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5. Title of Manuscript

ANTIBACTERIAL ACTIVITY OF NANOPARTICLES PRODUCED BY ETHANOL EXTRACT OF BOESENBERGIA ROTUNDA RHIZOME LOADED WITH CHITOSAN AND ALGINIC ACID AGAINST STREPTOCOCCUS MUTANS BY IN VITRO

6. Abstract

The objectives of this research is to analyze antibacterial activity of nanoparticles produced by ethanol extract Boesenbergia rotunda loaded with chitosan and alginic acid againts Streptococcus mutans. Antimicrobial activity of the extract was screened for activities againts pathogenic bacteria S. mutans by the disk-diffusion method. The assay was done in triplicate, and chloramphenicol was used as the positive control. Different concentrations, 500 to 0.5µg/mL, of each sample showed dose-dependant antibacterial activity. The results presented in this study showed the maximum of diameter zone of inhibition from nanoparticles produced by ethanol extract B. rotunda loaded with chitosan and alginic acid at concentration of 500 µg/mL, while ethanol extract of B. rotunda at concentration of 50 µg/mL, and chloramphenicol at 250 µg/mL. The optimal incubation time on the diameter zone of inhibition against S. mutans of each sample was 6 hours. The effect of incubation time on the diameter zone of inhibition against S. mutants showed that nanoparticles produced by ethanol extract B. rotunda loaded with chitosan and alginic acid were relatively more stable than by ethanol extract of B. rotunda. The minimum inhibitory concentration of each sample against S. Mutans was found to be 5 µg/mL.

7. Keywords (2-10)

nanoparticle; Alginic acid; chitosan; B. rotunda; antibacterial; Streptococcus mutans

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Editor
International Journal of Drug Delivery Technology
ISSN: 0975-4415
website: www.ijddt.com

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STREPTOCOCCUS MUTANS BY IN VITRO**

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Foundation project: This study was supported by an excellent research universities grant
(PUPT-IDB 2016-2017), Universitas Negeri Yogyakarta

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ABSTRACT

The objectives of this research is to analyze antibacterial activity of nanoparticles produced by ethanol extract *Boesenbergia rotunda* loaded with chitosan and alginic acid againts *Streptococcus mutans*. Antimicrobial activity of the extract was screened for activities againts pathogenic bacteria *S. mutans* by the disk-diffusion method. The assay was done in triplicate, and chloramphenicol was used as the positive control. Different concentrations, 500 to 0.5µg/mL, of each sample showed dose-dependant antibacterial activity. The results presented in this study showed the maximum of diameter zone of inhibition from nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid at concentration of 500 µg/mL, while ethanol extract of *B. rotunda* at concentration of 50 µg/mL, and chloramphenicol at 250 µg/mL. The optimal incubation time on the diameter zone of inhibition against *S. mutans* of each sample was 6 hours. The effect of incubation time on the diameter zone of inhibition against *S. mutants* showed that nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid were relatively more stable than by ethanol extract of *B. rotunda*. The minimum inhibitory concentration of each sample against *S. Mutans* was found to be 5 µg/mL.

Keyword: nanoparticle; Alginic acid; chitosan; B. rotunda; antibacterial; Streptococcus mutans

INTRODUCTION

Streptococcus mutans is one of the caries-causing microbes were many found in cariogenic biofilms or plaques. *S. mutans* was first isolated by Clark in 1924 from a carious human tooth¹. These bacteria play an important role in the metabolism of sucrose to lactic acid, which causes demineralization of tooth enamel. This bacterium is the most important bacteria as the cause of dental caries. Usually, to prevent dental caries or mouth disease we used antibiotics or antiseptics. However, the use of antibiotics often results in resistance, whereas the use of antiseptic, such as chlorhexidine which has side effects can change the color of the teeth and cause a sense disorder after use². Currently, the development of natural products as anti-microbial and antioxidants are safer and environmentally friendly. Thus, it is important to develop a phytochemical profile that represents the bioactive element of herbal medicine.

Boesenbergia rotunda (L.) MANSF. KULTURPFL (synonym with *Boesenbergia pandurata* (ROXB.) SCHLTR and also synonym with *Kaempferia pandurata* ROXB) is a perennial herb of the Zingiberaceae family mainly cultivated in 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 countries³. *B. rotunda* contains essential oils and also secondary metabolites such as pinostrobin, pinocembrin, cardamonin, panduratin A, and alpinetin⁴. Yoon⁵ showed that isopanduratin A and 4-hydroxypanduratin A isolated from *K. pandurata* are promising compounds that could be useful for treating hyperpigmentation as skin-whitening agents. The extract of *B. rotunda* is very effectively killing pathogenic bacteria *C. albicans* by in vitro⁶. Panduratin A showed a dose dependent effect in preventing and reducing the biofilm⁷.

Previous research has successfully synthesized nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginate⁸. The size range of the nanoparticles of chitosan were 389 to 877 nm, with a zeta potential of +41.87 mV, percentage nanoparticle

of 98.1%, and the nanoparticles of alginic acid were 197 to 877 nm, with a zeta potential of -82.1 mV, and percentage nanoparticle of 90.2%. The morphology of each nanoparticle product was spherical and a smooth surface. The nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid showed an antioxidant activity by DPPH (2,2-diphenyl-1-picrylhydrazyl) method⁸. In the present study, we evaluated the antibacterial effect of nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid against pathogenic bacteria *S. mutans*. In this study, we also tested the antibacterial activity of ethanol extract of *B. rotunda* and chloramphenicol as a positive control.

MATERIALS AND METHODS

Apparatus and reagent

General glassware, incubator, autoclave, LAF (Laminatory Air Flow), spectrophotometer UV-Vis, coloni counter, forceps, micro pipettes, shaker, water bath, deep freezer, ruler (millimeter scale), petri plate, and analytical balance were used in this work. Extract ethanol *B. rotunda*, nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid⁸, DMSO (dimethyl sulfoxide), chloramphenicol, Mueller-Hinton agar (MHA) was purchased from OXOID (Basingstoke, UK), Nutrient Broth (NB), Nutrient Agar (NA), plastic wrap, aluminium foil, paperdisk, ethanol, steril cotton swap, and aquadest were used in this work without further purification.

Plant Materials

Samples of the rizhoma of *B. rotunda* were collected in December 2015 from the Beringharjo market, Yogyakarta, Indonesia. The plant was identified by the Faculty of Biology, Gadjah Mada University, Indonesia and a voucher specimen (BR-01-2016) was deposited at the Organic Laboratory, Department of Chemistry Education, Universitas Negeri Yogyakarta, Indonesia.

Microorganism

The pathogenic bacterial isolates of *S. mutans* were obtained from the Faculty of Dentistry, Universitas Gadjah Mada, Indonesia. The microorganism were sub-cultured and stored in a semisolid medium (Mueller Hinton agar plates) at 4°C until needed.

Observation of growth curve of Streptococcus mutans

The growth curve of *S. mutans* was performed by preparing 25 mL Nutrient Broth (NB) media as starter and inoculation of *S. mutans* bacteria which has been previously rejuvenated, then stuck for 24 hours in a shaker at 120 rpm. The bacterial suspension of the starter was taken as much as 6 mL to be inoculated in 60 mL of Nutrient Broth medium and put in a shaker at 120 rpm for 48 hours. During shaking, the measurement of absorbance value was recorded every 3 hours using UV-Visible at 600 nm. The bacterial growth curve is made to determine the time at which the bacteria can grow rapidly in the logarithmic phase. In this phase, the nutritional needs of the bacteria will be met optimally so that bacteria can grow rapidly and can respond to external influences on it. Graph of the growth of *S. Mutans* was shown in Fig.1.

Antibacterial activity

Screening of antibacterial activity of samples was done using Kirby-Bauer test following agar diffusion method⁹. Pathogenic bacterial *S. mutans* were used in this study to determine the antimicrobial activity of the ethanol extracts *B. rotunda* and nanoparticle produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid. In the disc diffusion method, nutrient media was used as a culture media and the cavities were made aseptically over the bacterial culture on nutrient agar plates using borer and filled with standard chloramfenicol as positive control. The sample in DMSO and only DMSO as a negative control were incubated at 37°C for 24 hours, followed by observations every 6 hours. On every six-hour-observation after incubation, the zone of inhibition around the discs was measured in millimeter scale. Sample was dissolved in DMSO and was diluted from 500 to 0.5 µg/mL. All experiments used three discs and performed in triplicate round (Fig.2). Minimum inhibitory

concentration (MIC) values were determined by agar double dilution method. The MIC value of the sample was determined as the lowest concentration of the sample completely inhibited bacterial growth after 24 hrs of incubation at 37°C^{10,11}.

Statistical analysis

The data of all experiments were represented as Mean \pm SD and were analyzed with SPSS version of windows 16.0. The differences were considered significant at $p < 0.05$.

RESULT AND DISCUSSION

The antibacterial activity of the ethanolic extract of *B. rotunda* and nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid was examined against pathogenic bacteria *S. mutans* using Kirby-Bauer test following the agar diffusion method. The measurements of growth curve of *S. mutans* bacteria (Fig. 1) show the logarithmic phase at 3 to 15 hours, because at that time it shows a rapid increase of absorbance value. Observation in the diameter zone of inhibition around the discs of each sample was performed every 6 hours for 24 hours incubation. The data were then collected in Table 1. In this study the use of DMSO that was not diluted was to dissolve the sample, and the DMSO only was used as a negative control. The diameter zone of inhibition for DMSO after 6 hours was 7.98 ± 0.42 mm. These results suggest that each sample at various concentrations (0.5 to 500 $\mu\text{g/mL}$) showed a larger of zone inhibition, so the sample studied had an activity as antibacteria againsts *S. mutans*. Ethanol extract of *B. rotunda* and its nanoparticle products contain bioactive compounds such as panduratin A¹². Panduratin A has been reported to have the ability to reduce the biofilm of multispecies oral bacteria in vitro¹² and Methicillin-sensitive *Staphylococcus aureus* (MSSA), Methicillin-resistant coagulase-negative *Staphylococci* (MRCNS), *Bacillus subtilis*, and *Salmonella typhi*¹³⁻¹⁴.

In vitro antibacterial activity of ethanol extract of *B. rotunda* and its nanoparticle products were quantitatively investigated in terms of minimum inhibitory concentration (MIC).

From the data it also can be shown that the minimum inhibitory concentration (MIC) value of each sample against *S. mutans* is 5 µg/mL. At concentration of 0.5 µg/mL indicated a diameter zone of inhibition similar to DMSO (negative control). The MIC of sample was varied, depending on the type of bioactive compounds and antibiotic and the strain of bacteria tested. Furthermore, an early work reported that the ethanol extract of *B. rotunda* rhizome had antibacterial activity against MSSA, MRSA, Methicillin-resistant coagulase-negative *Staphylococci* (MRCNS), *Bacillus subtilis*, and *Salmonella typhi*, with MIC of 2-16 µg/mL¹³.

The measurement in diameter zone of inhibition from each sample was performed as 9 times, and the data shown is Mean ± SD. The result of statistical test using SPSS version of windows 16.0 shows that there was an effect of time to the diameter zone of inhibition against *S. mutans* (p <0.05). There was an effect of concentration to the diameter of zone of inhibition against *S. mutans* (p <0.05). There was an effect of sample type to the diameter zone of inhibition against *S. mutans* (p <0.05). The maximum zone inhibition of nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid was at concentration of 500 µg/mL, while the ethanol extract of *B. rotunda* was at concentration of 50 µg/mL. From this study it was also observed the effect of incubation time on diameter zone of inhibition showing the decrease of each sample (Fig.3). Observations were made every 6 hours for 24 hours. This indicated that all samples tested did not kill *S. mutans* bacteria but only inhibited their growth. The effect of incubation time on the diameter zone of inhibition against *S. mutants* shows that nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid are relatively more stable than ethanol extract of *B. rotunda*. Previous research showed that the bactericidal effect mechanism of chitosan-alginate nanoparticles was induced by *P. acnes* cell membrane, and that the antimicrobial activity of the nanoparticles was due to the chitosan and not the alginate¹⁶. The research of Bhawana¹⁷ showed that the aqueous dispersion of nanocurcumin was much more effective than curcumin against *Staphylococcus*

aureus, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Penicillium notatum*, and *Aspergillus niger*.

CONCLUSION

In conclusion, the results presented in this study showed that the maximum diameter zone of inhibition from nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid was at concentration of 500 µg/mL, while the ethanol extract of *B. rotunda* was at concentration of 50 µg/mL. The optimal incubation time on the diameter zone of inhibition against *S. mutans* of each sample was 6 hours. The effect of incubation time on the diameter zone of inhibition against *S. mutans* shows that the nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid were relatively more stable than ethanol extract of *B. rotunda*. The minimum inhibitory concentration of each sample against *S. mutans* was found to be 5 µg/mL.

ACKNOWLEDGEMENTS

We would like to thank Directorate of Higher Education, Indonesia for the research funding in an excellent research universities grant (PUPT-IDB) 2016-2017 and the Overseas Seminar Aid Program, Directorate General of Research and Development, Minister Research and Technology Directorate of Higher Education, Indonesia who has provided assistance to attend the ISTEK 2017 international seminar. We also express our gratitude to Prof.K.H. Sugiyarto Ph.D. from Dept. of Chem. Ed. Universitas Negeri Yogyakarta who has critical review on this manuscript.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest

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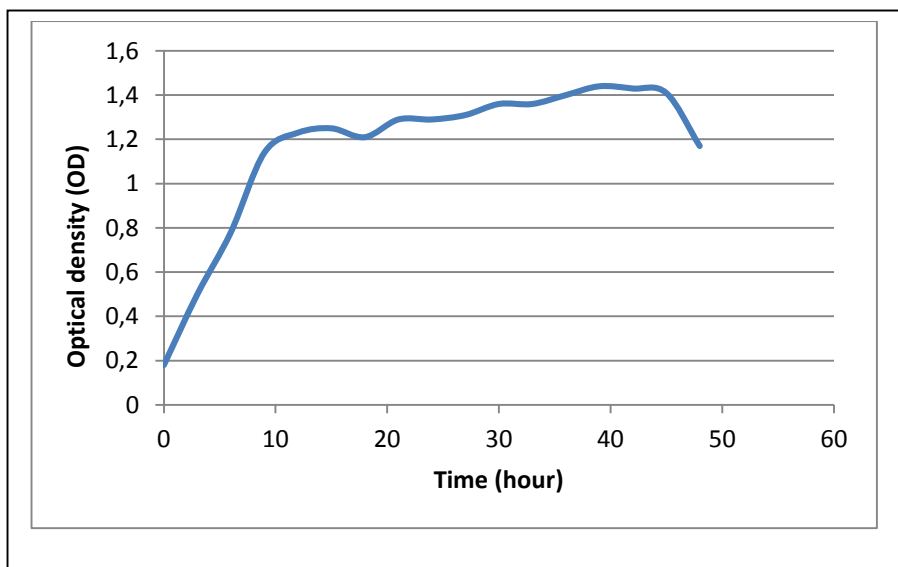
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Table 1: Diameter zone of inhibition ethanol extract of *B. rotunda* and nanoparticle produced of ethanol extract *B. rotunda* loaded of chitosan and alginic acid at various concentrations against *S. mutans*

No	sample	Concentration (µg/mL)	Diameter Zona of Inhibition (Mean±SD) mm*			
			Observed after			
			6 hour	12 hour	18 hour	24 hour
C	DMSO	-	7.98±0.42	8.12±0.61	7.46±0.39	8.17±0.69
1	Ethanol extract of <i>B. rotunda</i>	0.5	8.50±0.41	7.82±0.34	6.81±0.47	6.70±0.43
		5	9.46±0.56	8.97±0.73	7.18±0.53	6.88±0.41
		50	11.73±1.13	11.29±0.93	10.46±1.08	10.19±0.99
		250	9.96±0.43	9.76±0.36	8.98±0.45	8.56±0.62
		500	10.44±1.00	9.64±1.09	8.22±0.73	7.54±0.67
2	Nanoparticle produced of ethanol extract <i>B. rotunda</i> loaded of chitosan	0.5	9.20±1.09	7.87±0.33	7.60±0.39	7.33±0.29
		5	10.00±0.62	9.00±0.32	8.67±0.54	8.30±0.48
		50	10.22±0.34	9.66±0.44	8.94±0.26	8.64±0.74
		250	10.45±0.48	9.84±0.62	9.34±0.52	9.23±0.57
		500	10.66±0.98	9.98±0.72	9.68±0.37	8.62±0.65
3	Nanoparticle produced of ethanol extract <i>B. rotunda</i> loaded of alginic acid	0.5	7.89±0.60	8.09±0.62	8.26±0.69	8.03±0.60
		5	8.88±0.64	8.05 ±0.68	8.04±1.26	8.01±0.31
		50	8.13±0.47	8.56±0.63	7.91±0.91	7.37±2.83
		250	9.71±0.73	9.26±0.49	9.74±1.03	8.60±0.34
		500	10.54±1.04	10.29±1.23	8.62±0.59	8.12±1.27
4	Chloramphenicol (positive control)	0.5	8.21±0.46	7.98±0.44	7.42±0.45	7.18±0.69
		5	8.89±0.88	8.75±0.74	8.40±0.67	8.15±0.60
		50	11.54±0.75	11.05±0.44	10.17±0.47	7.37±0.90
		250	16.33±2.45	12.05±1.03	11.16±0.28	11.48±1.05
		500	10.19±0.49	9.50±0.87	8.98±0.85	9.16±1.40

*Average of 9 zones

*The result of statistical test using SPSS version of windows 16.0 shows that there was an effect of time to diameter zone of inhibition against *S. mutans* ($p < 0.05$); There was an effect of concentration on the diameter of zone of inhibition against *S. mutans* ($p < 0.05$); There was influence of sample type to zone diameter of inhibition against *S. mutans* ($p < 0.05$)



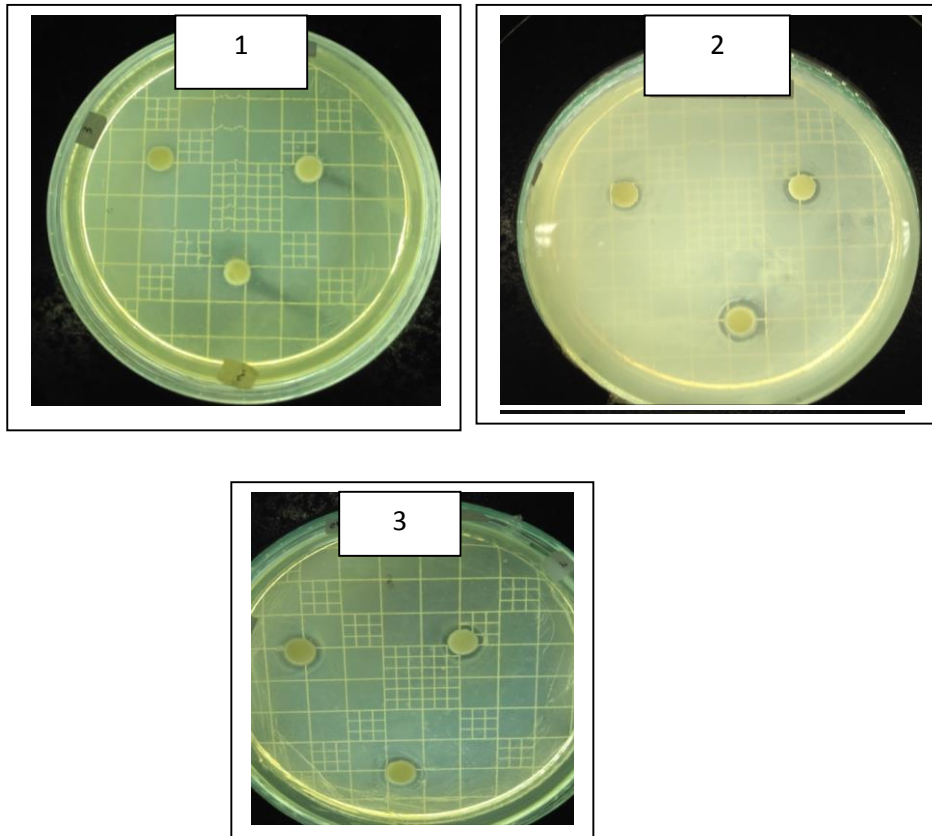


Figure 2. Screening of antibacterial activity of sample was performed using Kirby-Bauer test following the agar diffusion method. (1= DMSO (negative control); 2= sample; 3=Chloramphenicol (positive control))

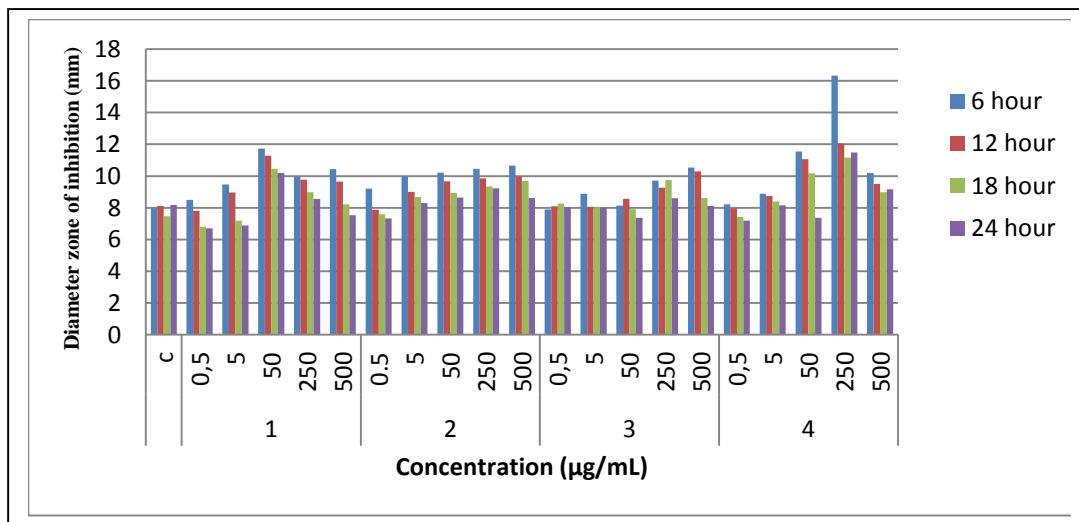


Figure 3. Graph of diameter zone of inhibition against *S. Mutans* sample at various concentrations and time Note : C=negative control (DMSO); 1= ethanol extract of *B. rotunda*; 2= Nanoparticle produced of ethanol extract *B. rotunda* loaded chitosan; 3= Nanoparticle produced of ethanol extract *B. rotunda* loaded alginic acid; 4= Chloramphenicol (positive control)

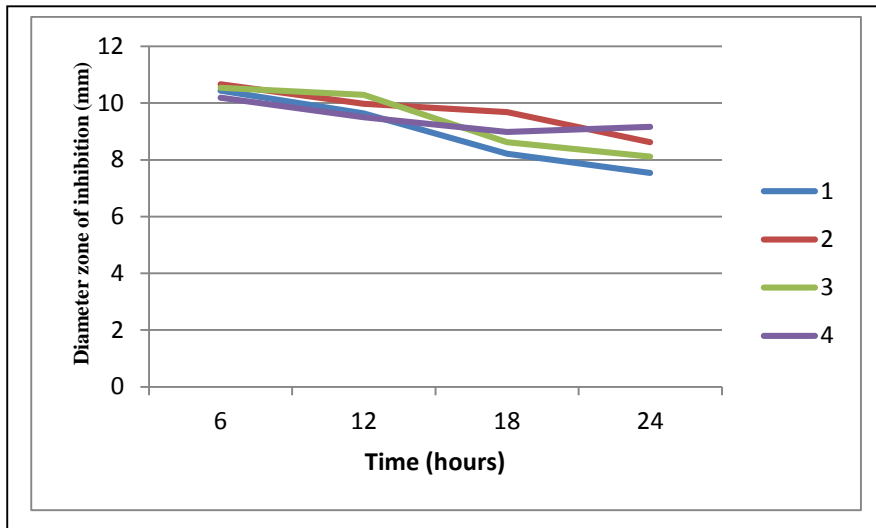


Figure 4. Graph of diameter zone of inhibition (mm) against *S. mutans* on various time Of sample at concentration of 500 µg/mL (1= ethanol extract of *B. rotunda*; 2 =Nanoparticle produced of ethanol extract *B. rotunda* loaded of chitosan; 3=Nanoparticle produced of ethanol extract *B. rotunda* loaded of alginic acid; 4=Chloramphenicol (positive control))

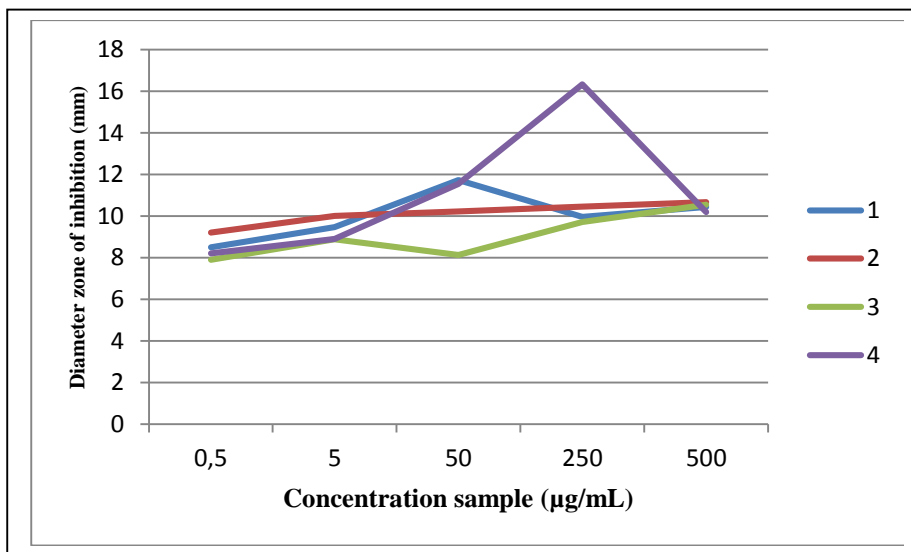


Figure 5. Graph of diameter zone of inhibition (mm) against *S. Mutans* in various concentration of sample at observation after 6 hours of sample (1= ethanol extract of *B. rotunda*; 2= nanoparticle produced of ethanol extract *B. rotunda* loaded of chitosan; 3=nanoparticle produced of ethanol extract *B. rotunda* loaded of alginic acid; 4=chloramphenicol (positive control))

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Title : Antibacterial activity of nanoparticle produced by ethanol extract of *Boesenbergia rotunda* rhizome loaded with chitosan and alginic acid against *Streptococcus mutans* by in vitro

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ANTIBACTERIAL ACTIVITY OF NANOPARTICLES PRODUCED BY ETHANOL EXTRACT OF
BOESENBERGIA ROTUNDA RHIZOME LOADED WITH CHITOSAN AND ALGINIC ACID AGAINST
STREPTOCOCCUS MUTANS BY IN VITRO

6. Abstract

The objectives of this research is to analyze antibacterial activity of nanoparticles produced by ethanol extract
Boesenbergia rotunda loaded with chitosan and alginic acid againts Streptococcus mutans. Antimicrobial

activity of the extract was screened for activities against pathogenic bacteria *S. mutans* by the disk-diffusion method. The assay was done in triplicate, and chloramphenicol was used as the positive control. Different concentrations, 500 to 0.5 µg/mL, of each sample showed dose-dependent antibacterial activity. The results presented in this study showed the maximum diameter zone of inhibition from nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginate at concentration of 500 µg/mL, while ethanol extract of *B. rotunda* at concentration of 50 µg/mL, and chloramphenicol at 250 µg/mL. The optimal incubation time on the diameter zone of inhibition against *S. mutans* of each sample was 6 hours. The effect of incubation time on the diameter zone of inhibition against *S. mutans* showed that nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginate were relatively more stable than by ethanol extract of *B. rotunda*. The minimum inhibitory concentration of each sample against *S. Mutans* was found to be 5 µg/mL.

7. Keywords (2-10)

nanoparticle; Alginate acid; chitosan; *B. rotunda*; antibacterial; *Streptococcus mutans*

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International Journal of Drug Delivery Technology

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**ANTIBACTERIAL ACTIVITY OF NANOPARTICLES PRODUCED
BY ETHANOL EXTRACT OF *BOESENBERGIA ROTUNDA* RHIZOME
LOADED WITH CHITOSAN AND ALGINIC ACID AGAINST
STREPTOCOCCUS MUTANS BY IN VITRO**

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Foundation project: This study was supported by an excellent research universities grant
(PUPT-IDB 2016-2017), Universitas Negeri Yogyakarta

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ABSTRACT

The objectives of this research is to analyze antibacterial activity of nanoparticles produced by ethanol extract *Boesenbergia rotunda* loaded with chitosan and alginic acid againts *Streptococcus mutans*. Antimicrobial activity of the extract was screened for activities againts pathogenic bacteria *S. mutans* by the disk-diffusion method. The assay was done in triplicate, and chloramphenicol was used as the positive control. Different concentrations, 500 to 0.5µg/mL, of each sample showed dose-dependant antibacterial activity. The results presented in this study showed the maximum of diameter zone of inhibition from nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid at concentration of 500 µg/mL, while ethanol extract of *B. rotunda* at concentration of 50 µg/mL, and chloramphenicol at 250 µg/mL. The optimal incubation time on the diameter zone of inhibition against *S. mutans* of each sample was 6 hours. The effect of incubation time on the diameter zone of inhibition against *S. mutants* showed that nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid were relatively more stable than by ethanol extract of *B. rotunda*. The minimum inhibitory concentration of each sample against *S. mutans* was found to be 5 µg/mL.

Keyword: nanoparticle; Alginic acid; chitosan; B. rotunda; antibacterial; Streptococcus mutans

INTRODUCTION

Streptococcus mutans is one of the caries-causing microbes were many found in cariogenic biofilms or plaques. *S. mutans* was first isolated by Clark in 1924 from a carious human tooth¹. These bacteria play an important role in the metabolism of sucrose to lactic acid, which causes demineralization of tooth enamel. This bacterium is the most important bacteria as the cause of dental caries. Usually, to prevent dental caries or mouth disease we used antibiotics or antiseptics. However, the use of antibiotics often results in resistance, whereas the use of antiseptic, such as chlorhexidine which has side effects can change the color of the teeth and cause a sense disorder after use². Currently, the development of natural products as anti-microbial and antioxidants are safer and environmentally friendly. Thus, it is important to develop a phytochemical profile that represents the bioactive element of herbal medicine.

Boesenbergia rotunda (L.) MANSF. KULTURPFL (synonym with *Boesenbergia pandurata* (ROXB.) SCHLTR and also synonym with *Kaempferia pandurata* ROXB) is a perennial herb of the Zingiberaceae family mainly cultivated in 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 countries³. *B. rotunda* contains essential oils and also secondary metabolites such as pinostrobin, pinocembrin, cardamonin, panduratin A, and alpinetin⁴. Yoon⁵ showed that isopanduratin A and 4-hydroxypanduratin A isolated from *K. pandurata* are promising compounds that could be useful for treating hyperpigmentation as skin-whitening agents. The extract of *B. rotunda* is very effectively killing pathogenic bacteria *C. albicans* by in vitro⁶. Panduratin A showed a dose dependent effect in preventing and reducing the biofilm⁷.

Previous research has successfully synthesized nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid⁸. The size range of the nanoparticles of chitosan were 389 to 877 nm, with a zeta potential of +41.87 mV, percentage nanoparticle

of 98.1%, and the nanoparticles of alginic acid were 197 to 877 nm, with a zeta potential of -82.1 mV, and percentage nanoparticle of 90.2%. The morphology of each nanoparticle product was spherical and a smooth surface. The nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid showed an antioxidant activity by DPPH (2,2-diphenyl-1-picrylhydrazyl) method⁸. In the present study, we evaluated the antibacterial effect of nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid against pathogenic bacteria *S. mutans*. In this study, we also tested the antibacterial activity of ethanol extract of *B. rotunda* and chloramphenicol as a positive control.

MATERIALS AND METHODS

Apparatus and reagent

General glassware, incubator, autoclave, LAF (Laminatory Air Flow), spectrophotometer UV-Vis, coloni counter, forceps, micro pipettes, shaker, water bath, deep freezer, ruler (millimeter scale), petri plate, and analytical balance were used in this work. Extract ethanol *B. rotunda*, nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid⁸, DMSO (dimethyl sulfoxide), chloramphenicol, Mueller-Hinton agar (MHA) was purchased from OXOID (Basingstoke, UK), Nutrient Broth (NB), Nutrient Agar (NA), plastic wrap, aluminium foil, paperdisk, ethanol, steril cotton swap, and aquadest were used in this work without further purification.

Plant Materials

Samples of the rizhoma of *B. rotunda* were collected in December 2015 from the Beringharjo market, Yogyakarta, Indonesia. The plant was identified by the Faculty of Biology, Gadjah Mada University, Indonesia and a voucher specimen (BR-01-2016) was deposited at the Organic Laboratory, Department of Chemistry Education, Universitas Negeri Yogyakarta, Indonesia.

Microorganism

The pathogenic bacterial isolates of *S. mutans* were obtained from the Faculty of Dentistry, Universitas Gadjah Mada, Indonesia. The microorganism were sub-cultured and stored in a semisolid medium (Mueller Hinton agar plates) at 4°C until needed.

Observation of growth curve of Streptococcus mutans

The growth curve of *S. mutans* was performed by preparing 25 mL Nutrient Broth (NB) media as starter and inoculation of *S. mutans* bacteria which has been previously rejuvenated, then stuck for 24 hours in a shaker at 120 rpm. The bacterial suspension of the starter was taken as much as 6 mL to be inoculated in 60 mL of Nutrient Broth medium and put in a shaker at 120 rpm for 48 hours. During shaking, the measurement of absorbance value was recorded every 3 hours using UV-Visible at 600 nm. The bacterial growth curve is made to determine the time at which the bacteria can grow rapidly in the logarithmic phase. In this phase, the nutritional needs of the bacteria will be met optimally so that bacteria can grow rapidly and can respond to external influences on it. Graph of the growth of *S. mutans* was shown in Fig.1.

Antibacterial activity

Screening of antibacterial activity of samples was done using Kirby-Bauer test following agar diffusion method⁹. Pathogenic bacterial *S. mutans* were used in this study to determine the antimicrobial activity of the ethanol extracts *B. rotunda* and nanoparticle produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid. In the disc diffusion method, nutrient media was used as a culture media and the cavities were made aseptically over the bacterial culture on nutrient agar plates using borer and filled with standard chloramfenicol as positive control. The sample in DMSO and only DMSO as a negative control were incubated at 37°C for 24 hours, followed by observations every 6 hours. On every six-hour-observation after incubation, the zone of inhibition around the discs was measured in millimeter scale. Sample was dissolved in DMSO and was diluted from 500 to 0.5 µg/mL. All experiments used three discs and performed in triplicate round (Fig.2). Minimum inhibitory

concentration (MIC) values were determined by agar double dilution method. The MIC value of the sample was determined as the lowest concentration of the sample completely inhibited bacterial growth after 24 hrs of incubation at 37°C^{10,11}.

Statistical analysis

The data of all experiments were represented as Mean \pm SD and were analyzed with SPSS version of windows 16.0. The differences were considered significant at $p < 0.05$.

RESULT AND DISCUSSION

The antibacterial activity of the ethanolic extract of *B. rotunda* and nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid was examined against pathogenic bacteria *S. mutans* using Kirby-Bauer test following the agar diffusion method. The measurements of growth curve of *S. mutans* bacteria (Fig. 1) show the logarithmic phase at 3 to 15 hours, because at that time it shows a rapid increase of absorbance value. Observation in the diameter zone of inhibition around the discs of each sample was performed every 6 hours for 24 hours incubation. The data were then collected in Table 1. In this study the use of DMSO that was not diluted was to dissolve the sample, and the DMSO only was used as a negative control. The diameter zone of inhibition for DMSO after 6 hours was 7.98 ± 0.42 mm. These results suggest that each sample at various concentrations (0.5 to 500 $\mu\text{g/mL}$) showed a larger of zone inhibition, so the sample studied had an activity as antibacteria againsts *S. mutans*. Ethanol extract of *B. rotunda* and its nanoparticle products contain bioactive compounds such as panduratin A¹². Panduratin A has been reported to have the ability to reduce the biofilm of multispecies oral bacteria in vitro¹² and Methicillin-sensitive *Staphylococcus aureus* (MSSA), Methicillin-resistant coagulase-negative *Staphylococci* (MRCNS), *Bacillus subtilis*, and *Salmonella typhi*¹³⁻¹⁴.

In vitro antibacterial activity of ethanol extract of *B. rotunda* and its nanoparticle products were quantitatively investigated in terms of minimum inhibitory concentration (MIC).

From the data it also can be shown that the minimum inhibitory concentration (MIC) value of each sample against *S. mutans* is 5 µg/mL. At concentration of 0.5 µg/mL indicated a diameter zone of inhibition similar to DMSO (negative control). The MIC of sample was varied, depending on the type of bioactive compounds and antibiotic and the strain of bacteria tested. Furthermore, an early work reported that the ethanol extract of *B. rotunda* rhizome had antibacterial activity against MSSA, MRSA, Methicillin-resistant coagulase-negative *Staphylococci* (MRCNS), *Bacillus subtilis*, and *Salmonella typhi*, with MIC of 2-16 µg/mL¹³.

The measurement in diameter zone of inhibition from each sample was performed as 9 times, and the data shown is Mean ± SD. The result of statistical test using SPSS version of windows 16.0 shows that there was an effect of time to the diameter zone of inhibition against *S. mutans* (p <0.05). There was an effect of concentration to the diameter of zone of inhibition against *S. mutans* (p <0.05). There was an effect of sample type to the diameter zone of inhibition against *S. mutans* (p <0.05). The maximum zone inhibition of nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid was at concentration of 500 µg/mL, while the ethanol extract of *B. rotunda* was at concentration of 50 µg/mL. From this study it was also observed the effect of incubation time on diameter zone of inhibition showing the decrease of each sample (Fig.3). Observations were made every 6 hours for 24 hours. This indicated that all samples tested did not kill *S. mutans* bacteria but only inhibited their growth. The effect of incubation time on the diameter zone of inhibition against *S. mutants* shows that nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid are relatively more stable than ethanol extract of *B. rotunda*. Previous research showed that the bactericidal effect mechanism of chitosan-alginate nanoparticles was induced by *P. acnes* cell membrane, and that the antimicrobial activity of the nanoparticles was due to the chitosan and not the alginate¹⁶. The research of Bhawana¹⁷ showed that the aqueous dispersion of nanocurcumin was much more effective than curcumin against *Staphylococcus*

aureus, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Penicillium notatum*, and *Aspergillus niger*.

CONCLUSION

In conclusion, the results presented in this study showed that the maximum diameter zone of inhibition from nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid was at concentration of 500 µg/mL, while the ethanol extract of *B. rotunda* was at concentration of 50 µg/mL. The optimal incubation time on the diameter zone of inhibition against *S. mutans* of each sample was 6 hours. The effect of incubation time on the diameter zone of inhibition against *S. mutans* shows that the nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid were relatively more stable than ethanol extract of *B. rotunda*. The minimum inhibitory concentration of each sample against *S. mutans* was found to be 5 µg/mL.

ACKNOWLEDGEMENTS

We would like to thank Directorate of Higher Education, Indonesia for the research funding in an excellent research universities grant (PUPT-IDB) 2016-2017 and the Overseas Seminar Aid Program, Directorate General of Research and Development, Minister Research and Technology Directorate of Higher Education, Indonesia who has provided assistance to attend the ISTEK 2017 international seminar. We also express our gratitude to Prof.K.H. Sugiyarto Ph.D. from Dept. of Chem. Ed. Universitas Negeri Yogyakarta who has critical review on this manuscript.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest

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Table 1: Diameter zone of inhibition ethanol extract of *B. rotunda* and nanoparticle produced of ethanol extract *B. rotunda* loaded of chitosan and alginic acid at various concentrations against *S. mutans*

No	sample	Concentration (µg/mL)	Diameter Zona of Inhibition (Mean±SD) mm*			
			Observed after			
			6 hour	12 hour	18 hour	24 hour
C	DMSO	-	7.98±0.42	8.12±0.61	7.46±0.39	8.17±0.69
1	Ethanol extract of <i>B. rotunda</i>	0.5	8.50±0.41	7.82±0.34	6.81±0.47	6.70±0.43
		5	9.46±0.56	8.97±0.73	7.18±0.53	6.88±0.41
		50	11.73±1.13	11.29±0.93	10.46±1.08	10.19±0.99
		250	9.96±0.43	9.76±0.36	8.98±0.45	8.56±0.62
		500	10.44±1.00	9.64±1.09	8.22±0.73	7.54±0.67
2	Nanoparticle produced of ethanol extract <i>B. rotunda</i> loaded of chitosan	0.5	9.20±1.09	7.87±0.33	7.60±0.39	7.33±0.29
		5	10.00±0.62	9.00±0.32	8.67±0.54	8.30±0.48
		50	10.22±0.34	9.66±0.44	8.94±0.26	8.64±0.74
		250	10.45±0.48	9.84±0.62	9.34±0.52	9.23±0.57
		500	10.66±0.98	9.98±0.72	9.68±0.37	8.62±0.65
3	Nanoparticle produced of ethanol extract <i>B. rotunda</i> loaded of alginic acid	0.5	7.89±0.60	8.09±0.62	8.26±0.69	8.03±0.60
		5	8.88±0.64	8.05 ±0.68	8.04±1.26	8.01±0.31
		50	8.13±0.47	8.56±0.63	7.91±0.91	7.37±2.83
		250	9.71±0.73	9.26±0.49	9.74±1.03	8.60±0.34
		500	10.54±1.04	10.29±1.23	8.62±0.59	8.12±1.27
4	Chloramphenicol (positive control)	0.5	8.21±0.46	7.98±0.44	7.42±0.45	7.18±0.69
		5	8.89±0.88	8.75±0.74	8.40±0.67	8.15±0.60
		50	11.54±0.75	11.05±0.44	10.17±0.47	7.37±0.90
		250	16.33±2.45	12.05±1.03	11.16±0.28	11.48±1.05
		500	10.19±0.49	9.50±0.87	8.98±0.85	9.16±1.40

*Average of 9 zones

*The result of statistical test using SPSS version of windows 16.0 shows that there was an effect of time to diameter zone of inhibition against *S. mutans* ($p < 0.05$); There was an effect of concentration on the diameter of zone of inhibition against *S. mutans* ($p < 0.05$); There was influence of sample type to zone diameter of inhibition against *S. mutans* ($p < 0.05$)

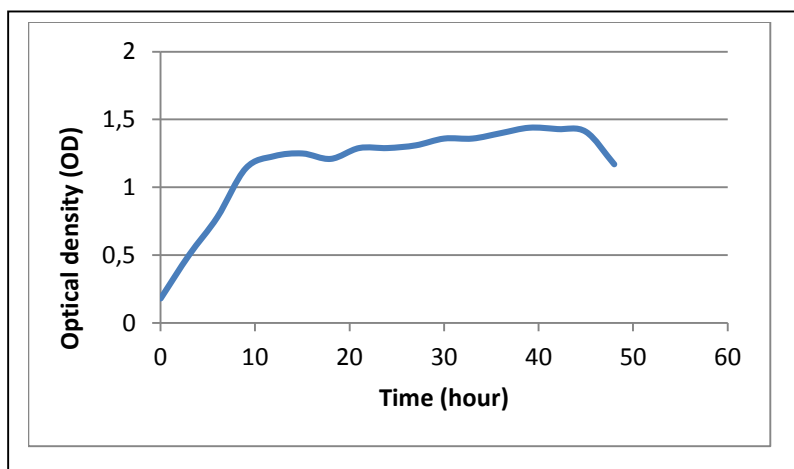


Figure 1. The growth curve of *Streptococcus mutans*

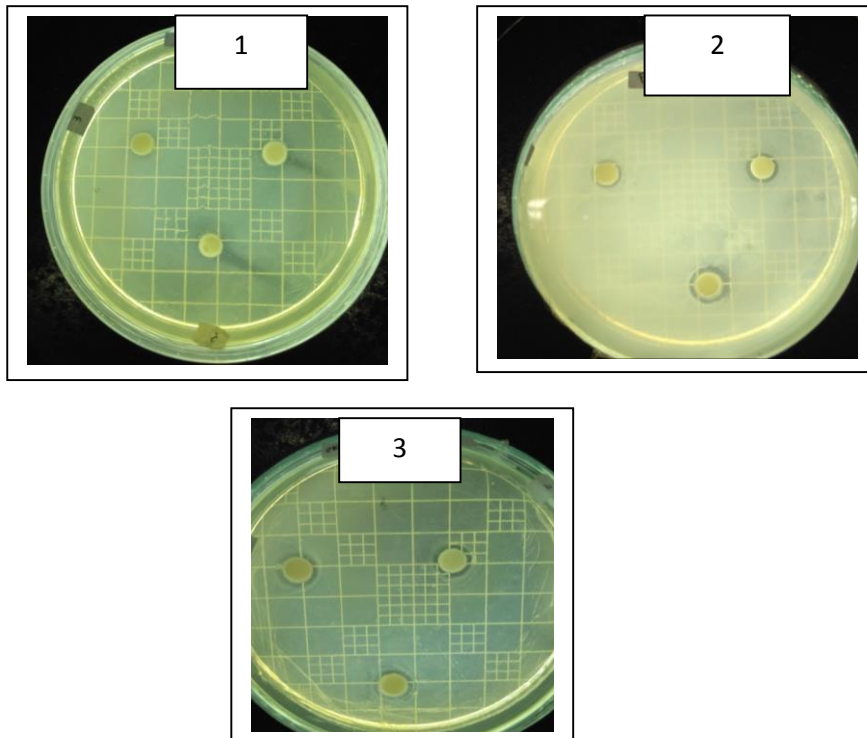


Figure 2. Screening of antibacterial activity of sample was performed using Kirby-Bauer test following the agar diffusion method. (1= DMSO (negative control); 2= sample; 3=Chloramphenicol (positive control))

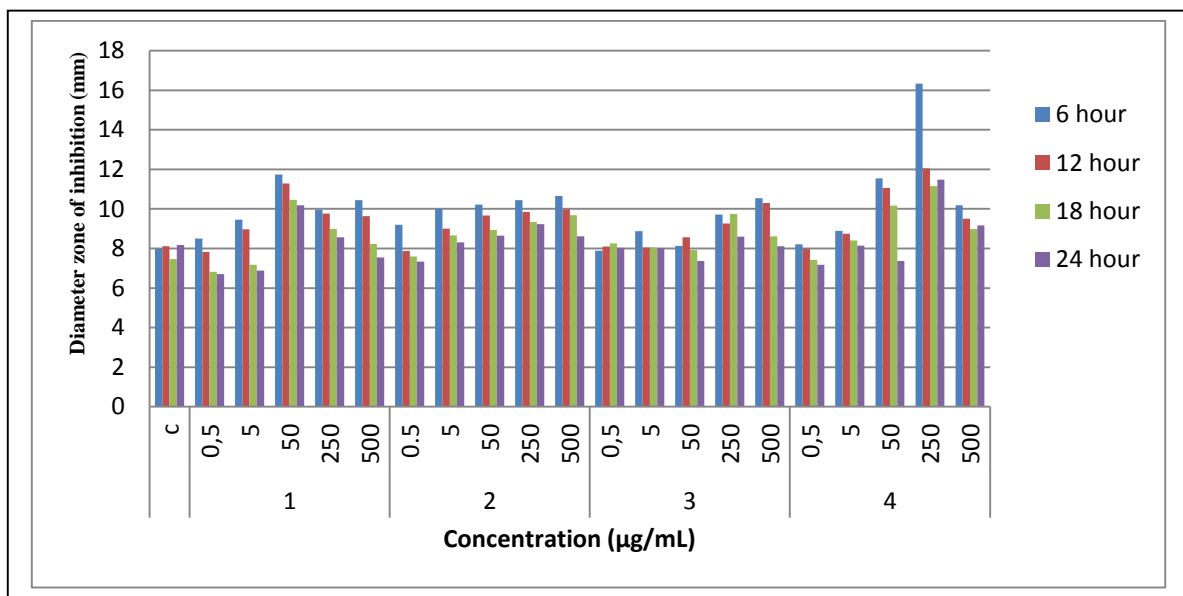


Figure 3. Graph of diameter zone of inhibition against *S. mutans* sample at various concentrations and time

Note : C= negative control (DMSO); 1= ethanol extract of *B. rotunda*; 2= Nanoparticle produced of ethanol extract *B. rotunda* loaded chitosan; 3= Nanoparticle produced of ethanol extract *B. rotunda* loaded alginic acid; 4= Chloramphenicol (positive control)

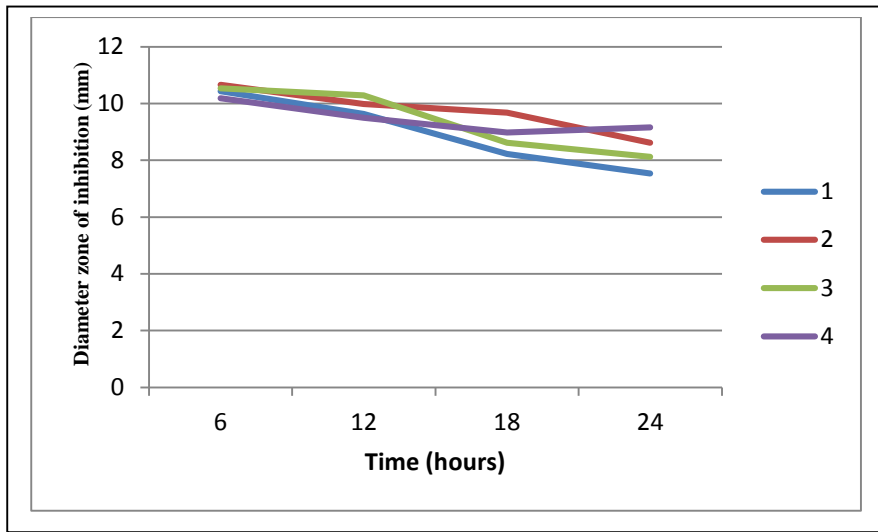


Figure 4. Graph of diameter zone of inhibition (mm) against *S. mutans* on various time Of sample at concentration of 500 $\mu\text{g}/\text{mL}$ (1= ethanol extract of *B. rotunda*; 2 =Nanoparticle produced of ethanol extract *B. rotunda* loaded of chitosan; 3=Nanoparticle produced of ethanol extract *B. rotunda* loaded of alginate acid; 4=Chloramphenicol (positive control))

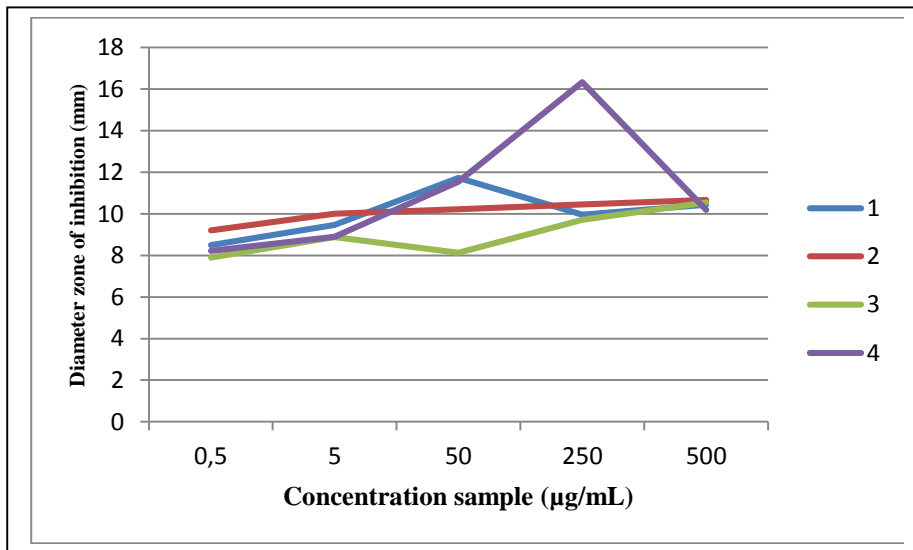


Figure 5. Graph of diameter zone of inhibition (mm) against *S. mutans* in various concentration of sample at observation after 6 hours of sample (1= ethanol extract of *B. rotunda*; 2= nanoparticle produced of ethanol extract *B. rotunda* loaded of chitosan; 3=nanoparticle produced of ethanol extract *B. rotunda* loaded of alginate acid; 4=chloramphenicol (positive control))



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Antibacterial Activity of Nanoparticles Produced by Ethanol Extract of *Boesenbergia rotunda* Rhizome Loaded with Chitosan and Alginic Acid Against *Streptococcus mutans* by In Vitro

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Received: 5th July, 17; Revised 14th July, 17, Accepted: 12th September, 7; Available Online: 25th September, 2017

ABSTRACT

The objectives of this research is to analyze antibacterial activity of nanoparticles produced by ethanol extract *Boesenbergia rotunda* loaded with chitosan and alginic acid against *Streptococcus mutans*. Antimicrobial activity of the extract was screened for activities against pathogenic bacteria *S. mutans* by the disk-diffusion method. The assay was done in triplicate, and chloramphenicol was used as the positive control. Different concentrations, 500 to 0.5 µg/mL, of each sample showed dose-dependant antibacterial activity. The results presented in this study showed the maximum of diameter zone of inhibition from nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid at concentration of 500 µg/mL, while ethanol extract of *B. rotunda* at concentration of 50 µg/mL, and chloramphenicol at 250 µg/mL. The optimal incubation time on the diameter zone of inhibition against *S. mutans* of each sample was 6 hours. The effect of incubation time on the diameter zone of inhibition against *S. mutans* showed that nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid were relatively more stable than by ethanol extract of *B. rotunda*. The minimum inhibitory concentration of each sample against *S. mutans* was found to be 5 µg/mL.

Keyword: nanoparticle; Alginic acid; chitosan; *B. rotunda*; antibacterial; *Streptococcus mutans*.

INTRODUCTION

Streptococcus mutans is one of the caries-causing microbes were many found in cariogenic biofilms or plaques. *S. mutans* was first isolated by Clark in 1924 from a carious human tooth¹. These bacteria play an important role in the metabolism of sucrose to lactic acid, which causes demineralization of tooth enamel. This bacterium is the most important bacteria as the cause of dental caries. Usually, to prevent dental caries or mouth disease we used antibiotics or antiseptics. However, the use of antibiotics often results in resistance, whereas the use of antiseptic, such as chlorhexidine which has side effects can change the color of the teeth and cause a sense disorder after use². Currently, the development of natural products as antimicrobial and antioxidants are safer and environmentally friendly. Thus, it is important to develop a phytochemical profile that represents the bioactive element of herbal medicine.

Boesenbergia rotunda (L.) MANSF. KULTURPFL (synonym with *Boesenbergia pandurata* (ROXB.) SCHLTR and also synonym with *Kaempferia pandurata* ROXB) is a perennial herb of the Zingiberaceae family mainly cultivated in 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 countries³. *B. rotunda* contains essential oils and also secondary metabolites such

as pinostrobin, pinocembrin, cardamomin, panduratin A, and alpinetin⁴. Yoon⁵ showed that isopanduratin A and 4-hydroxypanduratin A isolated from *K. pandurata* are promising compounds that could be useful for treating hyperpigmentation as skin-whitening agents. The extract of *B. rotunda* is very effectively killing pathogenic bacteria *C. albicans* by in vitro⁶. Panduratin A showed a dose dependent effect in preventing and reducing the biofilm⁷. Previous research has successfully synthesized nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid⁸. The size range of the nanoparticles of chitosan were 389 to 877 nm, with a zeta potential of +41.87 mV, percentage nanoparticle of 98.1%, and the nanoparticles of alginic acid were 197 to 877 nm, with a zeta potential of -82.1 mV, and percentage nanoparticle of 90.2%. The morphology of each nanoparticle product was spherical and a smooth surface. The nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid showed an antioxidant activity by DPPH (2,2-diphenyl-1-picrylhydrazyl) method⁸. In the present study, we evaluated the antibacterial effect of nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid against pathogenic bacteria *S. mutans*. In this study, we also tested the antibacterial activity of ethanol extract of *B. rotunda* and chloramphenicol as a positive control.

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MATERIALS AND METHODS

Apparatus and reagent

General glassware, incubator, autoclave, LAF (Laminatory Air Flow), spectrophotometer UV-Vis, coloni counter, forceps, micro pipettes, shaker, water bath, deep freezer, ruler (millimeter scale), petri plate, and analytical balance were used in this work. Extract ethanol *B. rotunda*, nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid⁸, DMSO (dimethyl sulfoxide), chloramphenicol, Mueller-Hinton agar (MHA) was purchased from OXOID (Basingstoke, UK), Nutrient Broth (NB), Nutrient Agar (NA), plastic wrap, aluminium foil, paperdisk, ethanol, steril cotton swap, and aquadest were used in this work without further purification.

Plant Materials

Samples of the rhizoma of *B. rotunda* were collected in December 2015 from the Beringharjo market, Yogyakarta, Indonesia. The plant was identified by the Faculty of Biology, Gadjah Mada University, Indonesia and a voucher specimen (BR-01-2016) was deposited at the Organic Laboratory, Department of Chemistry Education, Universitas Negeri Yogyakarta, Indonesia.

Microorganism

The pathogenic bacterial isolates of *S. mutans* were obtained from the Faculty of Dentistry, Universitas Gadjah Mada, Indonesia. The microorganism were sub-cultured and stored in a semisolid medium (Mueller Hinton agar plates) at 4°C until needed.

Observation of growth curve of *Streptococcus mutans*

The growth curve of *S. mutans* was performed by preparing 25 mL Nutrient Broth (NB) media as starter and inoculation of *S. mutans* bacteria which has been previously rejuvenated, then stuck for 24 hours in a shaker at 120 rpm. The bacterial suspension of the starter was taken as much as 6 mL to be inoculated in 60 mL of Nutrient Broth medium and put in a shaker at 120 rpm for 48 hours. During shaking, the measurement of absorbance value was recorded every 3 hours using UV-Visible at 600 nm. The bacterial growth curve is made to determine the time at which the bacteria can grow rapidly in the logarithmic phase. In this phase, the nutritional needs of the bacteria will be met optimally so that bacteria can grow rapidly and can respond to external influences on it. Graph of the growth of *S. mutans* was shown in Fig.1.

Antibacterial activity

Screening of antibacterial activity of samples was done using Kirby-Bauer test following agar diffusion method⁹. Pathogenic bacterial *S. mutans* were used in this study to determine the antimicrobial activity of the ethanol extracts *B. rotunda* and nanoparticle produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid. In the disc diffusion method, nutrient media was used as a culture media and the cavities were made aseptically over the bacterial culture on nutrient agar plates using borer and filled with standard chloramphenicol as positive control. The sample in DMSO and only DMSO as a negative control were incubated at 37°C for 24 hours, followed by observations every 6 hours. On every six-hour-observation after incubation, the zone of inhibition around the discs was measured in millimeter scale. Sample was dissolved

in DMSO and was diluted from 500 to 0.5 µg/mL. All experiments used three discs and performed in triplicate round (Fig.2). Minimum inhibitory concentration (MIC) values were determined by agar double dilution method. The MIC value of the sample was determined as the lowest concentration of the sample completely inhibited bacterial growth after 24 hrs of incubation at 37°C^{10,11}.

Statistical analysis

The data of all experiments were represented as Mean ± SD and were analyzed with SPSS version of windows 16.0. The differences were considered significant at p<0.05.

RESULT AND DISCUSSION

The antibacterial activity of the ethanolic extract of *B. rotunda* and nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid was examined against pathogenic bacteria *S. mutans* using Kirby-Bauer test following the agar diffusion method. The measurements of growth curve of *S. mutans* bacteria (Fig. 1) show the logarithmic phase at 3 to 15 hours, because at that time it shows a rapid increase of absorbance value. Observation in the diameter zone of inhibition around the discs of each sample was performed every 6 hours for 24 hours incubation. The data were then collected in Table 1. In this study the use of DMSO that was not diluted was to dissolve the sample, and the DMSO only was used as a negative control. The diameter zone of inhibition for DMSO after 6 hours was 7.98 ± 0.42 mm. These results suggest that each sample at various concentrations (0.5 to 500 µg/mL) showed a larger of zone inhibition, so the sample studied had an activity as antibacteria againts *S. mutans*. Ethanol extract of *B. rotunda* and its nanoparticle products contain bioactive compounds such as panduratin A¹². Panduratin A has been reported to have the ability to reduce the biofilm of multispecies oral bacteria in vitro¹² and Methicillin-sensitive *Staphylococcus aureus* (MSSA), Methicillin-resistant coagulase-negative *Staphylococci* (MRCNS), *Bacillus subtilis*, and *Salmonella typhi*¹³⁻¹⁴.

In vitro antibacterial activity of ethanol extract of *B. rotunda* and it's nanoparticle products were quantitatively investigated in terms of minimum inhibitory concentration (MIC). From the data it also can be shown that the minimum inhibitory concentration (MIC) value of each sample againts *S. mutans* is 5 µg /mL. At concentration of 0.5 µg/mL indicated a diameter zone of inhibition similar to DMSO (negative control). The MIC of sample was varied, depending on the type of bioactive compounds and antibiotic and the strain of bacteria tested. Furthermore, an early work reported that the ethanol extract of *B. rotunda* rhizome had antibacterial activity against MSSA, MRSA, Methicillin-resistant coagulase-negative *Staphylococci* (MRCNS), *Bacillus subtilis*, and *Salmonella typhi*, with MIC of 2-16 µg/mL¹³.

The measurement in diameter zone of inhibition from each sample was performed as 9 times, and the data shown is Mean ± SD. The result of statistical test using SPSS version of windows 16.0 shows that there was an effect of time to the diameter zone of inihition against *S. mutans* (p

Table 1: Diameter zone of inhibition ethanol extract of *B. rotunda* and nanoparticle produced of ethanol extract *B. rotunda* loaded of chitosan and alginic acid at various concentrations against *S. Mutans*.

No	sample	Concentration ($\mu\text{g/mL}$)	Diameter Zona of Inhibition (Mean \pm SD) mm*			
			Observed after 6 hour	12 hour	18 hour	24 hour
C	DMSO	-	7.98 \pm 0.42	8.12 \pm 0.61	7.46 \pm 0.39	8.17 \pm 0.69
1	Ethanol extract of <i>B. rotunda</i>	0.5	8.50 \pm 0.41	7.82 \pm 0.34	6.81 \pm 0.47	6.70 \pm 0.43
		5	9.46 \pm 0.56	8.97 \pm 0.73	7.18 \pm 0.53	6.88 \pm 0.41
		50	11.73 \pm 1.13	11.29 \pm 0.93	10.46 \pm 1.08	10.19 \pm 0.99
		250	9.96 \pm 0.43	9.76 \pm 0.36	8.98 \pm 0.45	8.56 \pm 0.62
		500	10.44 \pm 1.00	9.64 \pm 1.09	8.22 \pm 0.73	7.54 \pm 0.67
2	Nanoparticle produced of ethanol extract <i>B. rotunda</i> loaded of chitosan	0.5	9.20 \pm 1.09	7.87 \pm 0.33	7.60 \pm 0.39	7.33 \pm 0.29
		5	10.00 \pm 0.62	9.00 \pm 0.32	8.67 \pm 0.54	8.30 \pm 0.48
		50	10.22 \pm 0.34	9.66 \pm 0.44	8.94 \pm 0.26	8.64 \pm 0.74
		250	10.45 \pm 0.48	9.84 \pm 0.62	9.34 \pm 0.52	9.23 \pm 0.57
		500	10.66 \pm 0.98	9.98 \pm 0.72	9.68 \pm 0.37	8.62 \pm 0.65
3	Nanoparticle produced of ethanol extract <i>B. rotunda</i> loaded of alginic acid	0.5	7.89 \pm 0.60	8.09 \pm 0.62	8.26 \pm 0.69	8.03 \pm 0.60
		5	8.88 \pm 0.64	8.05 \pm 0.68	8.04 \pm 1.26	8.01 \pm 0.31
		50	8.13 \pm 0.47	8.56 \pm 0.63	7.91 \pm 0.91	7.37 \pm 2.83
		250	9.71 \pm 0.73	9.26 \pm 0.49	9.74 \pm 1.03	8.60 \pm 0.34
		500	10.54 \pm 1.04	10.29 \pm 1.23	8.62 \pm 0.59	8.12 \pm 1.27
4	Chloramphenicol (positive control)	0.5	8.21 \pm 0.46	7.98 \pm 0.44	7.42 \pm 0.45	7.18 \pm 0.69
		5	8.89 \pm 0.88	8.75 \pm 0.74	8.40 \pm 0.67	8.15 \pm 0.60
		50	11.54 \pm 0.75	11.05 \pm 0.44	10.17 \pm 0.47	7.37 \pm 0.90
		250	16.33 \pm 2.45	12.05 \pm 1.03	11.16 \pm 0.28	11.48 \pm 1.05
		500	10.19 \pm 0.49	9.50 \pm 0.87	8.98 \pm 0.85	9.16 \pm 1.40

*Average of 9 zones

*The result of statistical test using SPSS version of windows 16.0 shows that there was an effect of time to diameter zone of inhibition against *S. mutans* ($p < 0.05$); There was an effect of concentration on the diameter of zone of inhibition against *S. mutans* ($p < 0.05$); There was influence of sample type to zone diameter of inhibition against *S. mutans* ($p < 0.05$)

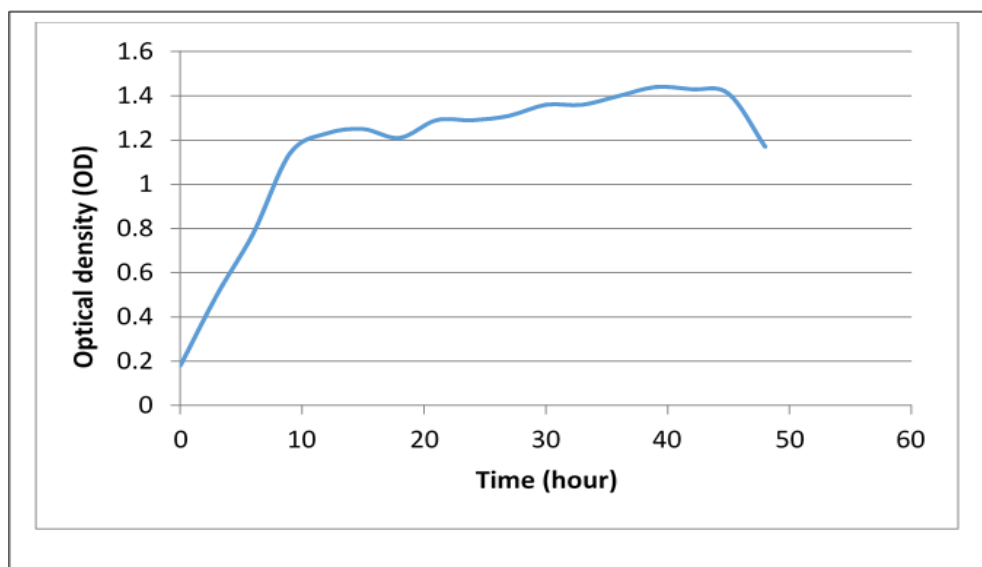


Figure 1: The growth curve of *Streptococcus mutans*.

< 0.05). There was an effect of concentration to the diameter of zone of inhibition against *S. mutans* ($p < 0.05$). There was an effect of sample type to the diameter zone of inhibition against *S. mutans* ($p < 0.05$). The maximum zone inhibition of nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid was at concentration of 500 $\mu\text{g/mL}$, while the ethanol extract of

B. rotunda was at concentration of 50 $\mu\text{g/mL}$. From this study it was also observed the effect of incubation time on diameter zone of inhibition showing the decrease of each sample (Fig.3). Observations were made every 6 hours for 24 hours. This indicated that all samples tested did not kill *S. mutans* bacteria but only inhibited their growth. The effect of incubation time on the diameter zone of inhibition

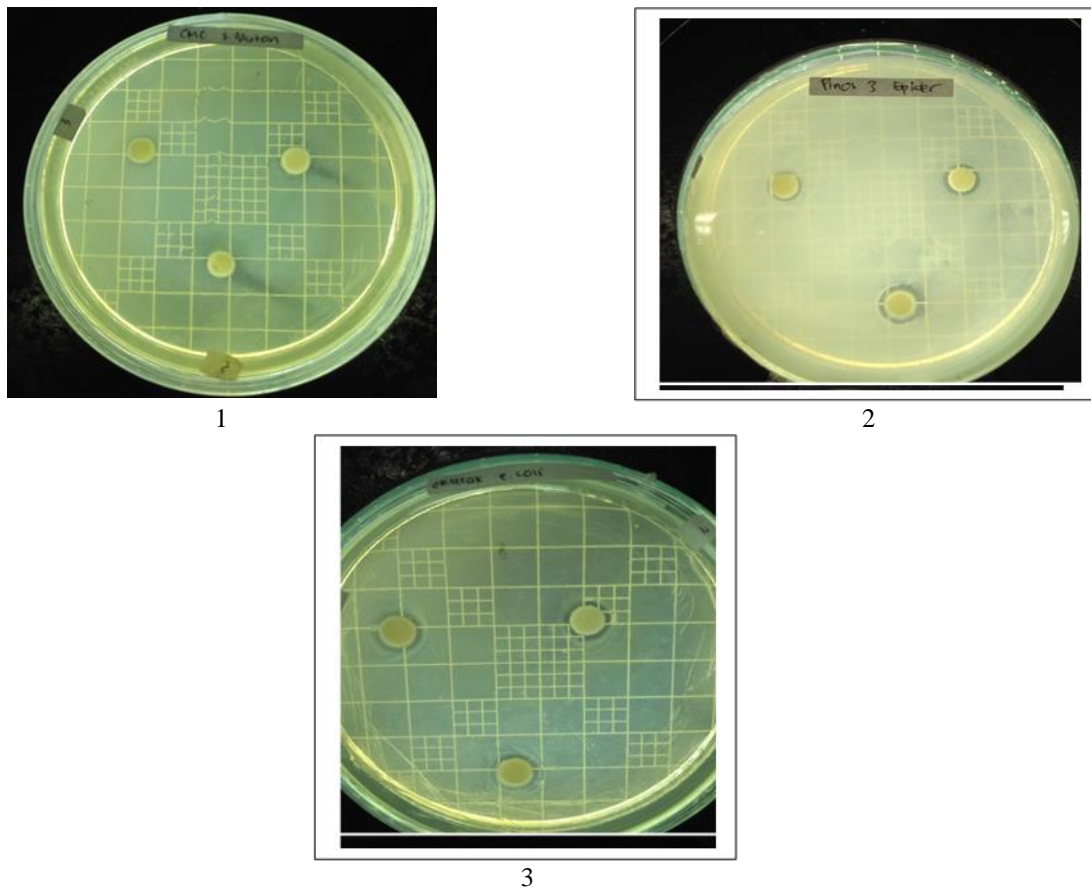


Figure 2: Screening of antibacterial activity of sample was performed using Kirby-Bauer test following the agar diffusion method. (1= DMSO (negative control); 2= sample; 3=Chloramphenicol (positive control).

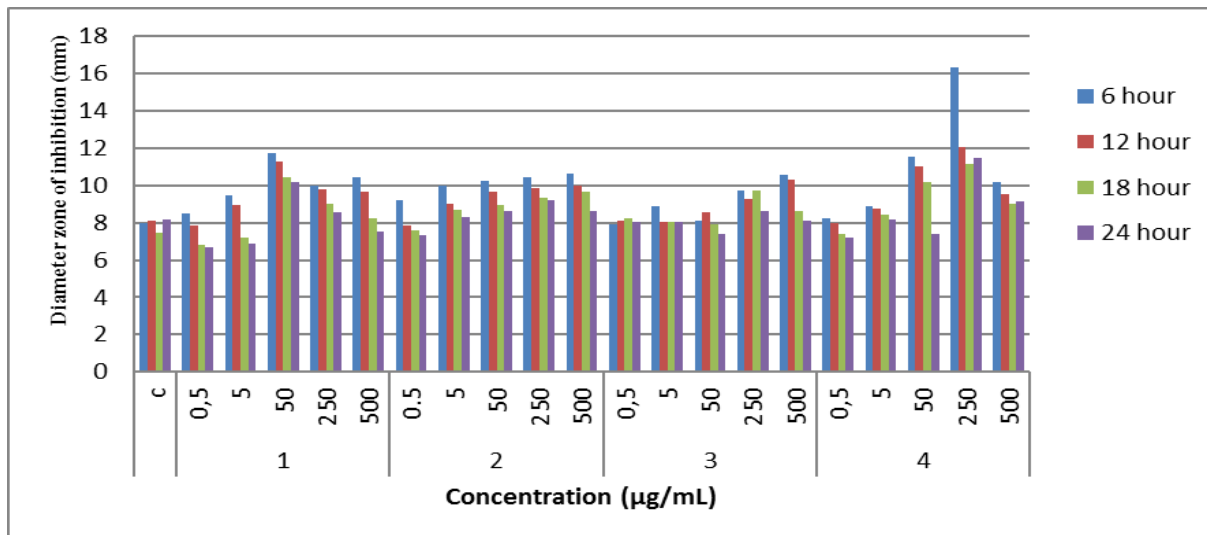


Figure 3: Graph of diameter zone of inhibition against *S. mutans* sample at various concentrations and time. Note : C=negative control (DMSO); 1= ethanol extract of *B. rotunda*; 2= Nanoparticle produced of ethanol extract *B. rotunda* loaded chitosan; 3= Nanoparticle produced of ethanol extract *B. rotunda* loaded alginate; 4= Chloramphenicol (positive control).

against *S. mutans* shows that nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginate are relatively more stable than ethanol extract of *B. rotunda*. Previous research showed that the bactericidal effect mechanism of chitosan-alginate nanoparticles was induced by *P. acnes* cell membrane, and that the

antimicrobial activity of the nanoparticles was due to the chitosan and not the alginate¹⁶. The research of Bhawana¹⁷ showed that the aqueous dispersion of nanocurcumin was much more effective than curcumin against *Staphylococcus aureus*, *Bacillus subtilis*,

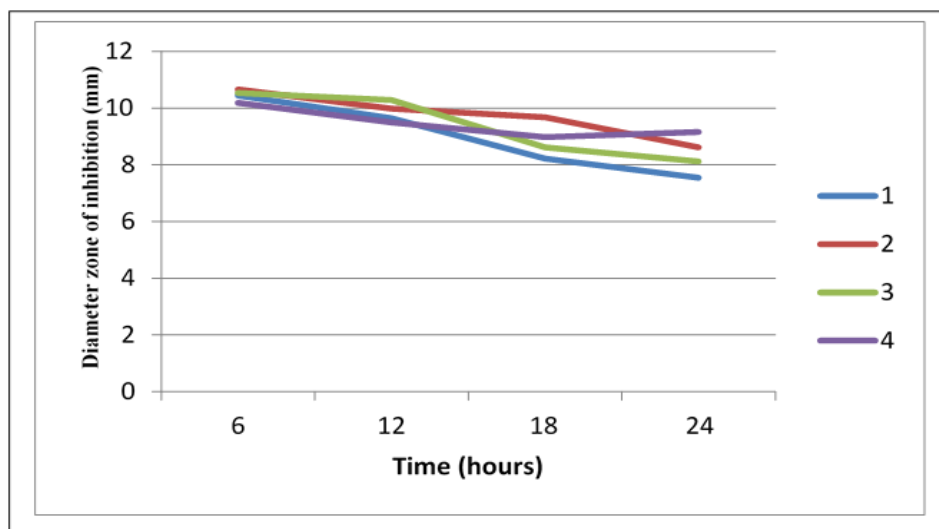


Figure 4: Graph of diameter zone of inhibition (mm) against *S. mutans* on various time of sample at concentration of 500 $\mu\text{g/mL}$ (1= ethanol extract of *B. rotunda*; 2= Nanoparticle produced of ethanol extract *B. rotunda* loaded of chitosan; 3= Nanoparticle produced of ethanol extract *B. rotunda* loaded of alginic acid; 4= Chloramphenicol (positive control)).

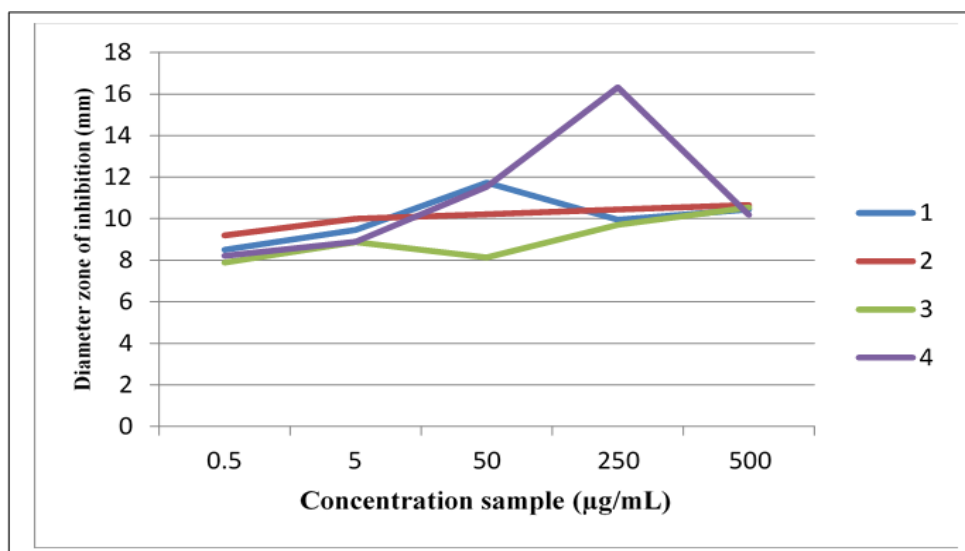


Figure 5: Graph of diameter zone of inhibition (mm) against *S. mutans* in various concentration of sample at observation after 6 hours of sample (1= ethanol extract of *B. rotunda*; 2= nanoparticle produced of ethanol extract *B. rotunda* loaded of chitosan; 3= nanoparticle produced of ethanol extract *B. rotunda* loaded of alginic acid; 4= chloramphenicol (positive control)).

Escherichia coli, *Pseudomonas aeruginosa*, *Penicillium notatum*, and *Aspergillus niger*.

CONCLUSION

In conclusion, the results presented in this study showed that the maximum diameter zone of inhibition from nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid was at concentration of 500 $\mu\text{g/mL}$, while the ethanol extract of *B. rotunda* was at concentration of 50 $\mu\text{g/mL}$. The optimal incubation time on the diameter zone of inhibition against *S. mutans* of each sample was 6 hours. The effect of incubation time on the diameter zone of inhibition against *S. mutans* shows that the nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid were

relatively more stable than ethanol extract of *B. rotunda*. The minimum inhibitory concentration of each sample against *S. mutans* was found to be 5 $\mu\text{g/mL}$.

ACKNOWLEDGEMENTS

We would like to thank Directorate of Higher Education, Indonesia for the research funding in an excellent research universities grant (PUPT-IDB) 2016-2017 and the Overseas Seminar Aid Program, Directorate General of Research and Development, Minister Research and Technology Directorate of Higher Education, Indonesia who has provided assistance to attend the ISTE 2017 international seminar. We also express our gratitude to Prof. K.H. Sugiyarto Ph.D. from Dept. of Chem. Ed.

Universitas Negeri Yogyakarta who has critical review on this manuscript.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest

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