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Synthesis of Nanoparticles Produced by Ethanol Extract of Boesenbergia rotunda Rhizome Loaded with Chitosan and Alginic Acid and its Biological Activity tes0074

Sri Atun*, Sri Handayani

ABSTRACT

This work reports the synthesis of nanoparticles produced by ethanol extract of Boesenbergia rotunda rhizome loaded with chitosan and alginic acid, and its biological activity test as antioxidant. The method of synthesis of nanoparticles used an ionic gelation. Activity of the nanoparticle products as antioxidant was tested by the DPPH method. Results of this work showed that nanoparticles chitosan produced by ethanol extract B. rotunda can be synthesized at a concentration (% w/v) of chitosan/ Na-TPP and ratio of 8: 1, the size range of the nanoparticles were 389 to 877 nm, with a zeta potential of +41.87 mV, and precentage nanoparticle 98.1%. The corresponding nanoparticles alginic acid can be synthesized at a concentration (% w/v) of alginic acid/ Ca^2+ and ratio of 5: 1, the size range of the nanoparticles 197 to 877 nm, with a zeta potential of -82.1 mV, and precentage nanoparticle 90.2%. Furthermore, nanoparticles result of the combination of alginic acid –chitosan can be synthesized at a concentration (% w/v) alginic acid/chitosan/ca^2+ and ratio of 6.7: 2: 1, the size range of the nanoparticle were 226 to 877 nm, and precentage nanoparticle 29.7%. The morphology of each nanoparticle products was spherical and a smooth surface. The chitosan-alginic acid nanoparticles show higher activity than the starting material ethanol extract of B. rotunda.

Keywords: Nanoparticles chitosan, alginic acid, chitosan-alginic acid, B. rotunda, antioxidant.

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INTRODUCTION

Dental caries and oral disease are often found in Indonesia. Both of them can affect the health of all sections of society, including vulnerable to gum disease. Dental caries is a multifactorial disease, caused by factors of the surface of the tooth itself, the substrate, microorganisms and time. The disease is also strongly associated as a cause of coronary heart disease, kidney failure, stomach cancer, colon cancer, and oral cancer. The negative impact is easily seen as a plaque attached to the tooth surface, which if not addressed could cause damage to the email that cause caries or cavities.[1,2] Some antibiotics have been found to prevent and reduce the formation of plaque, but they can cause side effects when used continuously, namely the emergence of a strain of bacteria resistant to antibiotics and cannot be absorbed by tissues.[3] The use of antibiotics often results in resistant, while the use of antiseptics, such as chlorhexidine can prevent even can remove plaque that has been formed, but the side effects is the discoloration of the teeth and tongue and taste disorders after use.[3] Therefore, it is necessary to find new antibacterial natural ingredients that have no side effects.

Nanotechnology is the study of particles in the size range of 1-1000 nm. The nano scale will bring up the physical, chemical, and biological characteristics. Nanotechnology has been applied in various fields such as electronics, energy, space, medicine, food, chemical sensors and molecular manufacturing. Today the use of nanotechnology has been developed in the medical field that focuses on the application of technology in the rapid diagnosis, drug delivery, imaging, and therapy. Nanoparticle based drug delivery systems have made an interestingly remarkable difference in the studies using chemotherapy agent, for the physical, chemical and biological aspects. Some products of nanoparticles have been developed and used clinically.[4,5] The use of nanotechnology in cancer drug discovery indicates that it can reduce damage to normal cells, power absorption and distribution; it can be set so that cancer drugs can work more optimally.[6]

Boesenbergia rotunda (L.) MANSF. KULTURPFL. is synonim with *Boesenbergia pandurata* (ROXB.)

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Table 1: Synthesis nanoparticle product produced by ethanol extract of B . rotunda loaded chitosan (Product I)

Code Formula	Ext (g)	Chito-san (% w/v)	NaTPP (% w/v)	MeanYield (mg) Colour	% Nano Particle	Size (nm)	% Micro Particle	Size (µm)	Zeta Potensial (mV)
A1	1	0.1	0.02	334 ± 5.7; yellow brown	1.3	877	98.7	1005-1510	*
A2	1	0.2	0.02	444 ± 4.8; yellow brown	68.7	510-877	31.3	1005-1510	+26.83
A3	1	0.3	0.02	395 ± 3.3; yellow brown	0	-	100	1005-1729	*
A4	1	0.4	0.02	523 ± 4.6; yellow brown	1	766-877	99	1005-2269	*
A5	1	0.1	0.01	440 ± 10.1; yellow brown	75.8	389-877	24.2	1005-1151	+14.40
A6	1	0.08	0.01	501 ± 12.8, yellow brown	98.1	389-877	1.9	1005	+41.87
A7	1	0.09	0.01	380 ± 20.2, yellow brown	70.1	296-877	29.9	1005-1729	*
A8	1	0.11	0.01	478 ± 8.9, yellow brown	19.0	766-877	81.0	1005-1151	*
A9	1	0.12	0.01	413 ± 5.8, yellow brown	53.7	339-877	46.3	1005-1510	*

*if % nanoparticle <70%, zeta potential very low and not be measured

Table 2: Synthesis nanoparticle product produced by ethanol extract of B. rotunda loaded alginic acid and Ca2+ (Product II)

Code Formula	Ext (g)	Alg (% w/v)	Ca2+ (% w/v)	MeanYield (mg) Colour	% Nano Particle	Size (nm)	% Micro Particle	Size (µm)	Zeta Potensial (mV)
B1	1	0.1	0.1	569±1.3; yellow brown	0	-	100	2269-3409	*
B2	1	0.3	0.1	576±2.5; yellow brown	0	-	100	1005-3409	*
B3	1	0.5	0.1	894±0.9; yellow brown	0	-	100	3905-5122	*
B6	1	0.1	0.4	597±2.2, yellow brown	0	-	100	1318-6000	*
B5	1	0.1	0.3	637±2.5; yellow brown	0	-	100	1151-1318	*
B5	1	0.1	0.3	637±2.5; yellow brown	0	-	100	1151-1318	*
B7	1	0.1	0.01	167±3.8, yellow brown	80.8	226-877	19.2	1005-1318	-89.5
B8	1	0.1	0.015	227±4.5, yellow brown	83.3	259-877	16.7	1005-1981	-84.7
B9	1	0.1	0.02	246±3.2, yellow brown	90.2	197-877	9.8	1005-1151	-82.1
B10	1	0.1	0.03	228±2.3, yellow brown	65.5	259-877	34.5	1005-1510	*
B11	1	0.1	0.04	182±2.3, yellow brown	95.2	339-877	4.8	2269-3905	-72.1

*if % nanoparticle <70%, zeta potential very low and not be measured Ext = ethanol extract of B. rotunda; Alg = alginic acid

SCHLTR. and also synonim with *Kaempferia pandurata* ROXB., belonging to the family of Zingiberaceae. It is a perennial herb distributed in some tropical countries including Indonesia, Malaysia, Myanmar, and Thailand. The local name in Indonesia is "Temu kunci" this plant is a common edible ingredient in many Asian countries. This herbal plant is also used as a traditional medicine to treat illnesses such as rheumatism, muscle pain, febrifuge, gout, gastrointestinal disorders, flatulence, carminative, stomach ache, dyspepsia, and peptic ulcer. In Indonesia, *B. rotunda* is typically used to prepare "jamu" a popular traditional tonic for women after childbirth as well as a beauty aid for teenage girls

Table 3: Synthesis nanoparticle product produced by ethanol extract of B. rotunda loaded Chitosan-alginic acid and Ca2+ (Product III)

Code Formula	Ext (g)	Alg (% w/v)	Chi (% w/v)	Ca2+ (% w/v)	MeanYield (mg) Colour	% Nano Particle	Size (nm)	% Micro Particle	Size (µm)
C1	1	0.1	0.05	0.015	592±2.2; yellow brown	0	-	100	2269-3409
C2	1	0.1	0.01	0.015	610±3.5; yellow brown	1.4	877	98.6	1005-6000
C3	1	0.1	0.03	0.015	427±3.4; yellow brown	29.7	226-877	70.3	1005-1729
C4	1	0.01	0.1	0.015	384±4.5; yellow brown	3.6	877	96.4	1005-6000
C5	1	0.05	0.1	0.015	504±1.8; yellow brown	0	-	100	1510-2269

Ext = ethanol extract of B. rotunda; Alg = alginic acid; Chi = Chitosan *if % nanoparticle <70%, zeta potential very low and not be measured

and to prevent leukorrhea. Essential oil of *B. rotunda* shows-antifungal properties against *Aspergillus niger, A. fumigatus* and *Mucor*[7]. Jantan *et al*[8] reported that essential oil of *B. rotunda* shows antifungal properties against *Saccharomyces cerevisiae, Cryptococcus neoformans, Candida albicans*, *Candida tropicalis*, and *Torulopsis glabrata*. The research of Taweechaisupapong *et al.*,[9] showed the extract *B. pandurata* very effectively kills pathogenic bacteria *C. albicans* by *in vitro*. In addition *B. rotunda* contains essential oils and also secondary metabolites such as pinostrombin, pinocembrin, cardamonin, panduratin A, and alpinetin. Yanti *et al.*,[10] demonstrated that panduratin A showed a dose-dependent effect in preventing and reducing the biofilm. These results suggest that panduratin A is applicable as a natural anti-biofilm. *B. rotunda* has similarities with *Kaempferia rotunda*, but it contains more essential oils, and shows a characteristic odour.[7] Previous research showed that several chemical compounds or extracts of *K. rotunda* has antibacterial activity, anti-inflammatory, analgesic, antipyretic, anti-tumor, anti-HIV, and antioxidants.[11] To improve the stability, solubility and activity of extracts and pure compounds of *B. rotunda* it is necessarily developed into a nanoparticle product.

The principles of the design of nanoparticles, including nano-emulsions, dendrimers, nano-gold, liposomes, conjugated drug-carrier, antibody complexes, and magnetic nanoparticles, are mainly based on the synthetic, natural, or biological components, including the use of synthetic polymers, ion metals, oils and lipids as the based material carrier group (delivery system). However, the potential success of these particles in the clinic depends on the consideration of important parameters such as nanoparticle fabrication strategies, efficiency of use of the drug, the potential release of the drug, and most importantly, minimal toxicity of the carrier group.[12] In recent years, the number of products containing nanoparticles of materials has increased because it shows the physical and chemical properties that-can be beneficial in drug delivery. Nanoparticles may consist of lipids, sugar, degradable or non-degradable polymers, metals and organic or inorganic compounds[13] [Some cancer drugs have been made in the form of nanoparticles and have been approved by the FDA, for example, Abraxane (FDA approved in January 2005), a breast cancer drug which is made from taxol (paclitaxel) tied with albumin and has a particle size of 130 nano meters. Doxil is also an ovarian cancer drug in the form of lipid nanoparticles with polyethylene glycol (PEG). In addition there is also a cancer drug with a cholesterol-lowering drug trade name Tricor (FDA approved December 2004) in the form of colloidal nano crystal.

The synthesis of nanoparticles can use several methods such as ionic gelation method, emulsifications method, coacervation or precipitation method, and spray drying method.[13] Ionic gelation method involves connecting a cross between polyelectrolyte in the presence of multivalent ion pairs. Ionic gelation is often followed by polyelectrolyte complexation with polyelectrolyte opposite. Formation the cross connecting bond will strengthen the mechanical strength of the particles formed. Polymer nanoparticles are usually made using biodegradable and hydrophilic polymers such as chitosan, gelatin and alginates.

Chitosan is a natural polysaccharide composed of [β (1 → 4) glucosamine (2-amino-2-deoxy-d-glucose) and N-acetyl-D-glucosamine (2-acetamido-2-deoxy-d-glucose)]. It is widely applied in the pharmaceutical industry, food and health. Chitosan has several beneficial properties; they are anti-microbial, wound healing, non-toxic, inexpensive, biocompatible, biodegradable, and water soluble. In the form of micro- or nanoparticles of chitosan they have many advantages that are non-toxic, unstable during use, high surface area, and can be used as a matrix for various types of drugs and extracts plants.[13] Chitosan has a capacity to increase epithelial permeation of macromolecules through the temporary opening of tight junctions of the epithelium. In addition, chitosan is known to be biocompatible and shows very low toxicity. Compared with many other natural polymers, chitosan has a positive charge and mucoadhesive.[14] The principle of this method is the existence of ionic interactions between the amino groups of a positively charged chitosan and substances negatively charged poly-anion to form a three-dimensional network structure. Cross-linker poly-anion which is the most widely used is sodium tripolyphosphate, because it is not toxic and has a multivalent. Chitosan nanoparticles can avoid the use of organic solvents as well as prevent the damage to the active ingredient of the drugs. Making products using chitosan nanoparticles as a drug delivery can be achieved as described by Wu *et al.*,[15]

Alginate is a linear polysaccharide that is soluble in water. This compound is extracted from brown seaweed and has a linear polymer structure composed by bonding (1-4) α-L-guluronic and β-D-mannuronic. Alginate has been reported to be mucoadhesive, biodegradable, and biocompatible and has the potential for a variety of pharmaceutical and biomedical applications such as drug delivery systems and cell encapsulation. Alginat micro- and nano-particles can be obtained easily by inducing gelation with calcium ions. It is an easy-gel which can be used to generate a pre-gel composed of aggregates which are very small particles of gel. A layer of polyelectrolyte complexes can be formed by the addition of an aqueous solution of polycationic such as Poly-L-lysine.[16]

This work has been made to produce nanoparticles of the ethanol extract of *B. rotunda* loaded with chitosan (product I), alginic acid (product II), and a combination of chitosan - alginic acid (product III). To make the

product I they were reacted with sodium tripolyphosphate (Na-TPP) at various compositions. The product II were prepared by reacting alginic acid and calcium ions at various compositions, while the product III were prepared by reacting chitosan and alginic acid and then calcium ion was added to the mixture. Chitosan is a polymer positively charged of amino groups, whereas alginic acid is a polymer of negatively charged of carboxyl groups.

MATERIALS AND METHODS

Apparatus and reagent

General glassware, analytical balance, evaporator Buchi Rotavapor R-114, magnetic stirer, sentrifuge, Scanning electron microscopy (SEM, Jeol T-300), particles size analysis (PSA, Horiba 550), Zeta potential (Malvern Zetasizer, UK), refrigerator, Spectronic 20 (Genesys) were used in this work.

Ethanol, aquabidest, chitosan (low molecular weight, Sigma), Sodium Tripoliphosphat (Na-TPP, Sigma-Aldrich), Acetic acid (p.a. Sigma), Alginic acid (p.a. Sigma), calsium cloride (p.a. Sigma), Rhizome of *B. rotunda,* 1,19-diphenyl-2-picrylhydrazyl (DPPH, Aldrich), ascorbat acid (Aldrich) were used in this work without further purification.

Preparation of ethanol extract of B. rotunda

The milled dried rhizoma of *B. rotunda* (5 kg) was maserated by ethanol at 24 hours at three times. The filtrate is separated by filtration, and evaporated using vacuum evaporator to dry to yield brown residue for about 147.6 g.

Preparation of nanoparticle product I

Nanoparticle chitosan produced by ethanol extract of B. rotunda was synthesized by ionic gelation. Ethanol extract of *B. rotunda* was dissolved in 35 mL ethanol and 35 mL aquadest. After homogen to the solution was added 100 ml chitosan (dissolved in acetic acid 1% v/v) at various concentrations (0.08 – 0.12 % w/v), while mixing with magnetic stirer until homogen. The resulting solution was further added with 350 mL Na-TPP (0.01-0.02% w/v in aquadest) at various concentration, and was kept for complete dissolution by magnetic stirer at medium speed for 2 hours. The mixture was stabilized overnight at refrigerator. The nanoparticles were collected by centrifugation at 12.000 rpm for 15 minute. The precipitate were washed with distilled water repeatedly and then freshdryed for 24 hours.

Preparation of nanoparticle product II

Ethanol extract *B. rotunda* was dissolved in 35 ml of ethanol and 35 mL of distilled water and to the solution was added 100 mL of alginic acid (dissolved in 0.1 M NaOH) at various concentrations (0.1 – 0.5 % w/v) below the magnetic stirrer until homogenous. Extract and alginic acid solution is then added a solution of calcium chloride at various concentrations (0.01 – 0.1 % w/v) slowly while stirring using a magnetic stirrer at low speed and mix stored for the complete dissolution with a magnetic stirrer at medium speed for 2 hours. The mixture was stabilized overnight at refrigerator. Nanoparticles were collected by centrifugation at 12.000 rpm for 15 minutes. The precipitate was washed with distilled water repeatedly and then freshdryed for 24 hours.

Preparation of nanoparticle product III

Ethanol extract of *B. rotunda* was dissolved in a well stiring of 35 mL ethanol and 35 mL aquadest and then into the solution was added 50 mL alginic acid (dissolved in NaOH 0.1M) at various concentrations (0.05 - 0.1 % w/v) and 50 mL chitosan (dissolved in acetic acid 1%) at various concentration (0.05 - 0.1% w/v); and the mixture was allowed to stand until homogen. The resulting solution was further added with 350 mL calsium chloride (0.015% w/v in aquadest), and was kept for complete dissolution by magnetic stirer for 2 hours. The mixture was stabilized overnight at refrigerator. The nanoparticles were collected by centrifugation at 12.000 rpm for 15 minute. The precipitate were washed with distilled water repeatedly and then freshdryed for 24 hours.

All resulting nanoparticles were dried and stored at refrigerator. Yield of the nanoparticles were calculated by the formula:

$$\% \text{ yield} = \frac{[\text{weight of nanoparticles obtained}]}{[\text{weight of sample fraction} + \text{weight of chitosan/alginic acid used for synthesis}]} \times 100\%$$

The characterization of this product was analyzed in term of particle size, zeta potential, and SEM (Scanning Electron Microscopy).

Biological activity as Antioxidant

Antioxidant activity was analyzed by DPPH (1.1-diphenyl-2-picrylhydrazyl) method. This method used DPPH as the source of free radical. About 5 mL of the nanoparticle was mixed with 5 ml methanolic solution of DPPH (0.12 mM) and kept in dark at room temperature for 30 minutes. The DPPH scavenging activity was determined using spectronic 20 (Genesys) at 516 nm against DPPH solution as control. The samples were tested in triplicates. The antioxidant activity was calculated as percentage of DPPH that was decreased in comparison with the control, and the inhibition activity could be calculated to determine IC_{50}.

Statistical analysis

The data of all experiments were represented as Mean ± SD and were analyzed using Microsoft Excel (Redmond, WA) software.

RESULTS AND DISCUSSION

The synthesised nanoparticles of ethanol extract of *B. rotunda* was conducted with ionic gelation method using chitosan (Product I), alginic

Table 4: The Inhibition activity (IC50) of nanoparticle product produced by ethanol extract from of B. rotunda and positive control as antioxidant

Sample Code	IC50	Note
A6. Chitosan nanoparticle(98.1%)	153.27	Less active
B9. Alginic acid nanoparticle (90.2%)	99.14	active
B11. Alginic acid nanoparticle (95.2%)	139.0	Less active
C3. Chitosan-alginic acid nanoparticle (29.7%)	27.05	active
Ethanol extract from B. rotunda	92.2	active
Positive control (Ascobat acid)	3.77	Very active

IC50 > 100 µg/mL less active; 100-10 µg/mL active; < 10 µg/mL very active

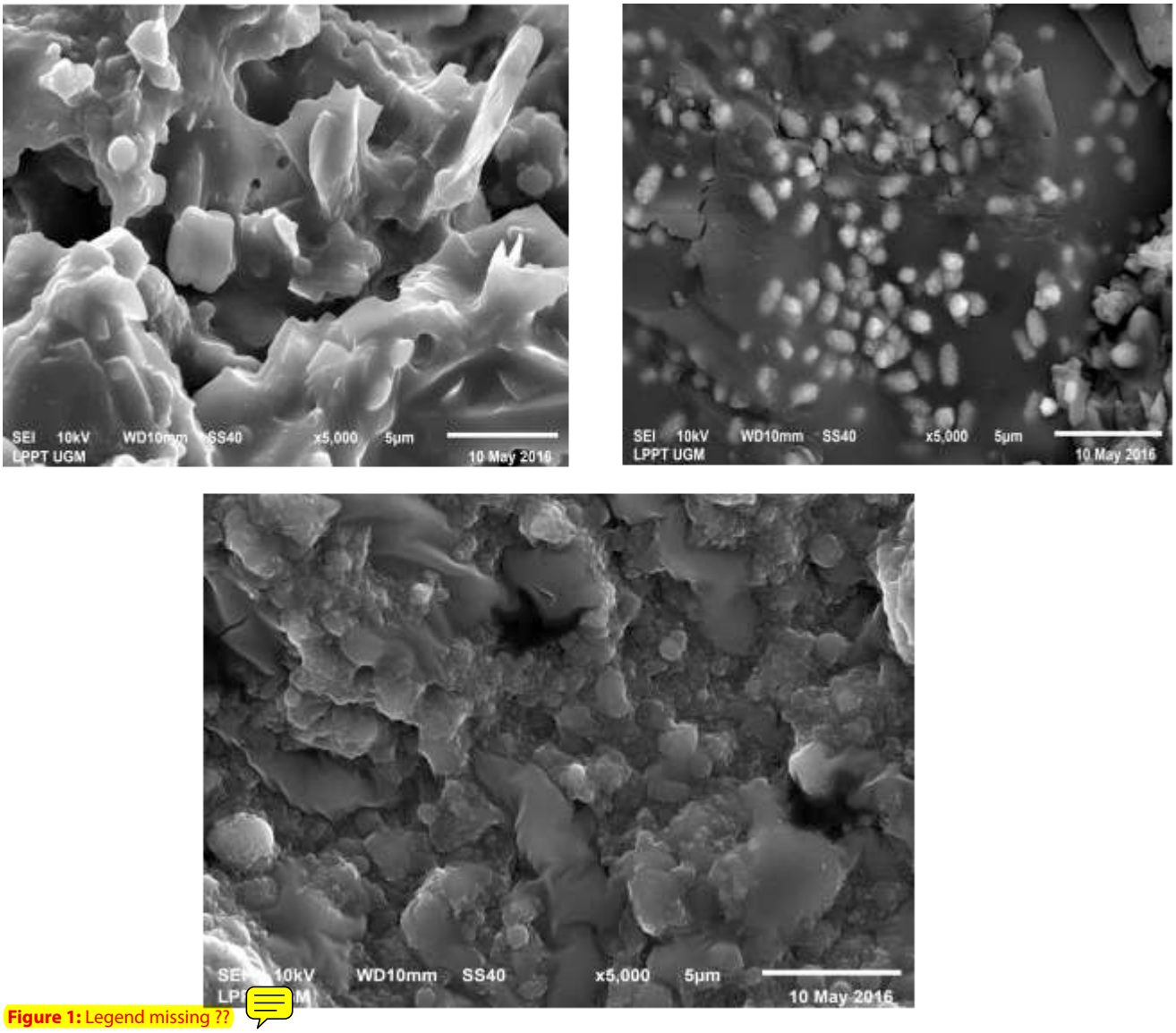

Figure 1: Legend missing ??

acid (Product II), and a combination of chitosan-alginic acid (Product III). This method are carried out at ambient temperature, and thus the preparation is relatively simple. Data of Table 1, 2 and 3 show particle size, zeta potential, yield, and physical properties of this products. In this work, the particle size was analyzed using PSA (Particle Size Analyser), it is the dynamic light scattering (DLS) system measurement. The instrument is capable of measuring particle size in the range of 1 nm to 6 µm at concentration up to 40% w/v.

The stability of nanoparticles was analised by zeta potential, that is to determine the surface charge of nanoparticles in solution or colloid. Nanoparticles have a surface charge that attracts a thin layer of ions of opposite charge to the nanoparticle surface. Two layers move with nanoparticles dispersed throughout the solution. Electrical potential on the second boundary layer called the zeta potential of particles and has a typical value between +100 mV to -100 mV. Zeta potential of nanoparticles with a value greater than +25 mV or less than -25 mV has a high degree of stability. Dispersions with a low zeta potential value will eventually aggregate due to Van Der Waal inter-particle attractions.[17] A colloidal solution containing a high percentage amount of nanoparticles would show the high value of the zeta potential, so that the solution will be difficult to form a precipitate. In this work nanoparticles produced by ethanol extract of *B. rotunda* loaded with chitosan (product I) show zeta potential positive charged, but alginic acid (product II) show zeta potential negatively charged. Product III contain components nanoparticles 29.7%, so the zeta potential not measured. Products with nanoparticles of less than 70% usually indicates a low zeta potential.

In the synthesis, the formation of nanoparticles of product I are influenced by the concentration ratio of the material forming the matrix. If the ratio is too low the product tend to form microparticles. Nanoparticles of product I are obtained at a concentration ratio of chitosan / Na-TPP 8: 1, with the precentage amount of nanoparticles 98.1%, and the size ranges of the nanoparticles were to be 389 to 877 nm, with a zeta potential of +41.87 mV. Previous research,[18] the nanoparticle of chitosan produced by chloroform fraction of *K. rotunda* were obtained at concentration ratio of chitosan / Na-TPP 10: 1 as much as 100%, and the size range of the nanoparticles were to be 172 to 877 nm, with a zeta potential of +28.06 to +38.03 mV. The difference is due to the composition and type of components in each of the different extracts, thus affecting the character of the nanoparticles produced. Similarly, other researcher has adjusted to get a chitosan/Na-TPP ratio of 6:1, and the nanoparticles

thus were obtained to be in the range of 300–400 nm with a positive surface charge ranging from +54 to +25 mV.[19] However, the nanoparticle product is highly dependent on the deacetylation of chitosan used, because it involves gelation of the protonated amino group of chitosan.

The nanoparticles of product II was synthesized using calcium ion as a cross junction to form pore size of the gel. The concentration of Ca^{2+} has a significant effect on the stability and pore size of the gel. The nanoparticles of Product II can be optimally synthesized at a concentration of alginic acid/ Ca^{2+} ratio of 5: 1, with the precentage amount of 90.2 %, the range size of 197 to 877 nm, and with a zeta potential of -82.1 mV. When the concentration of alginic acid/ Ca^{2+} ratio was 2.5: 1, it resulted in the precentage amount of 95.2 %, but the size ranged from 339 to 877 nm, with a zeta potential of -72.1 mV, and the mean yield was relatively low. When the concentration of calsium ion is high the gel microparticles were formed.

In this work the synthesis of product III resulted in more micro-sized gel. Unfortunately, the optimal combination to produce nanoparticles of more than 80% was not successesful. This is due to the interaction of the two polymers which have an opposite charge and this tends to produce large porous gel. Furthermore the combination-nanoparticles of alginic acid /chitosan /ca^{2+}, and ratio of 6.7 : 2: 1, only resulted in the percentage of about 29.7 %, and the size range of 226 to 877 nm.

The morphology of nanoparticles produced by ethanol extract of *B. rotunda* were identified using optical microscope by scanning electron microscopy (SEM). For SEM analysis, the working distance was 10 mm, beam energy was 20.0 kV, spot size was 5.0, and magnification was 5000. The nanoparticles were loaded on a double sided carbon tape and put on studs before being examined by SEM. Figure 1 shows the morphology of nanoparticles product I, II, and III, the surfaces are spherical and smooth.

The DPPH assay was used to study the free-radical scavenging capacity of these products. The results is shown in Table 4. The antioxidant activity of all nanoparticles products showed IC_{50} of 153.27; 139.0; and 27.05 µg/mL respectively. The antioxidant activity of ethanol extract of *B. rotunda* shows IC_{50} of 92.75 µg/mL. Thereby, the nanoparticles of product I and II have antioxidant activity weaker than the starting material ethanol extract of *B. rotunda*, while the nanoparticles of product III shows higher activity than the starting material ethanol extract of *B. rotunda*.

CONFLICT OF INTEREST

We declare that we have no conflict of interest

ACKNOWLEDGMENT??

ABBREVIATIONS USED??

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Comment Number (As marked in Galley Proof)	Page Number, Line Number	As in Galley Proof	Modification to be done	Remarks
1.	Page number 98-1	Type of article??	Original Article	
2.	98-2	Title :	**SYNTHESIS OF NANOPARTICLES PRODUCED BY ETHANOL EXTRACT OF *BOESENBERGIA ROTUNDA* RHIZOME LOADED WITH CHITOSAN AND ALGINIC ACID AND ITS BIOLOGICAL ACTIVITY TEST**	
3.	98-3	Abstract –line 1 Keywords	*Bosesenbergia rotunda* Nanoparticles chitosan; alginic acid; Combination of chitosan-alginic acid; *B. Rotunda;* antioxidant	

4.	99-1	Running title missing?	Synthesis of nanoparticles produced by ethanol extract of *B. rotunda*	
5.	99-2	Table-1	Synthesis nanoparticle product produced by ethanol extract of *B. rotunda* loaded chitosan	
6.	**99-3**	**Table-2-line 1 & 2**	Ca^{2+}	
7.	101-1	Matherials and Methods ....1,19-diphenyl-2-picrylhydrazyl (DPPH, Aldrich) ascorbat acid	.....2,2-diphenyl-1-picrylhydrazyl (DPPH, Aldrich) Ascorbic acid	
8.	101-2	B. rotunda	*B. rotunda*	
9.	101-3	Preparation of nanoparticle product I..... .....and then freshdryed for 24 hours	.....and were dried by a freeze dryer.	
10.		Preparation of nanoparticle product II..... .....and then freshdryed for 24 hours	.....and were dried by a freeze dryer.	
11.	101-4	Preparation of nanoparticle product III ....and then freshdryed for 24 hours	.....and were dried by a freeze dryer.	
12.	101-5	..... 1,1-diphenyl-2picrylhydrazyl	..... 2,2-diphenyl-1-picrylhydrazyl	
13.	101-6	Table 4.. title..... .....B. rotunda	IC_{50} .....*B. rotunda*	
14.	101-7	Positive control (Ascobat acid)	Positive control (Ascorbic acid)	
15.	102-1	Figure 1.....	Figure 1. SEM of the nanoparticles (A) chitosan (product I); (B) Alginic acid (product II); and (C) Combination of chitosan-alginic acid (product III) produced by ethanol extract of *B. rotunda*	

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ynthesis of Nanoparticles Produced by Ethanol Extract of Boesenbergia rotunda Rhizome Loaded with Chitosan and Alginic Acid and its Biological Activity tes0074

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ABSTRACT

This work reports the synthesis of nanoparticles produced by ethanol extract of Boesenbergia rotunda rhizome loaded with chitosan and alginic acid, and its biological activity test as antioxidant. The method of synthesis of nanoparticles used an ionic gelation. Activity of the nanoparticle products as antioxidant was tested by the DPPH method. Results of this work showed that nanoparticles chitosan produced by ethanol extract B. rotunda can be synthesized at a concentration (% w/v) of chitosan/ Na-TPP and ratio of 8: 1, the size range of the nanoparticles were 389 to 877 nm, with a zeta potential of +41.87 mV, and precentage nanoparticle 98.1%. The corresponding nanoparticles alginic acid can be synthesized at a concentration (% w/v) of alginic acid/ Ca^{2+} and ratio of 5: 1, the size range of the nanoparticles 197 to 877 nm, with a zeta potenti -82.1 mV, and precentage nanoparticle 90.2%. Furthermore, nanoparticles result of the combination of alginic acid –chitosan can be synthesized at a concentration (% w/v) alginic acid/chitosan/ca^{2+} and ratio of 6.7: 2: 1, the size range of the nanoparticle were 226 to 877 nm, and precentage nanoparticle 29.7%. The morphology of each nanoparticle products was spherical and a smooth surface. The chitosan-alginic acid nanoparticles show higher activity than the starting material ethanol extract of B. otunda.

Keywords: Nanoparticles chitosan, alginic acid, chitosan-alginic acid, B. rotunda, antioxidant.

INTRODUCTION

Dental caries and oral disease are often found in Indonesia. Both of them can affect the health of all sections of society, including vulnerable to gum disease. Dental caries is a multifactorial disease, caused by factors of the surface of the tooth itself, the substrate, microorganisms and time. The disease is also strongly associated as a cause of coronary heart disease, kidney failure, stomach cancer, colon cancer, and oral cancer. The negative impact is easily seen as a plaque attached to the tooth surface, which if not addressed could cause damage to the email that cause caries or cavities.[1,2] Some antibiotics have been found to prevent and reduce the formation of plaque, but they can cause side effects when used continuously, namely the emergence of a strain of bacteria resistant to antibiotics and cannot be absorbed by tissues.[3] The use of antibiotics often results in resistant, while the use of antiseptics, such as chlorhexidine can prevent even can remove plaque that has been formed, but the side effects is the discoloration of the teeth and tongue and taste disorders after use.[3] Therefore, it is necessary to find new antibacterial natural ingredients that have no side effects.

Nanotechnology is the study of particles in the size range of 1-1000 nm. The nano scale will bring up the physical, chemical, and biological characteristics. Nanotechnology has been applied in various fields such as electronics, energy, space, medicine, food, chemical sensors and molecular manufacturing. Today the use of nanotechnology has been developed in the medical field that focuses on the application of technology in the rapid diagnosis, drug delivery, imaging, and therapy. Nanoparticle based drug delivery systems have made an interestingly remarkable difference in the studies using chemotherapy agent, for the physical, chemical and biological aspects. Some products of nanoparticles have been developed and used clinically.[4,5] The use of nanotechnology in cancer drug discovery indicates that it can reduce damage to normal cells, power absorption and distribution; it can be set so that cancer drugs can work more optimally.[6]

Boesenbergia rotunda (L.) MANSF. KULTURPFL. is synonim with *Boesenbergia pandurata* (ROXB.)

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Table 1: Synthesis nanoparticle product produced by ethanol extract of ~~B. rotunda~~ loaded chitosan (Product I)

Code Formula	Ext (g)	Chito-san (% w/v)	NaTPP (% w/v)	MeanYield (mg) Colour	% Nano Particle	Size (nm)	% Micro Particle	Size (µm)	Zeta Potensial (mV)
A1	1	0.1	0.02	334 ± 5.7; yellow brown	1.3	877	98.7	1005-1510	*
A2	1	0.2	0.02	444 ± 4.8; yellow brown	68.7	510-877	31.3	1005-1510	+26.83
A3	1	0.3	0.02	395 ± 3.3; yellow brown	0	-	100	1005-1729	*
A4	1	0.4	0.02	523 ± 4.6; yellow brown	1	766-877	99	1005-2269	*
A5	1	0.1	0.01	440 ± 10.1; yellow brown	75.8	389-877	24.2	1005-1151	+14.40
A6	1	0.08	0.01	501 ± 12.8, yellow brown	98.1	389-877	1.9	1005	+41.87
A7	1	0.09	0.01	380 ± 20.2, yellow brown	70.1	296-877	29.9	1005-1729	*
A8	1	0.11	0.01	478 ± 8.9, yellow brown	19.0	766-877	81.0	1005-1151	*
A9	1	0.12	0.01	413 ± 5.8, yellow brown	53.7	339-877	46.3	1005-1510	*

*if % nanoparticle <70%, zeta potential very low and not be measured

Table 2: Synthesis nanoparticle product produced by ethanol extract of B. rotunda loaded alginic acid and ~~Ca2+~~ (Product II)

Code Formula	Ext (g)	Alg (% w/v)	~~Ca2+~~ (% w/v)	MeanYield (mg) Colour	% Nano Particle	Size (nm)	% Micro Particle	Size (µm)	Zeta Potensial (mV)
B1	1	0.1	0.1	569±1.3; yellow brown	0	-	100	2269-3409	*
B2	1	0.3	0.1	576±2.5; yellow brown	0	-	100	1005-3409	*
B3	1	0.5	0.1	894±0.9; yellow brown	0	-	100	3905-5122	*
B6	1	0.1	0.4	597±2.2, yellow brown	0	-	100	1318-6000	*
B5	1	0.1	0.3	637±2.5; yellow brown	0	-	100	1151-1318	*
B5	1	0.1	0.3	637±2.5; yellow brown	0	-	100	1151-1318	*
B7	1	0.1	0.01	167±3.8, yellow brown	80.8	226-877	19.2	1005-1318	-89.5
B8	1	0.1	0.015	227±4.5, yellow brown	83.3	259-877	16.7	1005-1981	-84.7
B9	1	0.1	0.02	246±3.2, yellow brown	90.2	197-877	9.8	1005-1151	-82.1
B10	1	0.1	0.03	228±2.3, yellow brown	65.5	259-877	34.5	1005-1510	*
B11	1	0.1	0.04	182±2.3, yellow brown	95.2	339-877	4.8	2269-3905	-72.1

*if % nanoparticle <70%, zeta potential very low and not be measured Ext = ethanol extract of B. rotunda; Alg = alginic acid

SCHLTR. and also synonim with *Kaempferia pandurata* ROXB., belonging to the family of Zingiberaceae. It is a perennial herb distributed in some tropical countries including Indonesia, Malaysia, Myanmar, and Thailand. The local name in Indonesia is "Temu kunci" this plant is a common edible ingredient in many Asian countries. This herbal plant is also used as a traditional medicine to treat illnesses such as rheumatism, muscle pain, febrifuge, gout, gastrointestinal disorders, flatulence, carminative, stomach ache, dyspepsia, and peptic ulcer. In Indonesia, *B. rotunda* is typically used to prepare "jamu" a popular traditional tonic for women after childbirth as well as a beauty aid for teenage girls

Table 3: Synthesis nanoparticle product produced by ethanol extract of B. rotunda loaded Chitosan-alginic acid and Ca2+ (Product III)

Code Formula	Ext (g)	Alg (% w/v)	Chi (% w/v)	Ca2+ (% w/v)	MeanYield (mg) Colour	% Nano Particle	Size (nm)	% Micro Particle	Size (µm)
C1	1	0.1	0.05	0.015	592±2.2; yellow brown	0	-	100	2269-3409
C2	1	0.1	0.01	0.015	610±3.5; yellow brown	1.4	877	98.6	1005-6000
C3	1	0.1	0.03	0.015	427±3.4; yellow brown	29.7	226-877	70.3	1005-1729
C4	1	0.01	0.1	0.015	384±4.5; yellow brown	3.6	877	96.4	1005-6000
C5	1	0.05	0.1	0.015	504±1.8; yellow brown	0	-	100	1510-2269

Ext = ethanol extract of B. rotunda; Alg = alginic acid; Chi = Chitosan *if % nanoparticle <70%, zeta potential very low and not be measured

and to prevent leukorrhea. Essential oil of *B. rotunda* shows-antifungal properties against *Aspergillus niger, A. fumigatus* and *Mucor*[7]. Jantan *et al*[8] reported that essential oil of *B. rotunda* shows antifungal properties against *Saccharomyces cerevisiae, Cryptococcus neoformans, Candida albicans*, *Candida tropicalis*, and *Torulopsis glabrata*. The research of Taweechaisupapong *et al.*,[9] showed the extract *B. pandurata* very effectively kills pathogenic bacteria *C. albicans* by *in vitro*. In addition *B. rotunda* contains essential oils and also secondary metabolites such as pinostrombin, pinocembrin, cardamonin, panduratin A, and alpinetin. Yanti *et al.*,[10] demonstrated that panduratin A showed a dose-dependent effect in preventing and reducing the biofilm. These results suggest that panduratin A is applicable as a natural anti-biofilm. *B. rotunda* has similarities with *Kaempferia rotunda*, but it contains more essential oils, and shows a characteristic odour.[7] Previous research showed that several chemical compounds or extracts of *K. rotunda* has antibacterial activity, anti-inflammatory, analgesic, antipyretic, anti-tumor, anti-HIV, and antioxidants.[11] To improve the stability, solubility and activity of extracts and pure compounds of *B. rotunda* it is necessarily developed into a nanoparticle product.

The principles of the design of nanoparticles, including nano-emulsions, dendrimers, nano-gold, liposomes, conjugated drug-carrier, antibody complexes, and magnetic nanoparticles, are mainly based on the synthetic, natural, or biological components, including the use of synthetic polymers, ion metals, oils and lipids as the based material carrier group (delivery system). However, the potential success of these particles in the clinic depends on the consideration of important parameters such as nanoparticle fabrication strategies, efficiency of use of the drug, the potential release of the drug, and most importantly, minimal toxicity of the carrier group.[12] In recent years, the number of products containing nanoparticles of materials has increased because it shows the physical and chemical properties that-can be beneficial in drug delivery. Nanoparticles may consist of lipids, sugar, degradable or non-degradable polymers, metals and organic or inorganic compounds[13] [Some cancer drugs have been made in the form of nanoparticles and have been approved by the FDA, for example, Abraxane (FDA approved in January 2005), a breast cancer drug which is made from taxol (paclitaxel) tied with albumin and has a particle size of 130 nano meters. Doxil is also an ovarian cancer drug in the form of lipid nanoparticles with polyethylene glycol (PEG). In addition there is also a cancer drug with a cholesterol-lowering drug trade name Tricor (FDA approved December 2004) in the form of colloidal nano crystal.

The synthesis of nanoparticles can use several methods such as ionic gelation method, emulsifications method, coacervation or precipitation method, and spray drying method.[13] Ionic gelation method involves connecting a cross between polyelectrolyte in the presence of multivalent ion pairs. Ionic gelation is often followed by polyelectrolyte complexation with polyelectrolyte opposite. Formation the cross connecting bond will strengthen the mechanical strength of the particles formed. Polymer nanoparticles are usually made using biodegradable and hydrophilic polymers such as chitosan, gelatin and alginates.

Chitosan is a natural polysaccharide composed of [β (1 → 4) glucosamine (2-amino-2-deoxy-d-glucose) and N-acetyl-D-glucosamine (2-acetamido-2-deoxy-d-glucose)]. It is widely applied in the pharmaceutical industry, food and health. Chitosan has several beneficial properties; they are anti-microbial, wound healing, non-toxic, inexpensive, biocompatible, biodegradable, and water soluble. In the form of micro- or nanoparticles of chitosan they have many advantages that are non-toxic, unstable during use, high surface area, and can be used as a matrix for various types of drugs and extracts plants.[13] Chitosan has a capacity to increase epithelial permeation of macromolecules through the temporary opening of tight junctions of the epithelium. In addition, chitosan is known to be biocompatible and shows very low toxicity. Compared with many other natural polymers, chitosan has a positive charge and mucoadhesive.[14] The principle of this method is the existence of ionic interactions between the amino groups of a positively charged chitosan and substances negatively charged poly-anion to form a three-dimensional network structure. Cross-linker poly-anion which is the most widely used is sodium tripolyphosphate, because it is not toxic and has a multivalent. Chitosan nanoparticles can avoid the use of organic solvents as well as prevent the damage to the active ingredient of the drugs. Making products using chitosan nanoparticles as a drug delivery can be achieved as described by Wu *et al.*,[15]

Alginate is a linear polysaccharide that is soluble in water. This compound is extracted from brown seaweed and has a linear polymer structure composed by bonding (1-4) α-L-guluronic and β-D-mannuronic. Alginate has been reported to be mucoadhesive, biodegradable, and biocompatible and has the potential for a variety of pharmaceutical and biomedical applications such as drug delivery systems and cell encapsulation. Alginat micro- and nano-particles can be obtained easily by inducing gelation with calcium ions. It is an easy-gel which can be used to generate a pre-gel composed of aggregates which are very small particles of gel. A layer of polyelectrolyte complexes can be formed by the addition of an aqueous solution of polycationic such as Poly-L-lysine.[16]

This work has been made to produce nanoparticles of the ethanol extract of *B. rotunda* loaded with chitosan (product I), alginic acid (product II), and a combination of chitosan - alginic acid (product III). To make the

product I they were reacted with sodium tripolyphosphate (Na-TPP) at various compositions. The product II were prepared by reacting alginic acid and calcium ions at various compositions, while the product III were prepared by reacting chitosan and alginic acid and then calcium ion was added to the mixture. Chitosan is a polymer positively charged of amino groups, whereas alginic acid is a polymer of negatively charged of carboxyl groups.

MATERIALS AND METHODS

Apparatus and reagent

General glassware, analytical balance, evaporator Buchi Rotavapor R-114, magnetic stirer, sentrifuge, Scanning electron microscopy (SEM, Jeol T-300), particles size analysis (PSA, Horiba 550), Zeta potential (Malvern Zetasizer, UK), refrigerator, Spectronic 20 (Genesys) were used in this work.

Ethanol, aquabidest, chitosan (low molecular weight, Sigma), Sodium Tripoliphosphat (Na-TPP, Sigma-Aldrich), Acetic acid (p.a. Sigma), Alginic acid (p.a. Sigma), calsium cloride (p.a. Sigma), Rhizome of *B. rotunda*, ~~1,19-diphenyl-2-picrylhydrazyl~~ (DPPH, Aldrich), ~~ascorbat acid~~ (Aldrich) were used in this work without further purification.

Preparation of ethanol extract of B. rotunda

The milled dried rhizoma of *B. rotunda* (5 kg) was maserated by ethanol at 24 hours at three times. The filtrate is separated by filtration, and evaporated using vacuum evaporator to dry to yield brown residue for about 147.6 g.

Preparation of nanoparticle product I

Nanoparticle chitosan produced by ethanol extract of ~~B. rotunda~~ was synthesized by ionic gelation. Ethanol extract of *B. rotunda* was dissolved in 35 mL ethanol and 35 mL aquadest. After homogen to the solution was added 100 ml chitosan (dissolved in acetic acid 1% v/v) at various concentrations (0.08 – 0.12 % w/v), while mixing with magnetic stirer until homogen. The resulting solution was further added with 350 mL Na-TPP (0.01-0.02% w/v in aquadest) at various concentration, and was kept for complete dissolution by magnetic stirer at medium speed for 2 hours. The mixture was stabilized overnight at refrigerator. The nanoparticles were collected by centrifugation at 12.000 rpm for 15 minute. The precipitate were washed with distilled water repeatedly and ~~then freshdryed for 24 hours.~~

Preparation of nanoparticle product II

Ethanol extract *B. rotunda* was dissolved in 35 ml of ethanol and 35 mL of distilled water and to the solution was added 100 mL of alginic acid (dissolved in 0.1 M NaOH) at various concentrations (0.1 – 0.5 % w/v) below the magnetic stirrer until homogenous. Extract and alginic acid solution is then added a solution of calcium chloride at various concentrations (0.01 – 0.1 % w/v) slowly while stirring using a magnetic stirrer at low speed and mix stored for the complete dissolution with a magnetic stirrer at medium speed for 2 hours. The mixture was stabilized overnight at refrigerator. Nanoparticles were collected by centrifugation at 12.000 rpm for 15 minutes. The precipitate was washed with distilled water repeatedly and ~~then freshdryed for 24 hours.~~

Preparation of nanoparticle product III

Ethanol extract of *B. rotunda* was dissolved in a well stiring of 35 mL ethanol and 35 mL aquadest and then into the solution was added 50 mL alginic acid (dissolved in NaOH 0.1M) at various concentrations (0.05 - 0.1 % w/v) and 50 mL chitosan (dissolved in acetic acid 1%) at various concentration (0.05 - 0.1% w/v); and the mixture was allowed to stand until homogen. The resulting solution was further added with 350 mL calsium chloride (0.015% w/v in aquadest), and was kept for complete dissolution by magnetic stirer for 2 hours. The mixture was stabilized overnight at refrigerator. The nanoparticles were collected by centrifugation at 12.000 rpm for 15 minute. The precipitate were washed with distilled water repeatedly and ~~then freshdryed for 24 hours.~~

All resulting nanoparticles were dried and stored at refrigerator. Yield of the nanoparticles were calculated by the formula:

$$\% \text{ yield} = \frac{[\text{weight of nanoparticles obtained}]}{[\text{weight of sample fraction + weight of chitosan/alginic acid used for synthesis}]} \times 100\%$$

The characterization of this product was analyzed in term of particle size, zeta potential, and SEM (Scanning Electron Microscopy).

Biological activity as Antioxidant

Antioxidant activity was analyzed by DPPH (~~1,1-diphenyl-2-picrylhydrazyl~~) method. This method used DPPH as the source of free radical. About 5 mL of the nanoparticle was mixed with 5 ml methanolic solution of DPPH (0.12 mM) and kept in dark at room temperature for 30 minutes. The DPPH scavenging activity was determined using spectronic 20 (Genesys) at 516 nm against DPPH solution as control. The samples were tested in triplicates. The antioxidant activity was calculated as percentage of DPPH that was decreased in comparison with the control, and the inhibition activity could be calculated to determine IC_{50}.

Statistical analysis

The data of all experiments were represented as Mean ± SD and were analyzed using Microsoft Excel (Redmond, WA) software.

RESULTS AND DISCUSSION

The synthesised nanoparticles of ethanol extract of *B. rotunda* was conducted with ionic gelation method using chitosan (Product I), alginic

Table 4: The Inhibition activity (~~IC50~~) of nanoparticle product produced by ethanol extract from of ~~B. rotunda~~ and positive control as antioxidant

Sample Code	~~IC50~~	Note
A6. Chitosan nanoparticle(98.1%)	153.27	Less active
B9. Alginic acid nanoparticle (90.2%)	99.14	active
B11. Alginic acid nanoparticle (95.2%)	139.0	Less active
C3. Chitosan-alginic acid nanoparticle (29.7%)	27.05	active
Ethanol extract from ~~B. rotunda~~	92.2	active
Positive control (~~Ascobat acid~~)	3.77	Very active

IC50 > 100 µg/mL less active; 100-10 µg/mL active; < 10 µg/mL very active

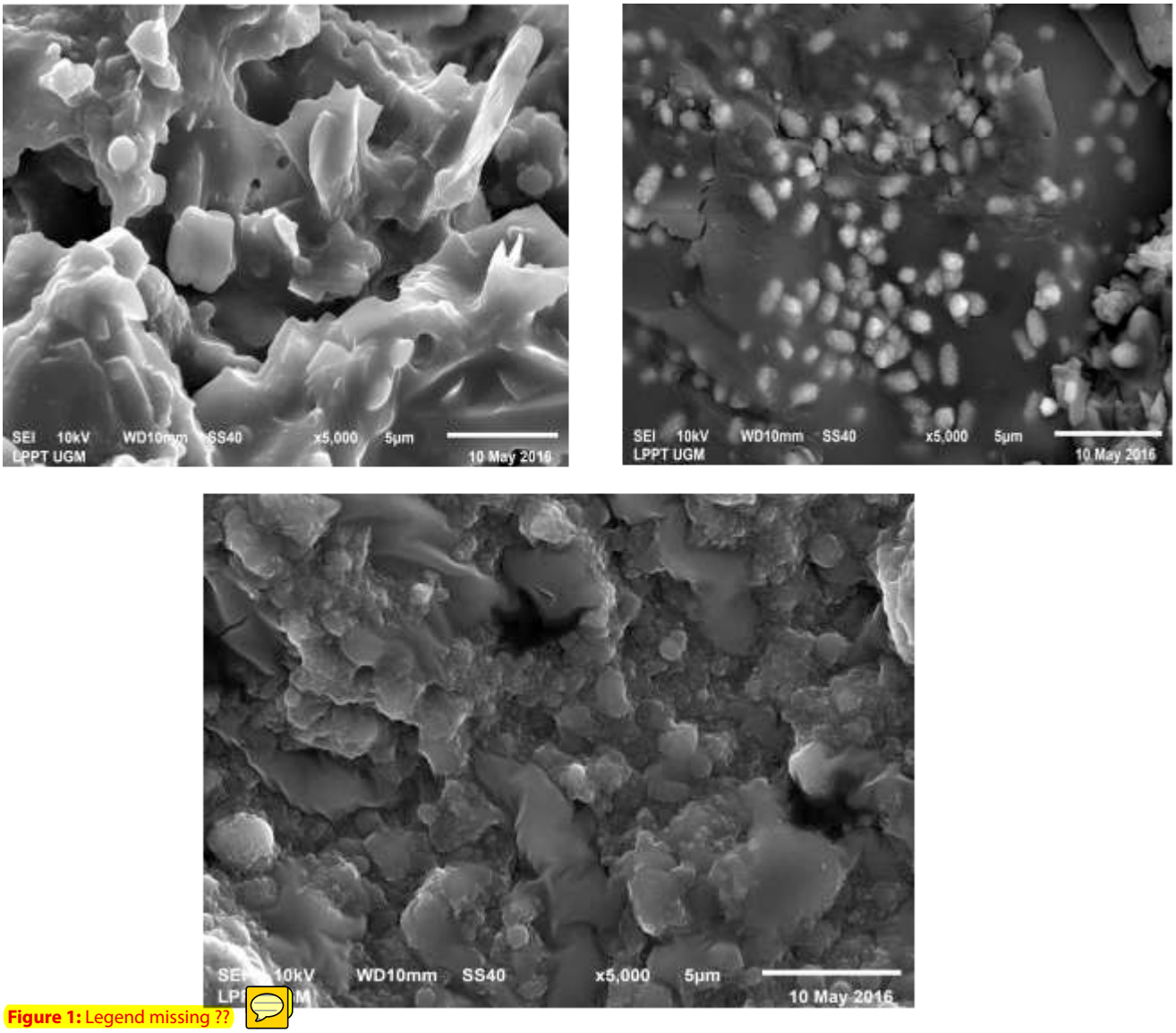

Figure 1: Legend missing ??

acid (Product II), and a combination of chitosan-alginic acid (Product III). This method are carried out at ambient temperature, and thus the preparation is relatively simple. Data of Table 1, 2 and 3 show particle size, zeta potential, yield, and physical properties of this products. In this work, the particle size was analyzed using PSA (Particle Size Analyser), it is the dynamic light scattering (DLS) system measurement. The instrument is capable of measuring particle size in the range of 1 nm to 6 µm at concentration up to 40% w/v.

The stability of nanoparticles was analised by zeta potential, that is to determine the surface charge of nanoparticles in solution or colloid. Nanoparticles have a surface charge that attracts a thin layer of ions of opposite charge to the nanoparticle surface. Two layers move with nanoparticles dispersed throughout the solution. Electrical potential on the second boundary layer called the zeta potential of particles and has a typical value between +100 mV to -100 mV. Zeta potential of nanoparticles with a value greater than +25 mV or less than -25 mV has a high degree of stability. Dispersions with a low zeta potential value will eventually aggregate due to Van Der Waal inter-particle attractions.[17] A colloidal solution containing a high percentage amount of nanoparticles would show the high value of the zeta potential, so that the solution will be difficult to form a precipitate. In this work nanoparticles produced by ethanol extract of *B. rotunda* loaded with chitosan (product I) show zeta potential positive charged, but alginic acid (product II) show zeta potential negatively charged. Product III contain components nanoparticles 29.7%, so the zeta potential not measured. Products with nanoparticles of less than 70% usually indicates a low zeta potential.

In the synthesis, the formation of nanoparticles of product I are influenced by the concentration ratio of the material forming the matrix. If the ratio is too low the product tend to form microparticles. Nanoparticles of product I are obtained at a concentration ratio of chitosan / Na-TPP 8: 1, with the precentage amount of nanoparticles 98.1%, and the size ranges of the nanoparticles were to be 389 to 877 nm, with a zeta potential of +41.87 mV. Previous research,[18] the nanoparticle of chitosan produced by chloroform fraction of *K. rotunda* were obtained at concentration ratio of chitosan / Na-TPP 10: 1 as much as 100%, and the size range of the nanoparticles were to be 172 to 877 nm, with a zeta potential of +28.06 to +38.03 mV. The difference is due to the composition and type of components in each of the different extracts, thus affecting the character of the nanoparticles produced. Similarly, other researcher has adjusted to get a chitosan/Na-TPP ratio of 6:1, and the nanoparticles

thus were obtained to be in the range of 300–400 nm with a positive surface charge ranging from +54 to +25 mV.[19] However, the nanoparticle product is highly dependent on the deacetylation of chitosan used, because it involves gelation of the protonated amino group of chitosan.

The nanoparticles of product II was synthesized using calcium ion as a cross junction to form pore size of the gel. The concentration of Ca^{2+} has a significant effect on the stability and pore size of the gel. The nanoparticles of Product II can be optimally synthesized at a concentration of alginic acid/ Ca^{2+} ratio of 5: 1, with the precentage amount of 90.2 %, the range size of 197 to 877 nm, and with a zeta potential of -82.1 mV. When the concentration of alginic acid/ Ca^{2+} ratio was 2.5: 1, it resulted in the precentage amount of 95.2 %, but the size ranged from 339 to 877 nm, with a zeta potential of -72.1 mV, and the mean yield was relatively low. When the concentration of calsium ion is high the gel microparticles were formed.

In this work the synthesis of product III resulted in more micro-sized gel. Unfortunately, the optimal combination to produce nanoparticles of more than 80% was not successesful. This is due to the interaction of the two polymers which have an opposite charge and this tends to produce large porous gel. Furthermore the combination-nanoparticles of alginic acid /chitosan /ca^{2+}, and ratio of 6.7 : 2: 1, only resulted in the percentage of about 29.7 %, and the size range of 226 to 877 nm.

The morphology of nanoparticles produced by ethanol extract of *B. rotunda* were identified using optical microscope by scanning electron microscopy (SEM). For SEM analysis, the working distance was 10 mm, beam energy was 20.0 kV, spot size was 5.0, and magnification was 5000. The nanoparticles were loaded on a double sided carbon tape and put on studs before being examined by SEM. Figure 1 shows the morphology of nanoparticles product I, II, and III, the surfaces are spherical and smooth.

The DPPH assay was used to study the free-radical scavenging capacity of these products. The results is shown in Table 4. The antioxidant activity of all nanoparticles products showed IC_{50} of 153.27; 139.0; and 27.05 µg/mL respectively. The antioxidant activity of ethanol extract of *B. rotunda* shows IC_{50} of 92.75 µg/mL. Thereby, the nanoparticles of product I and II have antioxidant activity weaker than the starting material ethanol extract of *B. rotunda*, while the nanoparticles of product III shows higher activity than the starting material ethanol extract of *B. rotunda*.

CONFLICT OF INTEREST

We declare that we have no conflict of interest

KNOWLEDGMENT??

ABBREVIATIONS USED??

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Original Article

Synthesis of Nanoparticles Produced by Ethanol Extract of *Bosenbergia rotunda* Rhizome Loaded with Chitosan and Alginic Acid and its Biological Activity tes

Sri Atun*, Sri Handayani

ABSTRACT

Introduction: *B. rotunda* used as a traditional medicine to treat illnesses such as rheumatism, muscle pain, febrifuge, gout, gastrointestinal disorders, flatulence, carminative, stomach ache, dyspepsia, and peptic ulcer. To enhance the stability, solubility and activity of the extract B. rotunda, should be developed into a product nanoparticles. **Objective:** This work reports the synthesis of nanoparticles produced by ethanol extract of *Boesenbergia rotunda* rhizome loaded with chitosan and alginic acid, and its biological activity test as antioxidant. **Method:** The synthesis of nanoparticles used an ionic gelation. Activity of the nanoparticle products as antioxidant was tested by the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. **Results:** This work showed that nanoparticles chitosan produced by ethanol extract *B. rotunda* can be synthesized at a concentration (% w/v) of chitosan/ Na-TPP (sodium tripolyphosphate) and ratio of 8: 1, the size range of the nanoparticles were 389 to 877 nm, with a zeta potential of + 41.87 mV, and precentage nanoparticle 98.1%. The corresponding nanoparticles alginic acid can be synthesized at a concentration (% w/v) of alginic acid/ Ca_2+ and ratio of 5: 1, the size range of the nanoparticles were 197 to 877 nm, with a zeta potential of -82.1 mV, and precentage nanoparticle 90.2%. Furthermore, nanoparticles result of the combination of alginic acid –chitosan can be synthesized at a concentration (% w/v) alginic acid/chitosan/ca_2+ and ratio of 6.7: 2: 1, the size range of the nanoparticle were 226 to 877 nm, and precentage nanoparticle 29.7%. The morphology of each nanoparticle products was spherical and a smooth surface. The chitosan-alginic acid nanoparticles show higher activity than the starting material ethanol extract of *B. rotunda.*

Keywords: Nanoparticles chitosan, alginic acid, chitosan-alginic acid combination, *B. rotunda*, antioxidant.

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INTRODUCTION

Dental caries and oral disease are often found in Indonesia. Both of them can affect the health of all sections of society, including vulnerable to gum disease. Dental caries is a multifactorial disease, caused by factors of the surface of the tooth itself, the substrate, microorganisms and time. The disease is also strongly associated as a cause of coronary heart disease, kidney failure, stomach cancer, colon cancer, and oral cancer. The negative impact is easily seen as a plaque attached to the tooth surface, which if not addressed could cause damage to the email that cause caries or cavities.[1,2] Some antibiotics have been found to prevent and reduce the formation of plaque, but they can cause side effects when used continuously, namely the emergence of a strain of bacteria resistant to antibiotics and cannot be absorbed by tissues.[3] The use of antibiotics often results in resistant, while the use of antiseptics, such as chlorhexidine can prevent even can remove plaque that has been formed, but the side effects is the discoloration of the teeth and tongue and taste disorders after use.[3] Therefore, it is necessary to find new antibacterial natural ingredients that have no side effects.

Nanotechnology is the study of particles in the size range of 1-1000 nm. The nano scale will bring up the physical, chemical, and biological characteristics. Nanotechnology has been applied in various fields such as electronics, energy, space, medicine, food, chemical sensors and molecular manufacturing. Today the use of nanotechnology has been developed

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Table 1: Synthesis nanoparticle product produced by ethanol extract of *B . rotunda* loaded chitosan (Product I)

Code Formula	Ext (g)	Chitosan (% w/v)	NaTPP (% w/v)	MeanYield (mg) Colour	% Nano Particle	Size (nm)	% Micro Particle	Size (μm)	Zeta Potensial (mV)
A1	1	0.1	0.02	334 ± 5.7; yellow brown	1.3	877	98.7	1005-1510	*
A2	1	0.2	0.02	444 ± 4.8; yellow brown	68.7	510-877	31.3	1005-1510	+26.83
A3	1	0.3	0.02	395 ± 3.3; yellow brown	0	-	100	1005-1729	*
A4	1	0.4	0.02	523 ± 4.6; yellow brown	1	766-877	99	1005-2269	*
A5	1	0.1	0.01	440 ± 10.1; yellow brown	75.8	389-877	24.2	1005-1151	+14.40
A6	1	0.08	0.01	501 ± 12.8, yellow brown	98.1	389-877	1.9	1005	+41.87
A7	1	0.09	0.01	380 ± 20.2, yellow brown	70.1	296-877	29.9	1005-1729	*
A8	1	0.11	0.01	478 ± 8.9, yellow brown	19.0	766-877	81.0	1005-1151	*
A9	1	0.12	0.01	413 ± 5.8, yellow brown	53.7	339-877	46.3	1005-1510	*

*if % nanoparticle <70%, zeta potential very low and not be measured

Table 2: Synthesis nanoparticle product produced by ethanol extract of *B. rotunda* loaded alginic acid and Ca^{2+} (Product II)

Code Formula	Ext (g)	Alg (% w/v)	Ca^{2+} (% w/v)	MeanYield (mg) Colour	% Nano Particle	Size (nm)	% Micro Particle	Size (μm)	Zeta Potensial (mV)
B1	1	0.1	0.1	569±1.3; yellow brown	0	-	100	2269-3409	*
B2	1	0.3	0.1	576±2.5; yellow brown	0	-	100	1005-3409	*
B3	1	0.5	0.1	894±0.9; yellow brown	0	-	100	3905-5122	*
B6	1	0.1	0.4	597±2.2, yellow brown	0	-	100	1318-6000	*
B5	1	0.1	0.3	637±2.5; yellow brown	0	-	100	1151-1318	*
B5	1	0.1	0.3	637±2.5; yellow brown	0	-	100	1151-1318	*
B7	1	0.1	0.01	167±3.8, yellow brown	80.8	226-877	19.2	1005-1318	-89.5
B8	1	0.1	0.015	227±4.5, yellow brown	83.3	259-877	16.7	1005-1981	-84.7
B9	1	0.1	0.02	246±3.2, yellow brown	90.2	197-877	9.8	1005-1151	-82.1
B10	1	0.1	0.03	228±2.3, yellow brown	65.5	259-877	34.5	1005-1510	*
B11	1	0.1	0.04	182±2.3, yellow brown	95.2	339-877	4.8	2269-3905	-72.1

*if % nanoparticle <70%, zeta potential very low and not be measured Ext = ethanol extract of B. rotunda; Alg = alginic acid

in the medical field that focuses on the application of technology in the rapid diagnosis, drug delivery, imaging, and therapy. Nanoparticle based drug delivery systems have made an interestingly remarkable difference in the studies using chemotherapy agent, for the physical, chemical and biological aspects. Some products of nanoparticles have been developed and used clinically.[4,5] The use of nanotechnology in cancer drug discovery indicates that it can reduce damage to normal cells, power absorption and distribution; it can be set so that cancer drugs can work more optimally.[6]

Table 3: Synthesis nanoparticle product produced by ethanol extract of *B. rotunda* loaded Chitosan-alginic acid and Ca^{2+} (Product III)

Code Formula	Ext (g)	Alg (% w/v)	Chi (% w/v)	Ca^{2+} (% w/v)	MeanYield (mg) Colour	% Nano Particle	Size (nm)	% Micro Particle	Size (µm)
C1	1	0.1	0.05	0.015	592±2.2; yellow brown	0	-	100	2269-3409
C2	1	0.1	0.01	0.015	610±3.5; yellow brown	1.4	877	98.6	1005-6000
C3	1	0.1	0.03	0.015	427±3.4; yellow brown	29.7	226-877	70.3	1005-1729
C4	1	0.01	0.1	0.015	384±4.5; yellow brown	3.6	877	96.4	1005-6000
C5	1	0.05	0.1	0.015	504±1.8; yellow brown	0	-	100	1510-2269

Ext = ethanol extract of B. rotunda; Alg = alginic acid; Chi = Chitosan *if % nanoparticle <70%, zeta potential very low and not be measured

Boesenbergia rotunda (L.) MANSF. KULTURPFL. is synonim with *Boesenbergia pandurata* (ROXB.) SCHLTR. and also synonim with *Kaempferia pandurata* ROXB., belonging to the family of Zingiberaceae. It is a perennial herb distributed in some tropical countries including Indonesia, Malaysia, Myanmar, and Thailand. The local name in Indonesia is "Temu kunci" this plant is a common edible ingredient in many Asian countries. This herbal plant is also used as a traditional medicine to treat illnesses such as rheumatism, muscle pain, febrifuge, gout, gastrointestinal disorders, flatulence, carminative, stomach ache, dyspepsia, and peptic ulcer. In Indonesia, *B. rotunda* is typically used to prepare "jamu" a popular traditional tonic for women after childbirth as well as a beauty aid for teenage girls and to prevent leukorrhea. Essential oil of *B. rotunda* shows antifungal properties against *Aspergillus niger, A. fumigatus* and *Mucor*[7]. Jantan *et al*[8] reported that essential oil of *B. rotunda* shows antifungal properties against *Saccharomyces cerevisiae, Cryptococcus neoformans, Candida albicans*, *Candida tropicalis*, and *Torulopsis glabrata*. The research of Taweechaisupapong *et al.*,[9] showed the extract *B. pandurata* very effectively kills pathogenic bacteria *C. albicans* by *in vitro*. In addition *B. rotunda* contains essential oils and also secondary metabolites such as pinostrombin, pinocembrin, cardamonin, panduratin A, and alpinetin. Yanti *et al.*,[10] demonstrated that panduratin A showed a dose-dependent effect in preventing and reducing the biofilm. These results suggest that panduratin A is applicable as a natural anti-biofilm. *B. rotunda* has similarities with *Kaempferia rotunda*, but it contains more essential oils, and shows a characteristic odour.[7] Previous research showed that several chemical compounds or extracts of *K. rotunda* has antibacterial activity, anti-inflammatory, analgesic, antipyretic, anti-tumor, anti-HIV, and antioxidants.[11] To improve the stability, solubility and activity of extracts and pure compounds of *B. rotunda* it is necessarily developed into a nanoparticle product.

The principles of the design of nanoparticles, including nano-emulsions, dendrimers, nano-gold, liposomes, conjugated drug-carrier, antibody complexes, and magnetic nanoparticles, are mainly based on the synthetic, natural, or biological components, including the use of synthetic polymers, ion metals, oils and lipids as the based material carrier group (delivery system). However, the potential success of these particles in the clinic depends on the consideration of important parameters such as nanoparticle fabrication strategies, efficiency of use of the drug, the potential release of the drug, and most importantly, minimal toxicity of the carrier group.[12] In recent years, the number of products containing nanoparticles of materials has increased because it shows the physical and chemical properties that-can be beneficial in drug delivery. Nanoparticles may consist of lipids, sugar, degradable or non-degradable polymers, metals and organic or inorganic compounds[13] [Some cancer drugs have been made in the form of nanoparticles and have been approved by the FDA, for example, Abraxane (FDA approved in January 2005), a breast cancer drug which is made from taxol (paclitaxel) tied with albumin and has a particle size of 130 nano meters. Doxil is also an ovarian cancer drug in the form of lipid nanoparticles with polyethylene glycol (PEG). In addition there is also a cancer drug with a cholesterol-lowering drug trade name Tricor (FDA approved December 2004) in the form of colloidal nano crystal.

The synthesis of nanoparticles can use several methods such as ionic gelation method, emulsifications method, coacervation or precipitation method, and spray drying method.[13] Ionic gelation method involves connecting a cross between polyelectrolyte in the presence of multivalent ion pairs. Ionic gelation is often followed by polyelectrolyte complexation with polyelectrolyte opposite. Formation the cross connecting bond will strengthen the mechanical strength of the particles formed. Polymer nanoparticles are usually made using biodegradable and hydrophilic polymers such as chitosan, gelatin and alginates.

Chitosan is a natural polysaccharide composed of [β (1 → 4) glucosamine (2-amino-2-deoxy-d-glucose) and N-acetyl-D-glucosamine (2-acetamido-2-deoxy-d-glucose)]. It is widely applied in the pharmaceutical industry, food and health. Chitosan has several beneficial properties; they are anti-microbial, wound healing, non-toxic, inexpensive, biocompatible, biodegradable, and water soluble. In the form of micro- or nanoparticles of chitosan they have many advantages that are non-toxic, unstable during use, high surface area, and can be used as a matrix for various types of drugs and extracts plants.[13] Chitosan has a capacity to increase epithelial permeation of macromolecules through the temporary opening of tight junctions of the epithelium. In addition, chitosan is known to be biocompatible and shows very low toxicity. Compared with many other natural polymers, chitosan has a positive charge and mucoadhesive.[14] The principle of this method is the existence of ionic interactions between the amino groups of a positively charged chitosan and substances negatively charged poly-anion to form a three-dimensional network structure. Cross-linker poly-anion which is the most widely used is sodium tripolyphosphate, because it is not toxic and has a multivalent. Chitosan nanoparticles can avoid the use of organic solvents as well as prevent the damage to the active ingredient of the drugs. Making products using chitosan nanoparticles as a drug delivery can be achieved as described by Wu *et al.*,[15]

Alginate is a linear polysaccharide that is soluble in water. This compound is extracted from brown seaweed and has a linear polymer structure composed by bonding (1-4) α-L-guluronic and β-D-mannuronic. Alginate has been reported to be mucoadhesive, biodegradable, and

biocompatible and has the potential for a variety of pharmaceutical and biomedical applications such as drug delivery systems and cell encapsulation. Alginat micro- and nano-particles can be obtained easily by inducing gelation with calcium ions. It is an easy-gel which can be used to generate a pre-gel composed of aggregates which are very small particles of gel. A layer of polyelectrolyte complexes can be formed by the addition of an aqueous solution of polycationic such as Poly-L-lysine.[16]

This work has been made to produce nanoparticles of the ethanol extract of *B. rotunda* loaded with chitosan (product I), alginic acid (product II), and a combination of chitosan - alginic acid (product III). To make the product I they were reacted with sodium tripolyphosphate (Na-TPP) at various compositions. The product II were prepared by reacting alginic acid and calcium ions at various compositions, while the product III were prepared by reacting chitosan and alginic acid and then calcium ion was added to the mixture. Chitosan is a polymer positively charged of amino groups, whereas alginic acid is a polymer of negatively charged of carboxyl groups.

MATERIALS AND METHODS

Apparatus and reagent

General glassware, analytical balance, evaporator Buchi Rotavapor R-114, magnetic stirer, sentrifuge, Scanning electron microscopy (SEM, Jeol T-300), particles size analysis (PSA, Horiba 550), Zeta potential (Malvern Zetasizer, UK), refrigerator, Spectronic 20 (Genesys) were used in this work.

Ethanol, aquabidest, chitosan (low molecular weight, Sigma), Sodium Tripoliphosphat (Na-TPP, Sigma-Aldrich), Acetic acid (p.a. Sigma), Alginic acid (p.a. Sigma), calsium cloride (p.a. Sigma), Rhizome of *B. rotunda,* 2,2 diphenyl-1-picrylhydrazyl (DPPH, Aldrich), ascobic acid (Aldrich) were used in this work without further purification.

Preparation of ethanol extract of B. rotunda

The milled dried rhizoma of *B. rotunda* (5 kg) was maserated by ethanol at 24 hours at three times. The filtrate is separated by filtration, and evaporated using vacuum evaporator to dry to yield brown residue for about 147.6 g.

Preparation of nanoparticle product I

Nanoparticle chitosan produced by ethanol extract of *B. rotunda* was synthesized by ionic gelation. Ethanol extract of *B. rotunda* was dissolved in 35 mL ethanol and 35 mL aquadest. After homogen to the solution was added 100 ml chitosan (dissolved in acetic acid 1% v/v) at various concentrations (0.08 – 0.12 % w/v), while mixing with magnetic stirer until homogen. The resulting solution was further added with 350 mL Na-TPP (0.01-0.02% w/v in aquadest) at various concentration, and was kept for complete dissolution by magnetic stirer at medium speed for 2 hours. The mixture was stabilized overnight at refrigerator. The nanoparticles were collected by centrifugation at 12.000 rpm for 15 minute. The precipitate were washed with distilled water repeatedly and were dried by a freeze dryer.

Preparation of nanoparticle product II

Ethanol extract *B. rotunda* was dissolved in 35 ml of ethanol and 35 mL of distilled water and to the solution was added 100 mL of alginic acid (dissolved in 0.1 M NaOH) at various concentrations (0.1 – 0.5 % w/v) below the magnetic stirrer until homogenous. Extract and alginic acid solution is then added a solution of calcium chloride at various concentrations (0.01 – 0.1 % w/v) slowly while stirring using a magnetic stirrer at low speed and mix stored for the complete dissolution with a magnetic stirrer at medium speed for 2 hours. The mixture was stabilized overnight at refrigerator. Nanoparticles were collected by centrifugation at 12.000 rpm for 15 minutes. The precipitate was washed with distilled water repeatedly and were dried by a freeze dryer.

Preparation of nanoparticle product III

Ethanol extract of *B. rotunda* was dissolved in a well stiring of 35 mL ethanol and 35 mL aquadest and then into the solution was added 50 mL alginic acid (dissolved in NaOH 0.1M) at various concentrations (0.05 - 0.1 % w/v) and 50 mL chitosan (dissolved in acetic acid 1%) at various concentration (0.05 - 0.1% w/v); and the mixture was allowed to stand until homogen. The resulting solution was further added with 350 mL calsium chloride (0.015% w/v in aquadest), and was kept for complete dissolution by magnetic stirer for 2 hours. The mixture was stabilized overnight at refrigerator. The nanoparticles were collected by centrifugation at 12.000 rpm for 15 minute. The precipitate were washed with distilled water repeatedly and then freshdryed for 24 hours.

All resulting nanoparticles were dried and stored at refrigerator. Yield of the nanoparticles were calculated by the formula:

$$\% \text{ yield} = \frac{[\text{weight of nanoparticles obtained}]}{[\text{weight of sample fraction} + \text{weight of chitosan/alginic acid used for synthesis}]} \times 100\%$$

The characterization of this product was analyzed in term of particle size, zeta potential, and SEM (Scanning Electron Microscopy).

Biological activity as Antioxidant

Antioxidant activity was analyzed by DPPH (2,2-diphenyl-1-picrylhydrazyl) method. This method used DPPH as the source of free radical. About 5 mL of the nanoparticle was mixed with 5 ml methanolic solution of DPPH (0.12 mM) and kept in dark at room temperature for 30 minutes. The DPPH scavenging activity was determined using spectronic 20 (Genesys) at 516 nm against DPPH solution as control. The samples were tested in triplicates. The antioxidant activity was calculated as percentage

Table 4: The Inhibition activity (IC_{50}) of nanoparticle product produced by ethanol extract from of *B. rotunda* and positive control as antioxidant

Sample Code	IC_{50}	Note
A6. Chitosan nanoparticle(98.1%)	153.27	Less active
B9. Alginic acid nanoparticle (90.2%)	99.14	active
B11. Alginic acid nanoparticle (95.2%)	139.0	Less active
C3. Chitosan-alginic acid nanoparticle (29.7%)	27.05	active
Ethanol extract from *B. rotunda*	92.2	active
Positive control (Ascobic acid)	3.77	Very active

IC50 > 100 µg/mL less active; 100-10 µg/mL active; < 10 µg/mL very active

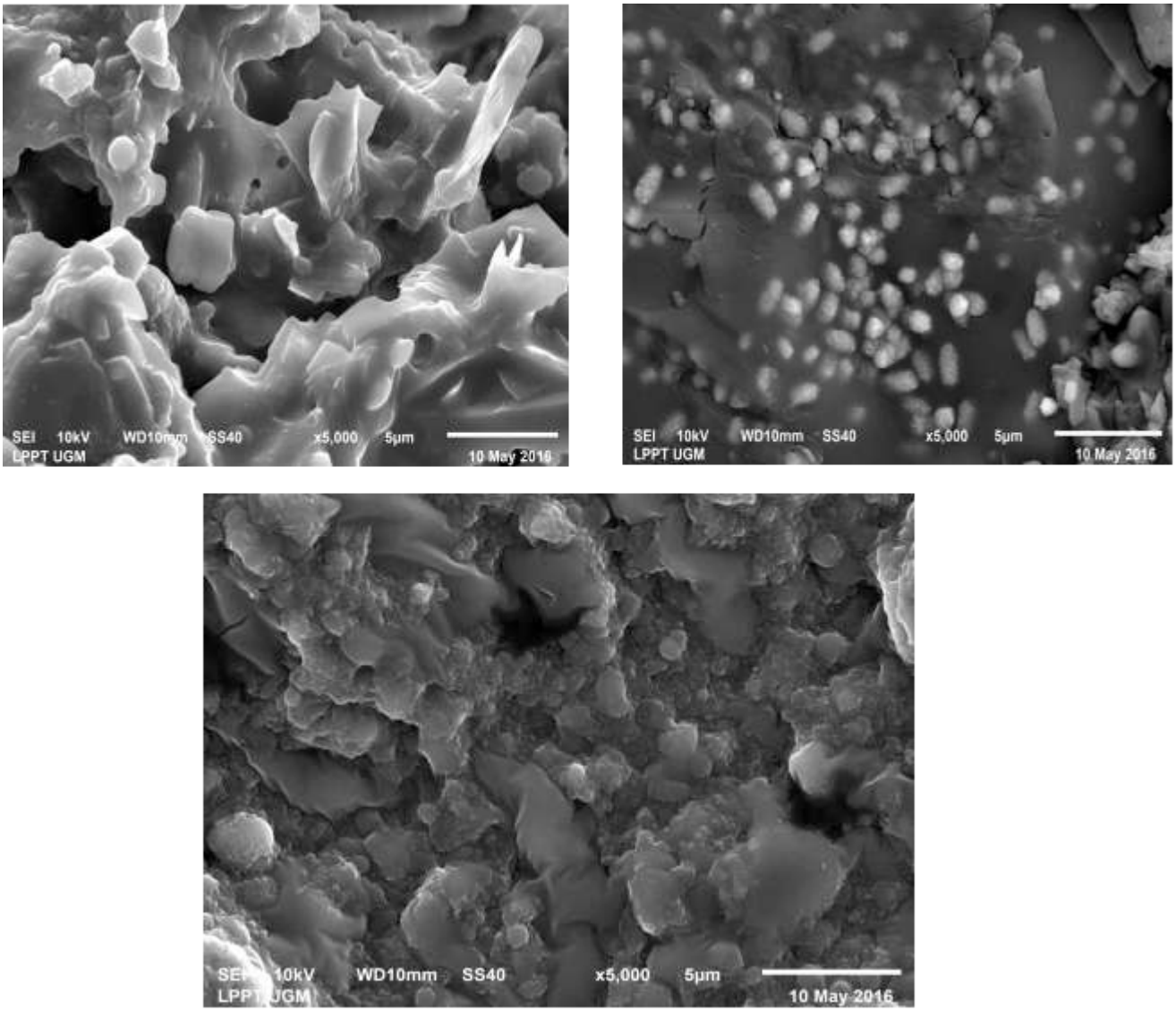

Figure 1: SEM of the nanoparticles (A) chitosan (product I); (B) Alginic acid (product II); and (C) Combination of chitosan-alginic acid (product III) produced by ethanol extract of *B. rotunda*.

of DPPH that was decreased in comparison with the control, and the inhibition activity could be calculated to determine IC_{50}.

Statistical analysis

The data of all experiments were represented as Mean ± SD and were analyzed using Microsoft Excel (Redmond, WA) software.

RESULTS AND DISCUSSION

The synthesised nanoparticles of ethanol extract of *B. rotunda* was conducted with ionic gelation method using chitosan (Product I), alginic acid (Product II), and a combination of chitosan-alginic acid (Product III). This method are carried out at ambient temperature, and thus the preparation is relatively simple. Data of Table 1, 2 and 3 show particle size, zeta potential, yield, and physical properties of this products. In this work, the particle size was analyzed using PSA (Particle Size Analyser), it is the dynamic light scattering (DLS) system measurement. The instrument is capable of measuring particle size in the range of 1 nm to 6 µm at concentration up to 40% w/v.

The stability of nanoparticles was analised by zeta potential, that is to determine the surface charge of nanoparticles in solution or colloid. Nanoparticles have a surface charge that attracts a thin layer of ions of opposite charge to the nanoparticle surface. Two layers move with nanoparticles dispersed throughout the solution. Electrical potential on the second boundary layer called the zeta potential of particles and has a typical value between +100 mV to -100 mV. Zeta potential of nanoparticles with a value greater than +25 mV or less than -25 mV has a high degree of stability. Dispersions with a low zeta potential value will eventually aggregate due to Van Der Waal inter-particle attractions.[17] A colloidal solution containing a high percentage amount of nanoparticles would show the high value of the zeta potential, so that the solution will be difficult to form a precipitate. In this work nanoparticles produced by ethanol extract of *B. rotunda* loaded with chitosan (product I) show zeta potential positive charged, but alginic acid (product II) show zeta potential negatively charged. Product III contain components nanoparticles 29.7%, so the zeta potential not measured. Products with nanoparticles of less than 70% usually indicates a low zeta potential.

In the synthesis, the formation of nanoparticles of product I are influenced by the concentration ratio of the material forming the matrix. If the ratio is too low the product tend to form microparticles. Nanoparticles of product I are obtained at a concentration ratio of chitosan / Na-

TPP 8: 1, with the precentage amount of nanoparticles 98.1%, and the size ranges of the nanoparticles were to be 389 to 877 nm, with a zeta potential of +41.87 mV. Previous research,[18] the nanoparticle of chitosan produced by chloroform fraction of *K. rotunda* were obtained at concentration ratio of chitosan / Na-TPP 10: 1 as much as 100%, and the size range of the nanoparticles were to be 172 to 877 nm, with a zeta potential of +28.06 to +38.03 mV. The difference is due to the composition and type of components in each of the different extracts, thus affecting the character of the nanoparticles produced. Similarly, other researcher has adjusted to get a chitosan/Na-TPP ratio of 6:1, and the nanoparticles thus were obtained to be in the range of 300–400 nm with a positive surface charge ranging from +54 to +25 mV.[19] However, the nanoparticle product is highly dependent on the deacetylation of chitosan used, because it involves gelation of the protonated amino group of chitosan.

The nanoparticles of product II was synthesized using calcium ion as a cross junction to form pore size of the gel. The concentration of Ca^{2+} has a significant effect on the stability and pore size of the gel. The nanoparticles of Product II can be optimally synthesized at a concentration of alginic acid/ Ca^{2+} ratio of 5: 1, with the precentage amount of 90.2 %, the range size of 197 to 877 nm, and with a zeta potential of -82.1 mV. When the concentration of alginic acid/ Ca^{2+} ratio was 2.5: 1, it resulted in the precentage amount of 95.2 %, but the size ranged from 339 to 877 nm, with a zeta potential of -72.1 mV, and the mean yield was relatively low. When the concentration of calsium ion is high the gel microparticles were formed.

In this work the synthesis of product III resulted in more micro-sized gel. Unfortunately, the optimal combination to produce nanoparticles of more than 80% was not successesful. This is due to the interaction of the two polymers which have an opposite charge and this tends to produce large porous gel. Furthermore the combination-nanoparticles of alginic acid /chitosan /ca^{2+}, and ratio of 6.7 : 2: 1, only resulted in the percentage of about 29.7 %, and the size range of 226 to 877 nm.

The morphology of nanoparticles produced by ethanol extract of *B. rotunda* were identified using optical microscope by scanning electron microscopy (SEM). For SEM analysis, the working distance was 10 mm, beam energy was 20.0 kV, spot size was 5.0, and magnification was 5000. The nanoparticles were loaded on a double sided carbon tape and put on studs before being examined by SEM. Figure 1 shows the morphology of nanoparticles product I, II, and III, the surfaces are spherical and smooth.

The DPPH assay was used to study the free-radical scavenging capacity of these products. The results is shown in Table 4. The antioxidant activity of all nanoparticles products showed IC_{50} of 153.27; 139.0; and 27.05 µg/mL respectively. The antioxidant activity of ethanol extract of *B. rotunda* shows IC_{50} of 92.75 µg/mL. Thereby, the nanoparticles of product I and II have antioxidant activity weaker than the starting material ethanol extract of *B. rotunda*, while the nanoparticles of product III shows higher activity than the starting material ethanol extract of *B. rotunda*.

CONFLICT OF INTEREST

We declare that we have no conflict of interest

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ABBREVIATIONS USED??

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Synthesis of Nanoparticles Produced by Ethanol Extract of *Boesenbergia rotunda* Rhizome Loaded with Chitosan and Alginic Acid and its Biological Activity test

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ABSTRACT

Introduction: *B. rotunda* used as a traditional medicine to treat illnesses such as rheumatism, muscle pain, febrifuge, gout, gastrointestinal disorders, flatulence, carminative, stomach ache, dyspepsia, and peptic ulcer. To enhance the stability, solubility and activity of the extract *B. rotunda*, should be developed into a product nanoparticles. **Objective:** This work reports the synthesis of nanoparticles produced by ethanol extract of *Boesenbergia rotunda* rhizome loaded with chitosan and alginic acid, and its biological activity test as antioxidant. **Method:** The synthesis of nanoparticles used an ionic gelation. Activity of the nanoparticle products as antioxidant was tested by the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. **Results:** This work showed that nanoparticles chitosan produced by ethanol extract *B. rotunda* can be synthesized at a concentration (% w/v) of chitosan/ Na-TPP (sodium tripolyphosphate) and ratio of 8: 1, the size range of the nanoparticles were 389 to 877 nm, with a zeta potential of + 41.87 mV, and precentage nanoparticle 98.1%. The corresponding nanoparticles alginic acid can be synthesized at a concentration (% w/v) of alginic acid/ Ca2+ and ratio of 5: 1, the size range of the nanoparticles were 197 to 877 nm, with a zeta potential of -82.1 mV, and precentage nanoparticle 90.2%. Furthermore, nanoparticles result of the combination of alginic acid –chitosan can be synthesized at a concentration (% w/v) alginic acid/chitosan/ca2+ and ratio of 6.7: 2: 1, the size range of the nanoparticle were 226 to 877 nm, and precentage nanoparticle 29.7%. The morphology of each nanoparticle products was spherical and a smooth surface. The chitosan-alginic acid nanoparticles show higher activity than the starting material ethanol extract of *B. rotunda*.

Key words: Nanoparticles chitosan, alginic acid, chitosan-alginic acid combination, *B. rotunda*, antioxidant.

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INTRODUCTION

Dental caries and oral disease are often found in Indonesia. Both of them can affect the health of all sections of society, including vulnerable to gum disease. Dental caries is a multifactorial disease, caused by factors of the surface of the tooth itself, the substrate, microorganisms and time. The disease is also strongly associated as a cause of coronary heart disease, kidney failure, stomach cancer, colon cancer, and oral cancer. The negative impact is easily seen as a plaque attached to the tooth surface, which if not addressed could cause damage to the email that cause caries or cavities.[1,2] Some antibiotics have been found to prevent and reduce the formation of plaque, but they can cause side effects when used continuously, namely the emergence of a strain of bacteria resistant to antibiotics and cannot be absorbed by tissues.[3] The use of antibiotics often results in resistant, while the use of antiseptics, such as chlorhexidine can prevent even can remove plaque that has been formed, but the side effects is the discoloration of the teeth and tongue and taste disorders after use.[3] Therefore, it is necessary to find new antibacterial natural ingredients that have no side effects.

Nanotechnology is the study of particles in the size range of 1-1000 nm. The nano scale will bring up the physical, chemical, and biological characteristics. Nanotechnology has been applied in various fields such as electronics, energy, space, medicine, food, chemical sensors and molecular manufacturing. Today the use of nanotechnology has been developed in the medical field that focuses on the application of technology in the rapid diagnosis, drug delivery, imaging, and therapy. Nanoparticle based drug delivery systems have made an interestingly remarkable difference in the studies using chemotherapy agent, for the physical, chemical and biological aspects. Some products of nanoparticles have been developed and used clinically.[4,5] The use of nanotechnology in cancer drug discovery indicates that it can reduce damage to normal cells, power absorption and distribution; it can be set so that cancer drugs can work more optimally.[6]

Boesenbergia rotunda (L.) MANSF. KULTURPFL. is synonim with *Boesenbergia pandurata* (ROXB.) SCHLTR. and also synonim with *Kaempferia pandurata* ROXB., belonging to the family of Zingiberaceae. It is a perennial herb distributed in some tropical countries including Indonesia, Malaysia, Myanmar, and Thailand. The local name in Indonesia is "Temu kunci" this plant is a common edible ingredient in many Asian countries. This herbal plant is also used as a traditional medicine to treat illnesses such as

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rheumatism, muscle pain, febrifuge, gout, gastrointestinal disorders, flatulence, carminative, stomach ache, dyspepsia, and peptic ulcer. In Indonesia, *B. rotunda* is typically used to prepare "jamu" a popular traditional tonic for women after childbirth as well as a beauty aid for teenage girls and to prevent leukorrhea. Essential oil of *B. rotunda* shows antifungal properties against *Aspergillus niger, A. fumigatus* and *Mucor*[7]. Jantan *et al*[8] reported that essential oil of *B. rotunda* shows antifungal properties against *Saccharomyces cerevisiae, Cryptococcus neoformans, Candida albicans*, *Candida tropicalis*, and *Torulopsis glabrata*. The research of Taweechaisupapong *et al.,*[9] showed the extract *B. pandurata* very effectively kills pathogenic bacteria *C. albicans* by *in vitro*. In addition *B. rotunda* contains essential oils and also secondary metabolites such as pinostrombin, pinocembrin, cardamonin, panduratin A, and alpinetin. Yanti *et al.,*[10] demonstrated that panduratin A showed a dose-dependent effect in preventing and reducing the biofilm. These results suggest that panduratin A is applicable as a natural anti-biofilm. *B. rotunda* has similarities with *Kaempferia rotunda*, but it contains more essential oils, and shows a characteristic odour.[7] Previous research showed that several chemical compounds or extracts of *K. rotunda* has antibacterial activity, anti-inflammatory, analgesic, antipyretic, anti-tumor, anti-HIV, and antioxidants.[11] To improve the stability, solubility and activity of extracts and pure compounds of *B. rotunda* it is necessarily developed into a nanoparticle product.

The principles of the design of nanoparticles, including nano-emulsions, dendrimers, nano-gold, liposomes, conjugated drug-carrier, antibody complexes, and magnetic nanoparticles, are mainly based on the synthetic, natural, or biological components, including the use of synthetic polymers, ion metals, oils and lipids as the based material carrier group (delivery system). However, the potential success of these particles in the clinic depends on the consideration of important parameters such as nanoparticle fabrication strategies, efficiency of use of the drug, the potential release of the drug, and most importantly, minimal toxicity of the carrier group.[12] In recent years, the number of products containing nanoparticles of materials has increased because it shows the physical and chemical properties that can be beneficial in drug delivery. Nanoparticles may consist of lipids, sugar, degradable or non-degradable polymers, metals and organic or inorganic compounds[13] [Some cancer drugs have been made in the form of nanoparticles and have been approved by the FDA, for example, Abraxane (FDA approved in January 2005), a breast cancer drug which is made from taxol (paclitaxel) tied with albumin and has a particle size of 130 nano meters. Doxil is also an ovarian cancer drug in the form of lipid nanoparticles with polyethylene glycol (PEG). In addition there is also a cancer drug with a cholesterol-lowering drug trade name Tricor (FDA approved December 2004) in the form of colloidal nano crystal.

The synthesis of nanoparticles can use several methods such as ionic gelation method, emulsifications method, coacervation or precipitation method, and spray drying method.[13] Ionic gelation method involves connecting a cross between polyelectrolyte in the presence of multivalent ion pairs. Ionic gelation is often followed by polyelectrolyte complexation with polyelectrolyte opposite. Formation the cross connecting bond will strengthen the mechanical strength of the particles formed. Polymer nanoparticles are usually made using biodegradable and hydrophilic polymers such as chitosan, gelatin and alginates.

Chitosan is a natural polysaccharide composed of [β (1 → 4) glucosamine (2-amino-2-deoxy-d-glucose) and N-acetyl-D-glucosamine (2-acetamido-2-deoxy-d-glucose)]. It is widely applied in the pharmaceutical industry, food and health. Chitosan has several beneficial properties; they are anti-microbial, wound healing, non-toxic, inexpensive, biocompatible, biodegradable, and water soluble. In the form of micro- or nanoparticles of chitosan they have many advantages that are non-toxic, unstable during use, high surface area, and can be used as a matrix for various types of drugs and extracts plants.[13] Chitosan has a capacity to increase epithelial permeation of macromolecules through the temporary opening of tight junctions of the epithelium. In addition, chitosan is known to be biocompatible and shows very low toxicity. Compared with many other natural polymers, chitosan has a positive charge and mucoadhesive.[14] The principle of this method is the existence of ionic interactions between the amino groups of a positively charged chitosan and substances negatively charged poly-anion to form a three-dimensional network structure. Cross-linker poly-anion which is the most widely used is sodium tripolyphosphate, because it is not toxic and has a multivalent. Chitosan nanoparticles can avoid the use of organic solvents as well as prevent the damage to the active ingredient of the drugs. Making products using chitosan nanoparticles as a drug delivery can be achieved as described by Wu *et al*.,[15]

Alginate is a linear polysaccharide that is soluble in water. This compound is extracted from brown seaweed and has a linear polymer structure composed by bonding (1-4) α-L-guluronic and β-D-mannuronic. Alginate has been reported to be mucoadhesive, biodegradable, and biocompatible and has the potential for a variety of pharmaceutical and biomedical applications such as drug delivery systems and cell encapsulation. Alginat micro- and nano-particles can be obtained easily by inducing gelation with calcium ions. It is an easy-gel which can be used to generate a pre-gel composed of aggregates which are very small particles of gel. A layer of polyelectrolyte complexes can be formed by the addition of an aqueous solution of polycationic such as Poly-L-lysine.[16]

This work has been made to produce nanoparticles of the ethanol extract of *B. rotunda* loaded with chitosan (product I), alginic acid (product II), and a combination of chitosan - alginic acid (product III). To make the product I they were reacted with sodium tripolyphosphate (Na-TPP) at various compositions. The product II were prepared by reacting alginic acid and calcium ions at various compositions, while the product III were prepared by reacting chitosan and alginic acid and then calcium ion was added to the mixture. Chitosan is a polymer positively charged of amino groups, whereas alginic acid is a polymer of negatively charged of carboxyl groups.

MATERIALS AND METHODS

Apparatus and reagent

General glassware, analytical balance, evaporator Buchi Rotavapor R-114, magnetic stirer, sentrifuge, Scanning electron microscopy (SEM, Jeol T-300), particles size analysis (PSA, Horiba 550), Zeta potential (Malvern Zetasizer, UK), refrigerator, Spectronic 20 (Genesys) were used in this work.

Ethanol, aquabidest, chitosan (low molecular weight, Sigma), Sodium Tripoliphosphat (Na-TPP, Sigma-Aldrich), Acetic acid (p.a. Sigma), Alginic acid (p.a. Sigma), calsium cloride (p.a. Sigma), Rhizome of *B. rotunda,* 2,2 diphenyl-1-picrylhydrazyl (DPPH, Aldrich), ascobic acid (Aldrich) were used in this work without further purification.

Preparation of ethanol extract of *B. rotunda*

The milled dried rhizoma of *B. rotunda* (5 kg) was maserated by ethanol at 24 hours at three times. The filtrate is separated by filtration, and evaporated using vacuum evaporator to dry to yield brown residue for about 147.6 g.

Preparation of nanoparticle product I

Nanoparticle chitosan produced by ethanol extract of *B. rotunda* was synthesized by ionic gelation. Ethanol extract of *B. rotunda* was dissolved in 35 mL ethanol and 35 mL aquadest. After homogen to the so-

lution was added 100 ml chitosan (dissolved in acetic acid 1% v/v) at various concentrations (0.08 – 0.12 % w/v), while mixing with magnetic stirer until homogen. The resulting solution was further added with 350 mL Na-TPP (0.01-0.02% w/v in aquadest) at various concentration, and was kept for complete dissolution by magnetic stirer at medium speed for 2 hours. The mixture was stabilized overnight at refrigerator. The nanoparticles were collected by centrifugation at 12.000 rpm for 15 minute. The precipitate were washed with distilled water repeatedly and were dried by a freeze dryer.

Preparation of nanoparticle product II

Ethanol extract *B. rotunda* was dissolved in 35 ml of ethanol and 35 mL of distilled water and to the solution was added 100 mL of alginic acid (dissolved in 0.1 M NaOH) at various concentrations (0.1 – 0.5 % w/v) below the magnetic stirrer until homogenous. Extract and alginic acid solution is then added a solution of calcium chloride at various concentrations (0.01 – 0.1 % w/v) slowly while stirring using a magnetic stirrer at low speed and mix stored for the complete dissolution with a magnetic stirrer at medium speed for 2 hours. The mixture was stabilized overnight at refrigerator. Nanoparticles were collected by centrifugation at 12.000 rpm for 15 minutes. The precipitate was washed with distilled water repeatedly and were dried by a freeze dryer.

Preparation of nanoparticle product III

Ethanol extract of *B. rotunda* was dissolved in a well stiring of 35 mL ethanol and 35 mL aquadest and then into the solution was added 50 mL alginic acid (dissolved in NaOH 0.1M) at various concentrations (0.05 - 0.1 % w/v) and 50 mL chitosan (dissolved in acetic acid 1%) at various concentration (0.05 - 0.1% w/v); and the mixture was allowed to stand until homogen. The resulting solution was further added with 350 mL calsium chloride (0.015% w/v in aquadest), and was kept for complete dissolution by magnetic stirer for 2 hours. The mixture was stabilized overnight at refrigerator. The nanoparticles were collected by centrifugation at 12.000 rpm for 15 minute. The precipitate were washed with distilled water repeatedly and then freshdryed for 24 hours.

All resulting nanoparticles were dried and stored at refrigerator. Yield of the nanoparticles were calculated by the formula:

$$\% \text{ yield} = \frac{[\text{weight of nanoparticles obtained}]}{[\text{weight of sample fraction} + \text{weight of chitosan/alginic acid used for synthesis}]} \times 100\%$$

The characterization of this product was analyzed in term of particle size, zeta potential, and SEM (Scanning Electron Microscopy).

Biological activity as Antioxidant

Antioxidant activity was analyzed by DPPH (2,2-diphenyl-1-picrylhydrazyl) method. This method used DPPH as the source of free radical. About 5 mL of the nanoparticle was mixed with 5 ml methanolic solution of DPPH (0.12 mM) and kept in dark at room temperature for 30 minutes. The DPPH scavenging activity was determined using spectronic 20 (Genesys) at 516 nm against DPPH solution as control. The samples were tested in triplicates. The antioxidant activity was calculated as percentage of DPPH that was decreased in comparison with the control, and the inhibition activity could be calculated to determine IC_{50}.

Statistical analysis

The data of all experiments were represented as Mean ± SD and were analyzed using Microsoft Excel (Redmond, WA) software.

RESULTS AND DISCUSSION

The synthesised nanoparticles of ethanol extract of *B. rotunda* was conducted with ionic gelation method using chitosan (Product I), alginic acid (Product II), and a combination of chitosan-alginic acid (Product III). This method are carried out at ambient temperature, and thus the preparation is relatively simple. Data of Table 1, 2 and 3 show particle size, zeta potential, yield, and physical properties of this products. In this work, the particle size was analyzed using PSA (Particle Size Analyser), it is the dynamic light scattering (DLS) system measurement. The instrument is capable of measuring particle size in the range of 1 nm to 6 μm at concentration up to 40% w/v.

The stability of nanoparticles was analised by zeta potential, that is to determine the surface charge of nanoparticles in solution or colloid. Nanoparticles have a surface charge that attracts a thin layer of ions of opposite charge to the nanoparticle surface. Two layers move with nanoparticles dispersed throughout the solution. Electrical potential on the second boundary layer called the zeta potential of particles and has a typical value between +100 mV to -100 mV. Zeta potential of nanoparticles with a value greater than +25 mV or less than -25 mV has a high degree of stability. Dispersions with a low zeta potential value will eventually aggregate due to Van Der Waal inter-particle attractions.[17] A colloidal solution containing a high percentage amount of nanoparticles would show the high value of the zeta potential, so that the solution will be difficult to form a precipitate. In this work nanoparticles produced by ethanol extract of *B. rotunda* loaded with chitosan (product I) show zeta potential positive charged, but alginic acid (product II) show zeta potential negatively charged. Product III contain components nanoparticles 29.7%, so the zeta potential not measured. Products with nanoparticles of less than 70% usually indicates a low zeta potential.

In the synthesis, the formation of nanoparticles of product I are influenced by the concentration ratio of the material forming the matrix. If the ratio is too low the product tend to form microparticles. Nanoparticles of product I are obtained at a concentration ratio of chitosan / Na-TPP 8: 1, with the precentage amount of nanoparticles 98.1%, and the size ranges of the nanoparticles were to be 389 to 877 nm, with a zeta potential of +41.87 mV. Previous research,[18] the nanoparticle of chitosan produced by chloroform fraction of *K. rotunda* were obtained at concentration ratio of chitosan / Na-TPP 10: 1 as much as 100%, and the size range of the nanoparticles were to be 172 to 877 nm, with a zeta potential of +28.06 to +38.03 mV. The difference is due to the composition and type of components in each of the different extracts, thus affecting the character of the nanoparticles produced. Similarly, other researcher has adjusted to get a chitosan/Na-TPP ratio of 6:1, and the nanoparticles thus were obtained to be in the range of 300–400 nm with a positive surface charge ranging from +54 to +25 mV.[19] However, the nanoparticle product is highly dependent on the deacetylation of chitosan used, because it involves gelation of the protonated amino group of chitosan.

The nanoparticles of product II was synthesized using calcium ion as a cross junction to form pore size of the gel. The concentration of Ca^{2+} has a significant effect on the stability and pore size of the gel. The nanoparticles of Product II can be optimally synthesized at a concentration of alginic acid/ Ca^{2+} ratio of 5: 1, with the precentage amount of 90.2 %, the range size of 197 to 877 nm, and with a zeta potential of -82.1 mV. When the concentration of alginic acid/ Ca^{2+} ratio was 2.5: 1, it resulted in the precentage amount of 95.2 %, but the size ranged from 339 to 877 nm, with a zeta potential of -72.1 mV, and the mean yield was relatively low. When the concentration of calsium ion is high the gel microparticles were formed.

In this work the synthesis of product III resulted in more micro-sized gel. Unfortunately, the optimal combination to produce nanoparticles of

Table 1: Synthesis nanoparticle product produced by ethanol extract of *B . rotunda* loaded chitosan (Product I)

Code Formula	Ext (g)	Chitosan (% w/v)	NaTPP (% w/v)	MeanYield (mg) Colour	% Nano Particle	Size (nm)	% Micro Particle	Size (µm)	Zeta Potensial (mV)
A1	1	0.1	0.02	334 ± 5.7; yellow brown	1.3	877	98.7	1005-1510	*
A2	1	0.2	0.02	444 ± 4.8; yellow brown	68.7	510-877	31.3	1005-1510	+26.83
A3	1	0.3	0.02	395 ± 3.3; yellow brown	0	-	100	1005-1729	*
A4	1	0.4	0.02	523 ± 4.6; yellow brown	1	766-877	99	1005-2269	*
A5	1	0.1	0.01	440 ± 10.1; yellow brown	75.8	389-877	24.2	1005-1151	+14.40
A6	1	0.08	0.01	501 ± 12.8, yellow brown	98.1	389-877	1.9	1005	+41.87
A7	1	0.09	0.01	380 ± 20.2, yellow brown	70.1	296-877	29.9	1005-1729	*
A8	1	0.11	0.01	478 ± 8.9, yellow brown	19.0	766-877	81.0	1005-1151	*
A9	1	0.12	0.01	413 ± 5.8, yellow brown	53.7	339-877	46.3	1005-1510	*

*if % nanoparticle <70%, zeta potential very low and not be measured

Table 2: Synthesis nanoparticle product produced by ethanol extract of *B. rotunda* loaded alginic acid and Ca^{2+} (Product II)

Code Formula	Ext (g)	Alg (% w/v)	Ca^{2+} (% w/v)	MeanYield (mg) Colour	% Nano Particle	Size (nm)	% Micro Particle	Size (µm)	Zeta Potensial (mV)
B1	1	0.1	0.1	569±1.3; yellow brown	0	-	100	2269-3409	*
B2	1	0.3	0.1	576±2.5; yellow brown	0	-	100	1005-3409	*
B3	1	0.5	0.1	894±0.9; yellow brown	0	-	100	3905-5122	*
B6	1	0.1	0.4	597±2.2, yellow brown	0	-	100	1318-6000	*
B5	1	0.1	0.3	637±2.5; yellow brown	0	-	100	1151-1318	*
B5	1	0.1	0.3	637±2.5; yellow brown	0	-	100	1151-1318	*
B7	1	0.1	0.01	167±3.8, yellow brown	80.8	226-877	19.2	1005-1318	-89.5
B8	1	0.1	0.015	227±4.5, yellow brown	83.3	259-877	16.7	1005-1981	-84.7
B9	1	0.1	0.02	246±3.2, yellow brown	90.2	197-877	9.8	1005-1151	-82.1
B10	1	0.1	0.03	228±2.3, yellow brown	65.5	259-877	34.5	1005-1510	*
B11	1	0.1	0.04	182±2.3, yellow brown	95.2	339-877	4.8	2269-3905	-72.1

*if % nanoparticle <70%, zeta potential very low and not be measured Ext = ethanol extract of *B. rotunda*; Alg = alginic acid

more than 80% was not successesful. This is due to the interaction of the two polymers which have an opposite charge and this tends to produce large porous gel. Furthermore the combination-nanoparticles of alginic acid /chitosan /ca^{2+}, and ratio of 6.7 : 2: 1, only resulted in the percentage of about 29.7 %, and the size range of 226 to 877 nm.

The morphology of nanoparticles produced by ethanol extract of *B. rotunda* were identified using optical microscope by scanning electron microscopy (SEM). For SEM analysis, the working distance was 10 mm, beam energy was 20.0 kV, spot size was 5.0, and magnification was 5000. The nanoparticles were loaded on a double sided carbon tape and put

Table 3: Synthesis nanoparticle product produced by ethanol extract of *B. rotunda* loaded Chitosan-alginic acid and Ca^{2+} (Product III)

Code Formula	Ext (g)	Alg (% w/v)	Chi (% w/v)	Ca^{2+} (% w/v)	MeanYield (mg) Colour	% Nano Particle	Size (nm)	% Micro Particle	Size (µm)
C1	1	0.1	0.05	0.015	592±2.2; yellow brown	0	-	100	2269-3409
C2	1	0.1	0.01	0.015	610±3.5; yellow brown	1.4	877	98.6	1005-6000
C3	1	0.1	0.03	0.015	427±3.4; yellow brown	29.7	226-877	70.3	1005-1729
C4	1	0.01	0.1	0.015	384±4.5; yellow brown	3.6	877	96.4	1005-6000
C5	1	0.05	0.1	0.015	504±1.8; yellow brown	0	-	100	1510-2269

Ext = ethanol extract of *B. rotunda*; Alg = alginic acid; Chi = Chitosan *if % nanoparticle <70%, zeta potential very low and not be measured

on studs before being examined by SEM. Figure 1 shows the morphology of nanoparticles product I, II, and III, the surfaces are spherical and smooth.

The DPPH assay was used to study the free-radical scavenging capacity of these products. The results is shown in Table 4. The antioxidant activity of all nanoparticles products showed IC_{50} of 153.27; 139.0; and 27.05 µg/mL respectively. The antioxidant activity of ethanol extract of *B. rotunda* shows IC_{50} of 92.75 µg/mL. Thereby, the nanoparticles of product I and II have antioxidant activity weaker than the starting material ethanol extract of *B. rotunda*, while the nanoparticles of product III shows higher activity than the starting material ethanol extract of *B. rotunda*.

Table 4: The Inhibition activity (IC_{50}) of nanoparticle product produced by ethanol extract from of *B. rotunda* and positive control as antioxidant

Sample Code	IC_{50}	Note
A6. Chitosan nanoparticle(98.1%)	153.27	Less active
B9. Alginic acid nanoparticle (90.2%)	99.14	active
B11. Alginic acid nanoparticle (95.2%)	139.0	Less active
C3. Chitosan-alginic acid nanoparticle (29.7%)	27.05	active
Ethanol extract from *B. rotunda*	92.2	active
Positive control (Ascobic acid)	3.77	Very active

IC_{50} > 100 µg/mL less active; 100-10 µg/mL active; < 10 µg/mL very active

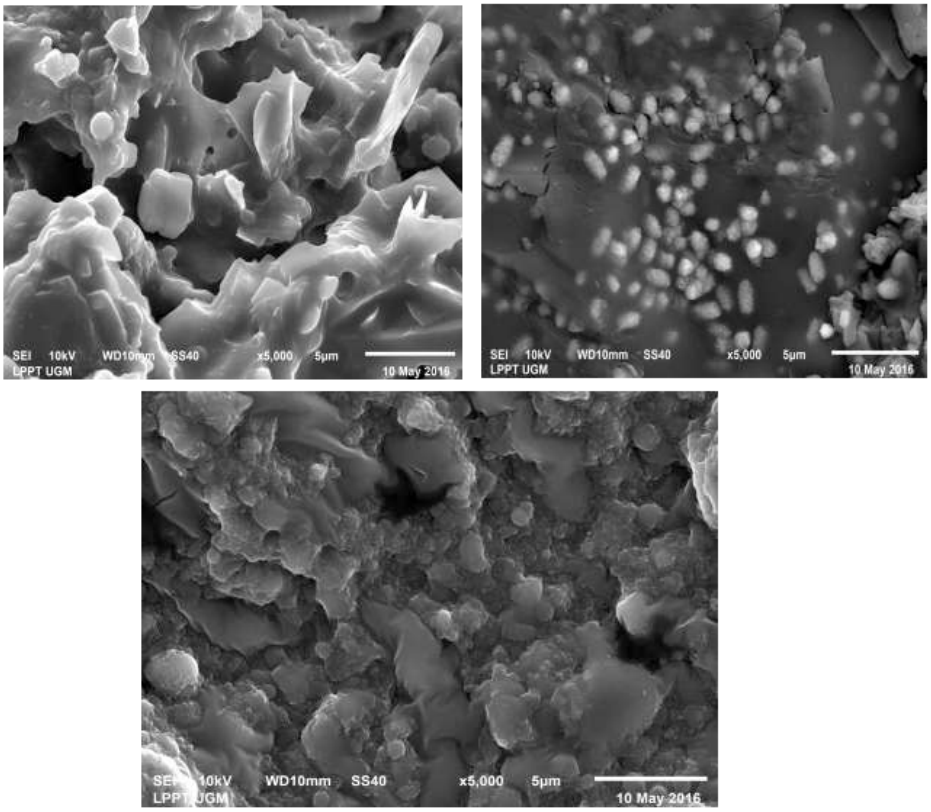

Figure 1: SEM of the nanoparticles (A) chitosan (product I); (B) Alginic acid (product II); and (C) Combination of chitosan-alginic acid (product III) produced by ethanol extract of *B. rotunda*.

CONFLICT OF INTEREST

We declare that we have no conflict of interest

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