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Research Article

# 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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**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 tooth[1]. 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 use[2]. 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 (synonim with *Boesenbergia pandurata* (ROXB.) SCHLTR and also synonim 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 countries[3]. *B. rotunda* contains essential oils and also secondary metabolites such as pinostrobin, pinocembrin, cardamonin, panduratin A, and alpinetin[4]. Yoon[5] 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 vitro[6]. Panduratin A showed a dose dependent effect in preventing and reducing the biofilm[7].

Previous research has successfully synthesized nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid[8]. The size range of the nanoparticles of chitosan were 389 to 877 nm, with a zeta potential of +41.87 mV, precentage nanoparticle of 98.1%, and the nanoparticles of alginic acid were 197 to 877 nm, with a zeta potential of -82.1 mV, and precentage 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) method[8]. In the present study, we evaluated the antibacterial effect of nanoparticles produced by ethanol extract *B. rotunda* loaded with chitosan and alginic acid againts 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 acid[8], 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 $4^0$C until needed.

*Observation of growth curve of Strepococcus 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 method[9]. 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 $37^0$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 A[12]. Panduratin A has been reported to have the ability to reduce the biofilm of multispecies oral bacteria in vitro[12] 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/mL[13].

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 inhition 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 chitosanand alginic acidat 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 *B. rotunda* | 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 *B. rotunda* 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 *B. rotunda* 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 inhition against *S. mutans* ($p < 0.05$); There was an effect of concentration on the diameter of zone of inhibition against *S. mutans* ($p < 0.05$); Therewas influence of sample type to zone diameter of inhibition against *S. mutans* ($p < 0.05$)

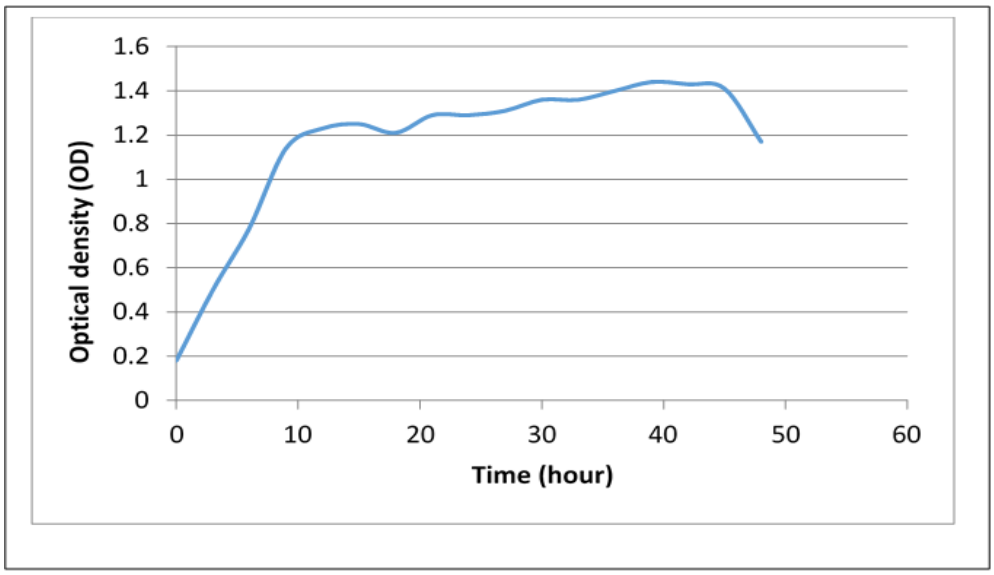
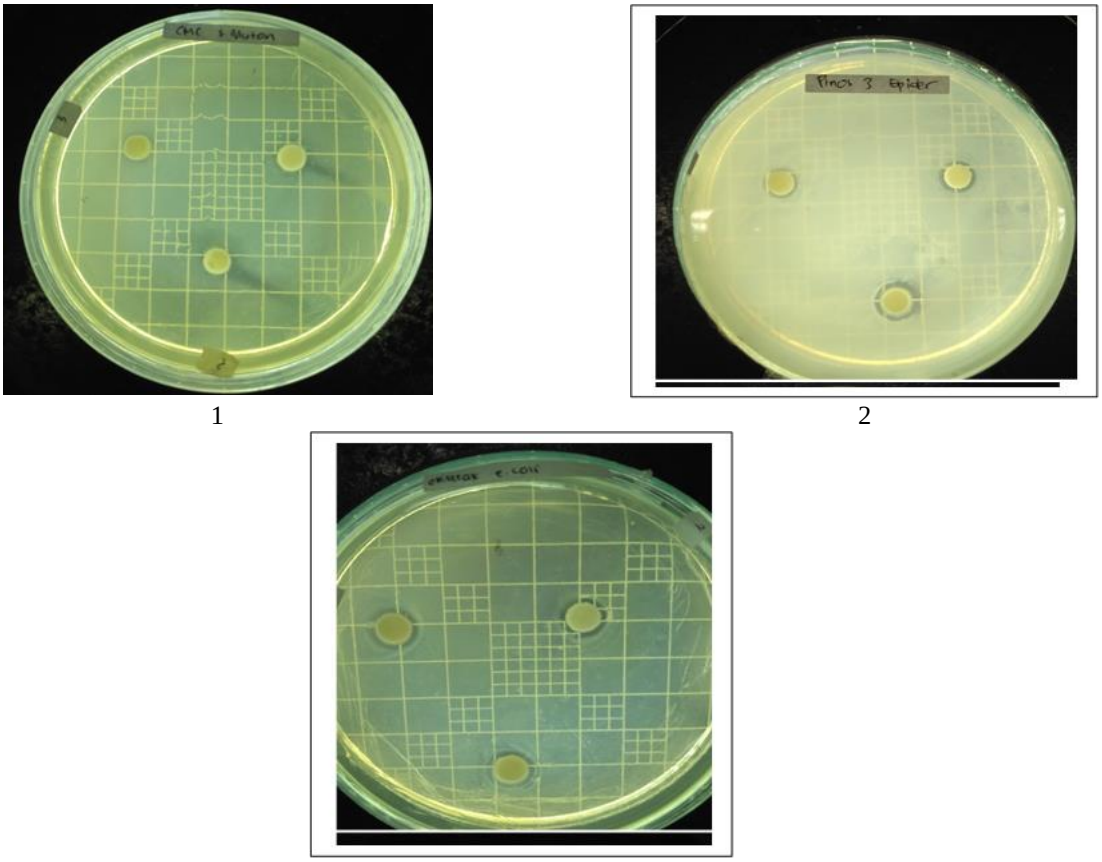



Figure 1: The growth curve of *Strepococcus mutans.*

<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 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



1 2 3

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).

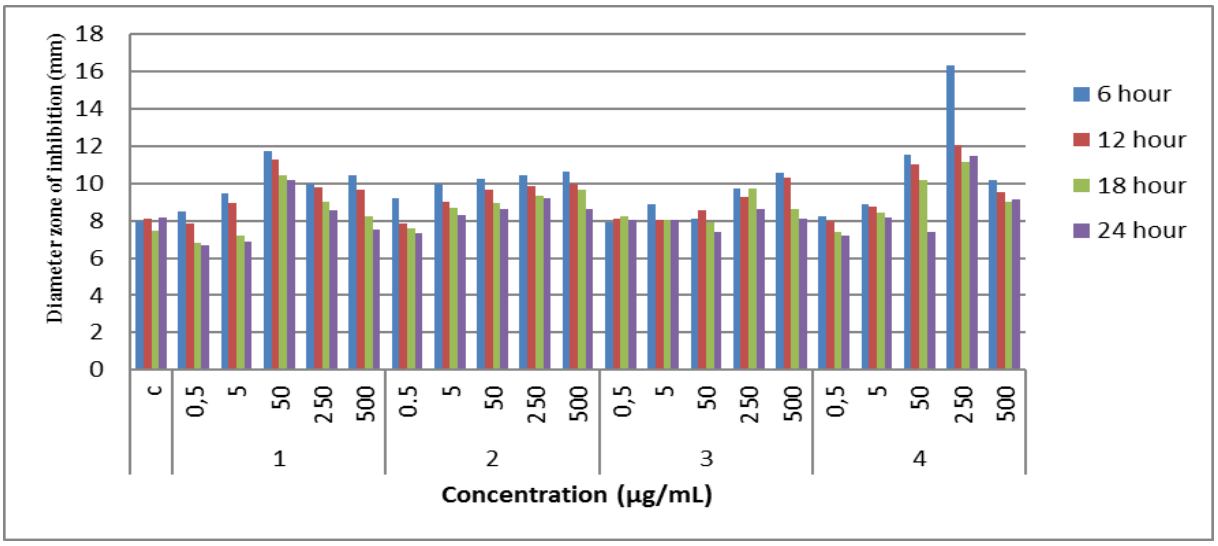



Figure 3: Graph of diameter zone of inhibition against *S. mutans* sample at various concentrations and time.
Note : C=negative control (DMSO); 1= ethanol extract of *B. rotunda*; 2= Nanoparticle produced of ethanol extract *B. rotunda* loaded chitosan; 3= Nanoparticle produced of ethanol extract *B.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

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 alginate[16]. The research of Bhawana[17]showed that the aqueous dispersion of nanocurcumin was much more effective than curcumin against *Staphylococcus aureus*, *Bacillus subtilis*,

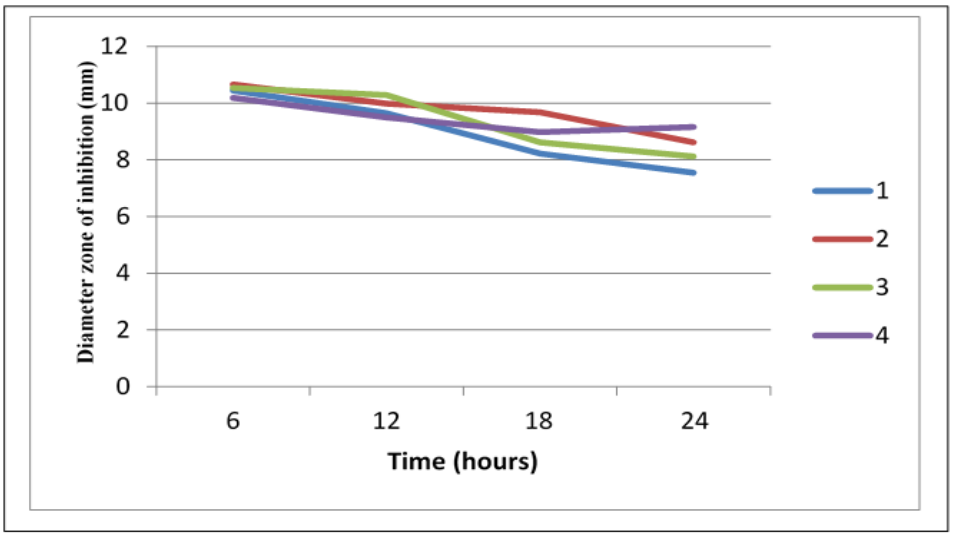



Figure 4: Graph of diameter zone of inhibition (mm) against *S. mutans* on various time Of sample at concentration of 500 µg/mL (1= ethanol extract of *B. rotunda*; 2 =Nanoparticle produced of ethanol extract *B. rotunda* loaded of chitosan; 3=Nanoparticle produced of ethanol extract *B. rotunda* loaded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

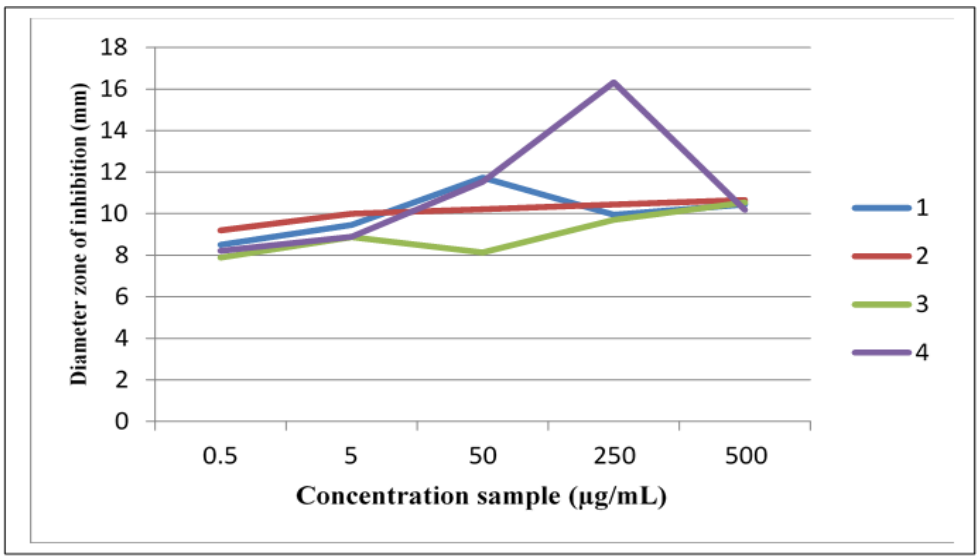



Figure 5: Graph of diameter zone of inhibition (mm) against *S. mutans* in various concentration of sample at observation after 6 hours of sample (1= ethanol extract of *B. rotunda*; 2= nanoparticle produced of ethanol extract *B. rotunda* loaded of chitosan; 3=nanoparticle produced of ethanol extract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

*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 fromnanoparticles 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. mutants* 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.

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**CONFLICT OF INTEREST STATEMENT**

We declare that we have no conflict of interest

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