The formulated RIP MJ on to chitosan-pectin nanoparticles could increase the cytotoxic effects of RIP MJ on T47D and Vero cells-lines. The conjugated antiEpCAM antibody on RIP MJ nanoparticles has not yet affected its cytotoxic activity on T47D cell-lines.

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