Original Article

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Green synthesis and inhibitory effects against oral pathogens of silver nanoparticles mediated by rice extracts

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Summary **Rice is staple food for people in many countries for centuries. It is therefore considered as safe and environmental friendly material for pharmaceutical formulations. In the present study, aqueous extracts of three different parts of rice grain; rice bran (RB), rice husk (RH), and rice germ (RG) were compared for their use as reducing agents in synthesis of silver nanoparticles (AgNPs). AgNPs from those three different parts of rice, RB-AgNPs, RH-AgNPs, and RG-AgNPs, respectively showed different reducing activity, which the highest capacity was RB. RG-AgNPs and RB-AgNPs showed the maximum absorption of AgNPs at 440 nm whereas that of RH-AgNPs was at 480 nm. FTIR spectra of all AgNPs indicated the presence of different functional groups from rice attached to the nanoparticles and these groups prevented the particle agglomeration. Size analysis using dynamic light scattering revealed that RB-AgNPs was the smallest particles (346.4 ± 36.8 nm) and possessed the highest negative zeta potential. Antimicrobial test showed that the AgNPs obtained from green synthesis mediated by rice extracts have great antimicrobial activity against *Streptococcus mutans*, the severe oral pathogenic bacteria causing dental caries. These results suggest that aqueous extracts of RB, RH, and RG have potential to be used as reducing agents in synthesis of silver nanoparticles.**

Keywords: AgNPs, rice grain, reducing agent, antimicrobial, *Streptococcus mutans*

1. Introduction

Nanoparticles of certain metals, such as titanium, zinc, magnesium, gold, copper, and silver have been developed for various fields. Among them, silver nanoparticles (AgNPs) have gained interest for commercialization applications since they have considerably versatile properties, such as a variable surface area to volume ratio, which is very useful for many biomedical and technological applications. They have been used extensively in electronic industry and as excellent catalyst. In medical applications, many reports demonstrate their biological activities, such as anticancer (*1*), antioxidant (*2*), and antimicrobial activities (*3*). AgNPs have been used as antibacterial agent for many kinds of applications including home appliances and water treatment (*4*). The biological activity of metal nanoparticles is closely related to their size. The smaller size usually gives the higher activity. Thus, control the size and size distribution of these nanoparticles is an important issue. Generally, specific control of shape, size, and size distribution is often achieved by varying the synthesis methods, reducing agents, and stabilizers (*5*). Recently, the ability of AgNPs on inhibition of certain viruses, such as human immunodeficiency virus 1 (HIV-1) has been reported. AgNPs showed a half maximal inhibitory concentration against the virus of 11.2 ± 0.6 μg/mL ($p < 0.0001$) with no significant toxicity against normal cells (*6*). Silver has long been historical used since ancient times and it has been demonstrated that, in low concentrations, silver is nontoxic to human cells (*7*).

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In former time, AgNPs were generally synthesized by chemical reaction based on the reduction of silver nitrate ($AgNO_3$) by chemical reducing agent (*8*). In global efforts to reduce generated hazardous chemical waste, the use of chemicals is decreased in the synthesis protocols and green or biological synthesis of AgNPs has been increased. The biological methods are using eco-friendly resources, such as plant (*9*), algae (*10*), bacteria (*11*), and fungi (*12*). The extracts from many plants have been reported to act as reducing agent in the green synthesis of AgNPs (*9,13,14*). Synthesis of AgNPs using microorganisms is readily scalable and of course eco-friendly, however, production of microorganisms is more expensive than the production of plant extracts (*15*).

Rice (*Oryza sativa* L.) is a cereal plant in family Poaceae. It is the predominant dietary energy source for many countries in the world. Rice is low in fat and high in starchy carbohydrate, packed full of vitamins and minerals. Dietary minerals and trace elements play a significant role in maintenance of optimal health (*16*). Rice grain has a hard cover called rice husk (RH) to protect the kernel inside. After RH is removed, the remaining product contains the inside endosperm and the outside rice bran (RB) and rice germ (RG). Many parts of rice grain contain high amount of compositions having antioxidant activity and high reducing property (*17,18*). RB and RG are commercial available for health care consumers. The commercial rice-milling process separates RH from the kernel inside because this part of rice grain is inedible and used in non-food applications.

The aim of present study is to synthesize AgNPs by eco-friendly method using RB, RH, and RG extracts as reducing agents. The reducing property of the extracts was confirmed using ferric reducing antioxidant power (FRAP) assay. For AgNPs synthesizing, the extracts were reacted with $AgNO_3$ as a precursor in a certain condition. The obtained AgNPs were characterized and investigated for antimicrobial activity against oral pathogens.

2. Materials and Methods

2.1. *Materials*

RB, RH, and RG of Jasmine rice was purchased from the local producer in Chiang Mai, Thailand. 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) was purchased from Sigma-Aldrich, Inc (St. Louis, MO, USA). $AgNO_3$ was supplied by RCI Lab-scan Co., Ltd. (Bangkok, Thailand). Potassium bromide (KBr) for infrared spectroscopy purchased from Fisher Scientific (Loughborough, UK). Tryptic soy agar (TSA) and broth (TSB) were supplied by Difco™ (Balti-more, Maryland, USA). Brain heart infusion agar (BHI-A) and broth (BHI-B) were purchased from Becton, Dickinson and Company (Franklin Lakes, New Jersey, USA). Sabouraud dextrose agar (SDA) and broth (SDB) were purchased from BBL™ (Baltimore, Maryland, USA). All other chemicals and solvents were of analytical reagent grade or the highest grade available.

2.2. *Microbial strains*

Two aerobic bacterial strains, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 which represent for Gram positive and Gram negative bacteria, respectively, and one facultative Gram-positive bacteria, *Streptococcus mutans* DMST 18777 were used as the oral pathogenic bacteria. *Candida albicans* ATCC 10231 was used as the oral pathogenic fungi.

2.3. *Preparation of rice extracts*

The obtained RB, RH, and RG powders were sieved through a 14-mesh sieve in order to remove the large particles. The sieved samples were pulverized and 2 g of RB, RH, and RG powder samples were dispersed in deionized water to obtain 2% aqueous dispersions. The dispersions were macerated with continuous stirring at room temperature for 24 h. Subsequently, they were filtered through Whatman No.1 filter paper and the filtrates were stored in the refrigerator at 4°C for further use.

2.4. *FRAP assay*

Reducing property of the rice extracts was determined using FRAP assay previously described (*19*) with some modification. The FRAP reagent was freshly prepared by mixing 2.5 mL of 10 mM TPTZ solution in 40 mM HCl with 2.5 mL of 20 mM FeCl3 and 25 mL of 0.3 M acetate buffer, pH 3.6. An amount of 20 µL rice extracts were mixed with 180 µL of FRAP reagent in 96 well plate. Then, they were incubated for 10 min at room temperature and the absorbance of was determined at 595 nm using a microplate reader (Bio-Rad, Model 680, Hercules, California, USA). All data were run in triplicate. The reducing power of the samples was evaluated by calculating the amount of Fe^{+2} produced by the rice extract samples using the calibration curved of $FeSO_4$.

2.5. *Synthesis of AgNPs*

The synthesis of AgNPs was done by the following procedure. The rice extract solution was filled into the 125 mL Erlenmeyer flasks and heated until 75°C. An exact amount of $AgNO_3$ solution (0.1 M) was added drop wise until the volume ratio of the rice solution to $AgNO_3$ solution was 100:1. The mixture was kept at 75°C under continuous stirring for 60 min. The obtained mixture was added thrice with deionized water and subjected to centrifugation (Heraeus™

Megafuge™ 40 Centrifuge Series, ThermoFisher Scientific, Waltham, Massachusettes, USA) at 8,000 rpm for 15 min to remove any traces of un-utilized phyto-constituents. After removing the liquid phase, the AgNPs obtained were kept in the refrigerator for further study. The AgNPs obtained from RB, RH, and RG were named as RB-AgNPs, RH-AgNPs, and RG-AgNPs, respectively.

2.6. *Characterization of AgNPs*

The obtained AgNPs were characterized using UV-vis and Fourier transform infrared (FTIR) spectroscopy measurement. Their particle size and zeta potential were measured using dynamic light scattering (DLS).

2.6.1. *Visualization and UV-vis spectroscopy*

The outer color appearance of the obtained AgNPs was early observed by visualization. The obtained particles were confirmed by UV-vis spectra using UV 2450 double-beam sspectrophotometer (Shimadzu-2450, Kyoto, Japan). The rice-AgNPs samples were diluted to 100 folds with deionized water before subjecting to this investigation. The optical property of the AgNPs solution was observed in the wavelength range of 300-700 nm. The UV-vis absorbance spectra were recorded at room temperature.

2.6.2. *FTIR*

In this experiment, the lyophilized RB-AgNPs, RH-AgNPs, and RG-AgNPs were used. The obtained AgNPs in the powder form were characterized using FTIR in order to investigate the possible role of the phyto-constituents presented in the rice extracts on the surface modification of the synthesized AgNPs. The KBr disc of the lyophilized AgNPs were prepared. The IR spectra in the range of 4,000-400 cm^{-1} of the samples were recorded using a Nicolet Nexus 470 FT-IR (Minneapolis, Minnesota, USA) in the diffuse reflectance mode at room temperature at a resolution of 4 cm^{-1}. The spectra were collected against a KBr disc background at room temperature. The instrument was maintained with the automatic dehumidifier to diminish water vapor interference.

2.6.3. *DLS*

The obtained AgNPs were investigated for their particle size and size distribution as well as zeta potential using DLS (Malvern Zeta sizer Nano-ZS, Malvern Instruments, Worcestershire, UK) at 25°C. Each sample was diluted to 100 folds with deionized water before investigation. The disposable plastic cuvettes and folded capillary zeta cell were used in the measurement. The measurement was done in triplicate of three independent AgNPs batches.

2.7. *Antimicrobial activity of AgNPs*

The antimicrobial activity of the obtained RB-AgNPs, RH-AgNPs, and RG-AgNPs against oral pathogenic microorganisms; *S. aureus*, *E. coli*, *S. mutans*, and *C. albicans* was tested based on Kirby-Bauer method (*20*). The aerobic and facultative bacteria were grown in TSA and BHI-A, respectively at 37°C for 24 h. The bacterial strains were diluted in TSB and BHI-B, respectively to a final density of 1.5×10^6 colony forming unit (CFU)/mL. *C. albicans* were cultured in SDA at 37°C for 36-48 h. The fungal suspension was prepared to a final concentration of $1\text{-}2 \times 10^5$ CFU/mL in SDB. The density of the microbial suspension was adjusted with 0.5 McFarland constant by observing the OD at 600 nm under a UV-vis spectrophotometer. The bacterial and fungal suspensions were swabbed on the surface of their corresponding agars. Freshly prepared lyophilized RB-AgNPs, RH-AgNPs, and RG-AgNPs suspensions (40 µL) were added onto the 6 mm-diameter filter paper discs which would be put onto the surface of the seeded agar plates. The discs filled with deionized water and 0.1 M $AgNO_3$ solution at the same amount (40 µL) were used as negative controls. The plates were incubated at 37°C for 24 h. The antimicrobial activity of the samples was evaluated by determining the diameter of the clear zone of inhibition around the paper discs. All samples were done in triplicate.

2.8. *Statistical analysis*

Descriptive statistics for continuous variables were calculated and reported as a mean ± standard deviation. Data were analyzed using a One-way analysis of variance and Duncan's multiple range test using Statistic a software version 17 (SPSS Inc., Chicago, Illinois, USA). The values were presented as means ± standard deviation which a *p*-value less than 0.05 was considered as significant difference.

3. Results

3.1. *Reducing property of rice extracts*

In the present study, the reducing property of three different parts of rice grain, RB, RH, and RG were compared. The values of FRAP assay showed wide variation among the samples (Figure 1). The significantly highest ($p < 0.05$) ferric reduction ability was obtained from RB extract (524.0 ± 7.68 µmol Fe^{2+}/g sample), followed by RH and RG with the reducing values of 247.1 ± 8.49 and 152.1 ± 4.24 µmol Fe^{2+}/g sample, respectively. Our results show that RG also possessed the reducing property, even less than RB and RH, respectively.

3.2. *Synthesis and characterization of AgNPs*

Reduction of silver ion to produce AgNPs during exposure to the rice extracts could be easily detected by color change. The color of RB, RH and RG extract solutions was pale yellow and the color of the precursor $AgNO_3$ was colorless. However, when AgNPs were formed, the color of the solution began to change. After complete reduction, the color of the system were yellow-brown as presented in Figure 2.

The intensity of the resultant color was observed during 0-60 min. It was found that the color change of the reaction mixtures of all rice extracts and $AgNO_3$ was gradually appeared as pale yellow-brown around 10-30 min of reaction, depending on the type of the rice extracts. It was noted that the color change in the formation of RB-AgNPs was observed within 10 min, significantly faster than that in the systems of RH-AgNPs (25 min) and RG-AgNPs (30 min). After 60 min, all systems turned to intense yellow-brown color, indicating the complete formation was occurred.

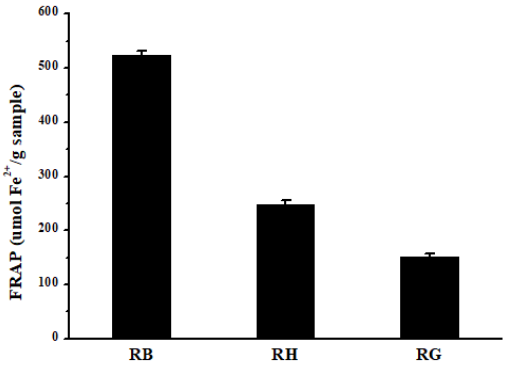

Figure 1. Reducing activities of RB, RH, and RG.

The UV-vis spectra of RB-AgNPs and RG-AgNPs showed the maximum absorption at 440 nm, whereas that of RH-AgNPs appeared at 480 nm, as shown in Figure 3. The absorption of the precursor $AgNO_3$ was at 216 nm, however the UV-vis spectra of all AgNPs did not exhibit the absorption at this wavelength, indicating that there was no trace $AgNO_3$ left in the obtained AgNPs systems.

After the synthesis of AgNPs, the dispersions containing nanoparticles were centrifuged to separate AgNPs from other rice compositions in the solutions. The FTIR of RB-AgNPs, RH-AgNPs, and RG-AgNPs are shown in Figure 4. The results showed the peaks at 965, 1,160, 1,445-1,453, 1,591-1,599, and 2,980 cm^{-1}.

Analysis using DLS reveals that the size of the AgNPs obtained from different part of rice grain is different. RB-AgNPs showed average diameter of 346.4 ± 36.8 nm whereas RH-AgNPs and RG-AgNPs showed similar particle size of 587.3 ± 49.6 and 510.9 ± 84.4 nm, respectively. Particle size distribution expressed as polydispersity index (PdI) of RB-AgNPs, RH-AgNPs, and RG-AgNPs were similar with the PdI values of 0.271, 0.260, and 0.266, respectively. The zeta potential of all AgNPs obtained was negative values of 32 ± 2.6,

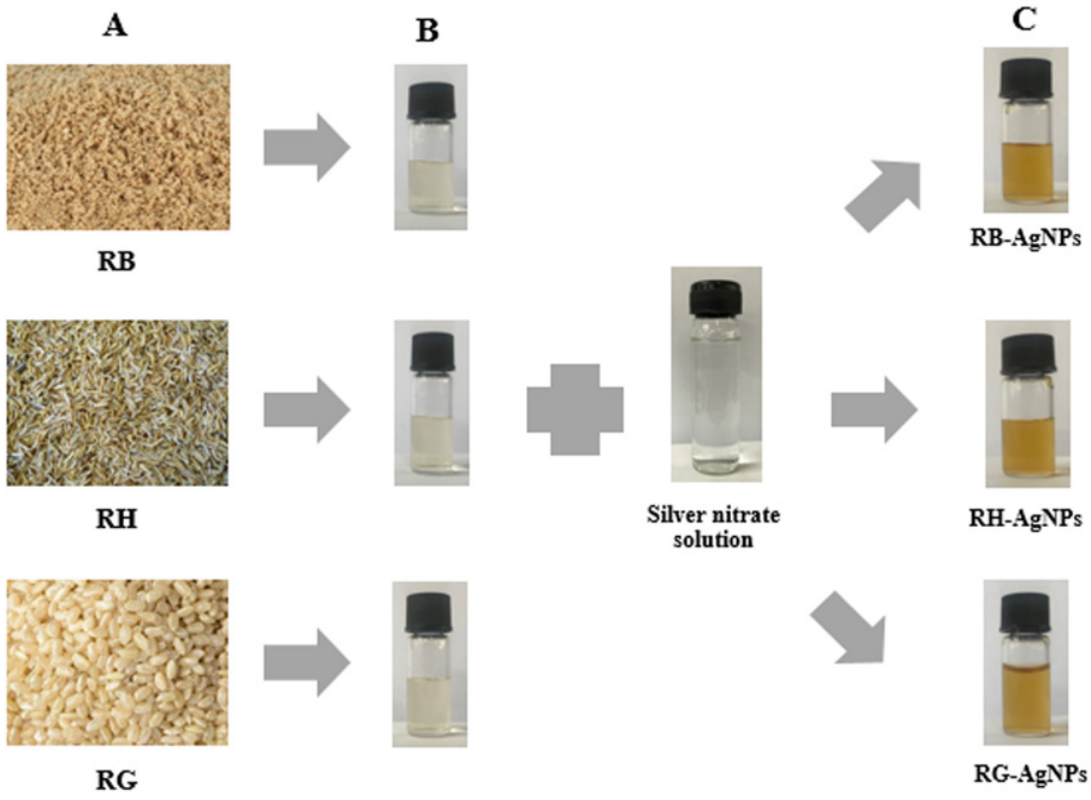

Figure 2. Outer appearance of three parts of rice grains (A), rice extracts (B), and the obtained AgNPs (C).

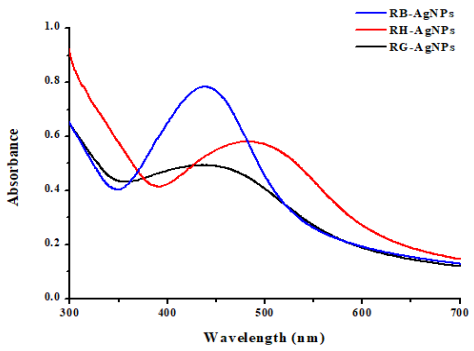

Figure 3. UV-vis spectra of the obtained AgNPs.

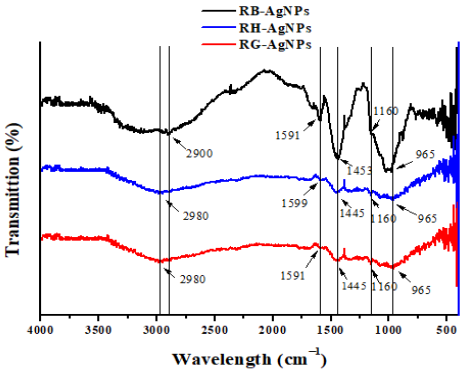

Figure 4. FTIR of the obtained AgNPs.

24.5 ± 3.1, 18.6 ± 2.4 mV for RB-AgNPs, RH- AgNPs, and RG-AgNPs, respectively.

3.3. *Antimicrobial activity*

The synthesized AgNPs were investigated for their antimicrobial activity against oral pathogenic bacteria and fungi. The results of our study showed that all AgNPs obtained possessed antimicrobial activity against all tested oral pathogens as shown in Figure 5. The inhibition potential of different AgNPs was similar, particularly on the inhibition of the facultative *S. mutans* and the pathogenic fungi *C. albicans*. Among the four tested pathogens, the inhibition zones of RB-AgNPs, RH-AgNPs, and RG-AgNPs against *S. mutans* were the largest diameters of 17.7 ± 0.6, 17.5 ± 0.5 and 17.7 ± 0.6 mm, respectively, indicating the highest potential antibacterial activity of the obtained AgNPs against this strain. RB-AgNPs showed similar activity against the aerobic *S. aureus* and *E. coli* with the inhibition zones of 14.3 ± 0.3 and 14.5 ± 1.8 mm, respectively. RH-AgNPs exhibited stronger inhibition against these strains with the inhibition zones of 12.7 ± 0.6 and 14.7 ± 0.3 mm, respectively. In contrast, RG-AgNPs exhibited stronger inhibition against both strains with the inhibition zones of 14.3 ± 0.5 and 13.3 ± 0.6, mm, respectively. All AgNPs exhibited the same potential inhibition of *C. albicans* with the inhibition zones of 11.2 ± 2.1, 11.5 ± 0.5, and 11.3 ± 2.1 mm for

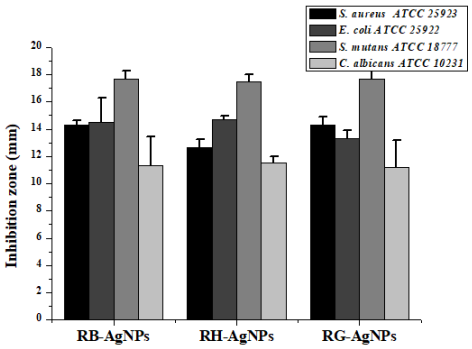

Figure 5. Inhibition zone diameters of the obtained AgNPs against four oral pathogens.

RB-AgNPs, RH-AgNPs, and RG-AgNPs, respectively. The negative controls show no inhibition zone against both bacterial strains and *C. albicans*.

4. Discussion

Phytochemicals, including phenolics and flavonoids, in rice have been reported. Oryzanol and other important compounds, such as tocols (topherols and tocotrienols) and phytosterols are the important bioactives in rice grain due to their antioxidant properties (*21*). The content of these compounds is different in different rice varieties (*22*). Moreover, different part of rice grain yields different type and amount of these compounds (*23,24*). RB extracts from many rice varieties contain high content of total phenolic and total flavonoid (*25*). These compounds are considered to play the important role on reducing property in RB. RH also contains high total phenolic content that can be an effective source of natural antioxidants (*26*). RG contains high amount of alpha tocopherol, small amount of gamma tocotrienol and alpha tocotrienol (*27*). These compounds are considered to support the reducing property of RG.

The present study reveals that rice extracts from different part of rice grain can act as a reducing agent in green synthesis of AgNPs. To detect AgNPs formation during the process of synthesis, color change can be observed. This visual signature was the easiest way for basic characterization of AgNPs. The intensity of the resultant color is dependent on the concentration of the reactants (*28*). The reaction time was also have an important role on the resultant color intensity (*29*). Therefore, in the current study, the reactant concentration and the reaction time were fixed. The spectra of AgNPs can be attributed to the surface plasmon resonance due to collective oscillation of surface electrons (*30*). The UV-vis absorption spectra of AgNPs generally appear in the range of approximately 350-600 nm, depending on the type of reducing agent. For example the gelatin-AgNPs showed their absorption at 400-450 nm (*31*), whereas those obtained from the extracts of *Plectranthus amboinicus* leaf and *Lantana camara* berry were at 428 nm (*32*) and in the range of

390-520 nm (*33*), respectively. The obtained AgNPs in the current study were confirmed by UV-vis spectra. Our results showed good agreement with the previous studies on the UV absorption range.

FTIR study was carried out in order to determine the possibility of the residual bio-reducing functional groups of the rice extracts involved in reduction process and their possible unique interactions with the surface of AgNPs. The organic functional groups like OH or C=O interacted to the surface of AgNPs can be detected by FTIR (*15*). The IR peaks of the obtained AgNPs in the current study indicate that many functional groups are involved. The peaks at 965 and 1160 cm^{-1} are considered to be the stretching vibrations of C–OCH_3, C–H stretching of alkenes and C–O stretching aromatic side chain of proteins (*34*). The peaks in 1,445-1,453 cm^{-1} are relevant to the N–O stretching of nitro groups (*35*). The peaks located at 1,591-1,599 cm^{-1} are assigned to C=O stretching vibrations of amides characteristic of rice proteins. The broad peaks at 2,980 cm^{-1} are assigned as –OH stretching in alcohols and phenolic compounds with strong hydrogen bonds (*36*). The presence of these peaks confirmed that the obtained AgNPs were covered by certain rice phytochemicals, including flavonoids and phenols, with functional groups, such as ketone, aldehyde, and carboxylic acid. The presence of these groups on the surface of AgNPs is considered to support the stability of the nanoparticles. They can prevent the pairing and agglomerating of the nanoparticles. If the amount of these compounds is high in the rice extracts, they can cover wide surface area of the obtained AgNPs. This can cause the size of the synthesized AgNPs to be extremely small since high agglomeration cannot occur.

The size of the nanoparticles obtained from DLS is hydrodynamic size which is slightly bigger than that measured by a transmission electron microscope due to the hydrodynamic radius (*37*). However, the size of the obtained nanoparticles can be compared by using the same equipment. In this study, the size of all AgNPs obtained was measured using DLS which their zeta potential could also be detected. The results reveal that among the three kinds of AgNPs, the smallest size and the highest negative value of zeta potential were obtained from RB-AgNPs. This was considered to be influenced by the high phytochemical content existed in the RB extract.

The oral cavity is the hub of an extremely diverse microflora consisting of about 500 species of microorganisms (*38*). The oral pathogenic bacteria (*S. aureus*, *E. coli*, and *S. mutans*) and fungi (*C. albicans*) used in the current study are the most common microorganisms found in oral cavity. The overgrowth of these microorganisms, particularly *S. mutans* and *C. albicans* can cause severe diseases in oral cavity. *S. mutans* is the major cause of dental caries (*39*). While *C. albicans* is the major cause of oral candidiasis (*40*), which the symptoms include pain, oral discomfort, and loss of taste (*41*). Several mechanisms of action on antibacterial activity of AgNPs have been proposed, such as the ability of AgNPs to attach bacterial cell wall and cause structure change in cell membrane, the ability to damage and make porous in the cell membrane resulted from free radicals of AgNPs, and the ability of silver ion that can be released to the inner cell and destroy several function in the cell (*42,43*). The mechanism of action on antifungal activity of AgNPs against *C. albicans* was previously proposed that AgNPs have high potential to disrupt cell membrane and arrest the cell cycle at the G2/M phase of *C. albicans* (*44*). The current study demonstrates that rice can be used as natural reducing agent to synthesize AgNPs. We explore the different reducing potential and advantages of many parts of rice grains in green synthesis of AgNPs without the use of any chemical stabilize and reducer. We also demonstrate the potential of the synthesized AgNPs on many important oral pathogens including aerobic bacteria, facultative bacteria, and fungi. The AgNPs obtained can inhibit all tested microorganisms especially *S. mutans* which is the most important oral pathogenic bacteria causing dental carries and oral infections. Among the three parts of rice grains, RB is the most effective and suitable part for the synthesis of AgNPs.

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