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Research article

Green synthesized copper nanoparticles and their anti-bacterial properties against bullfrog multidrug resistant gram negative bacteria

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Abstract

The emergence of multidrug-resistant (MDR) gram-negative bacteria in bullfrog (*Hoplobatrachus rugulosus*) farming has been increasing dramatically which resulted in searching for new types of antimicrobial agents. Although some new drugs against MDR bacteria have been introduced or presently in clinical trials, the efficiency of them are still limited by species of pathogens. Therefore, copper nanoparticles (CuNPs) has been immerged in MDR treatment due to their greater exhibition in broad-spectrum bactericidal properties. To prepare green synthesized CuNPs, the high antioxidant property of plant aqueous extracts assessed by scavenging free radicals of DPPH and Reducing Power (RP) of ferric(III)chloride were used. It was indicated that the IC_{50} value of extract was greatest in *Garcinia mangostana* following *Camellia sinensis*, *Phyllanthus urinaria*, *P. amarus* and *P. virgatus*, respectively ranging from 230±10 to 490±10 (µg/mL) and positive related to RP antioxidant activities. The formation CuNPs were characterized using UV-visible spectroscopy revealed a maximum absorbance at 340 nm. CuNPs using *G. mangostana* (GM-CuNPs) exhibited the greatest significant bactericidal activity against multi-drug resistant gram negative bacterial strains such as *Aeromonas sorbia*, *Edwardsiella tarda*, *Enterobacter* spp., *Klebsiella pneumoniae*, and *Pseudomonas* spp. by agar well diffusion method. Moreover, the results from Dynamic light scattering (DLS) demonstrated that only size of GM-CuNPs was in the nano-size range of 254±144.9 nm whereas the zeta potential was in the range of -0.37±11.3 mV. It can be concluded that GM-CuNPs exhibit greatest antibacterial properties for MDR treatment and should be a candidate for future bullfrog MDR therapeutic application. *In vivo* research and cost-effectiveness analysis will be also investigated for further study.

Keywords: Antioxidant, Antibacterial susceptibility, Bullfrog, Copper nanoparticles, Multidrug resistance

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INTRODUCTION

During the last decades, the incidence of bacterial infection in fresh water aquatic animals has increased dramatically especially the critical pathogen in family Enterobacteriaceae (WHO, 2017). Thus, the anti-bacterial drug has been employed for treating infections resulted in the emergence of resistance among the pathogens. The outbreak of these multidrug resistant (MDR) bacteria has been seriously affecting productivity and led to economically devastating for aquatic animal farmers. Moreover, it has been associated with human diseases and has been significantly triggered using of more expensive agents (Tanwar et al., 2014). Therefore, nanomaterials as antibacterial agents had been issued due to their effectiveness and eco-friendly approach that aims to reduce the usage of those substances. The process of eco-friendly synthesis has focused on the nanoparticles such as silver, zinc, manganese, gold, copper, etc (Yadav et al., 2018). However, the cost of synthesis of some metal nanoparticles is quite challenged. Among them, copper has been used as effective antibacterial agent for decades (Rajesh et al., 2018) and has been developed by synthesized into copper nanoparticles (CuNPs) recently for increasing the antibacterial property. CuNPs was considered to be interesting one of alternative which was cheaper and manifest antibacterial activity. Moreover, CuNPs had also been demonstrated superior antibacterial activity compared to silver nanoparticles (Yoon et al., 2007) and was reported as effective antimicrobial agent against bacteria such as *Bacillus* spp., *Escherichia coli*, *Pseudomonas* spp. and *Staphylococcus aureus* (Rajesh et al., 2018; DeAlba-Montero et al.,2017, Yadav et al., 2017).

CuNPs can be synthesized using various techniques, chemical/biological starters, temperature conditions, pH conditions and reducing power sources. Among them, green synthetic method employed natural reducing power sources has been the most efficient and convenient due to require simple equipment with low cost, low toxicity and allowed the eco-friendly reaction conditions. However, most of the properties of nano-materials were influenced by their size (Murphy and Jana, 2002). The smaller nano-sized range should be more effectiveness in antibacterial properties as indicated by Rajesh et al. (2018). Thus, the development of proper green synthesis method for CuNPs in nano-sized range formation using various plant materials which possess high antioxidant properties was an important task.

In the present study, tea (*Camellia sinensis*) has been used as material for synthesizing CuNPs from copper sulphate starter due to high property in antioxidant power (Mandava et al., 2017). Furthermore, tea has been also used as supplement for against aquatic pathogen *Aeromonas hydrophila* (Abdel-Tawwab et al., 2010). Another material such as mangosteen (*Garcinia mangostana*) leaf had also documented as a reducing agent for metal nanoparticles synthesis such as silver due to their high antioxidant properties (Veerasamy et al., 2011). Interestingly, the plants of the genus Phyllanthus which have been used as a folk medicine over decades in the Southeast Asian countries (Mao et al., 2016). Among them, *Phyllanthus amarus*, *P. urinaria*, and *P. virgatus* share the name of look tai bai in Thailand shown the remarkable as antioxidant (Poompachee and Chudapongse, 2012). Plant extracts from *Phyllanthus amarus* was also reported to has reducing agents, which reduced Cu^{2+} to CuNPs

(Ajitha et al., 2018). Therefore, *Phyllanthus urinaria* and *P. virgatus* were new challenged plants for green synthesized CuNPs production. Thus, it should be also exerted this property for CuNPs formation and against 5 MDR bacteria in this research.

In this work, five local plants containing high antioxidant properties were taken and were investigated therefore reducing Cu^{2+} to Cu. The antibacterial property of CuNPs was also investigated in MDR bacteria isolated from bullfrog by well diffusion method as indicated below.

MATERIALS and METHODS

Plant materials

Tea leaf powder (*Camellia sinensis*) afforded from commercial tea leaf no.3 Lot no. 760 (Three horse tea Co., Ltd, Thailand) was bought from the local market in Mueang Lampang, Lampang Thailand. Mangosteen leaf powder (*Garcinia mangostana*) was available from voucher specimen from Dr. Pat Pranamornkith collected in Suan Khuean (18.126197, 100.316127), Mueang Phrae District, Phrae Thailand. The fruits of *Phyllanthus amarus*, *Phyllanthus urinaria*, and *Phyllanthus virgatus* were collected in Rajamangala University of Technology Lanna, Lampang (18.367053, 99.596299), Mueang Lampang District, Lampang Thailand. Mature leaves and fruits were collected between September 28th and October 8th, 2017. Specimens were then identified and authenticated by an herbalist from Rajamangala University of Technology Lanna, Lampang (RMUTLL).

Preparation of extract

Fresh leaves of mangosteen were washed with tap water followed by distilled water prior drying in hot air oven at 55 °C. Dried leaves of mangosteen and tea were grounded into a fine powder and meshed. The samples were boiled at 95 °C for 15 min. After cooling, the aqueous solutions were filtered by 0.2 micron Nylon syringe filter (Filtrex, Singapore) before further analysis. Dried fruit powders of *Phyllanthus amarus*, *Phyllanthus urinaria* and *Phyllanthus virgatus* were also performed in similar manner.

DPPH assay

The 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radical-scavenging activity was determined using modified method described of Brand-Williams (1995). DPPH (Sigma–Aldrich, Steinheim, Germany) solution (oxidized form) was prepared in absolute ethanol (RCI Labscan, Bangkok, Thailand) to get final absorbance of 0.8-1.0. One hundred µL of various concentrations of samples was added to 900 µL of DPPH radical solution. The mixtures were shaken and then incubated for 30 min in the dark at the ambient temperature. The presence of antioxidant which can donate an electron to DPPH radical decays resulted in solution color change into yellow. The radical scavenging capacity was determined by measuring absorbance at 517 nm. The percentage of inhibition of samples was calculated using equation:

% inhibition = [(Abs control-Abs sample)/Abs control] x 100

The linear curves were constructed by plotting percentage of inhibition against concentration in µg/mL (R^2 = 0.99). The equation of linear curve was calculated for the IC_{50} which corresponding to the sample concentration that reduced the initial DPPH· absorbance of 50%. The smallest IC_{50} value indicated to highest antioxidant properties. All samples were carried out in triplicate.

Reducing power (RP) assay

Reducing power assay is an important method for quantitative analysis of metal reducing potential of compounds according to the method of Zhang et al., (2011) with some modifications (Ahmad et al., 2014). The volume of 10% trichloroacetic acid was taken 2-fold to react completely and centrifugation of mixture was done in 3,000 rpm, because of no interference for spectroscopy measuring. Briefly, Different concentrations of extract (25, 50, and 100 µg/mL) and standard antioxidant gallic acid (Bio Basic Inc., Markham, Canada) were added to 1.0 mL of 0.2 M phosphate buffer (pH 6.6) and 1.0 mL of 1% potassium ferricyanide (Univar, Ajax Finechem, Australia). The mixture was vortexed well then was incubated in water bath at 50 °C for 20 min. At the end of incubation, 1.0 mL of 10% trichloroacetic acid (Merck, Damstadt, Germany) was added and the mixture was centrifuged at 3,000 rpm for 10 min. The 0.5 mL of supernatant was mixed with equal volume of distilled water and 50 µL of 0.1% ferric(III) chloride (Univar, Ajax Finechem, Australia). After incubation for 10 min, the colored solution was read at 700 nm against a reagent blank.

Total phenolic content

The total phenolic content was determined using Folin-Ciocalteu (FC) colorimetric method. Twenty µL of extract was mixed with 100 µL of FC (Merck, Damstadt, Germany) reagent in 1,980 µL of DI water. Afterwards, the mixture was incubated for 5 min at the ambient temperature and then was added 300 µL of 7% of Na_2NO_3 (Univar, Ajax Finechem, Australia). After incubation for 60 min in the dark at the ambient temperature, the absorbance was measured at 765 nm using V-1200 spectrophotometer (Dshing Instrument Co., Ltd., China) with UV-Professional analysis software. The gallic acid at different concentrations was used as standard for calibration curve (R^2 = 0.99). All experiments were carried out in triplicates. TPC was expressed as microgram gallic acid equivalent (GAE) per milligram dried weight (Sassa-deepaeng et al., 2017).

The flavonoids content

The flavonoids content was determined by aluminium trichloride ($AlCl_3$) colorimetric method with some modified (Chang et al., 2002). Twenty µL of extract was added to 380 µL of DI water and then 100 µL of 5% $NaNO_2$ (Univar, Ajax Finechem, Australia) was added. After incubating for 5 min at the ambient temperature, 100 µL of 10% aluminium trichloride (lobachemie, Mumbai, India) was added and was allowed to stand for 6 min at the ambient temperature. Finally, 400 µL of 1M NaOH (RCI Labscan, Bangkok, Thailand) was added. After incubation for 15 min in the dark, the mixture turned to yellow or orange and the absorbance was measured at 415 nm using V-1200 spectrophotometer with UV-Professional analysis software. The flavonoids quantitation was carried out in triplicates and the content was expressed as microgram quercetin (Sigma–Aldrich, Steinheim, Germany) equivalent (QE) per milligram dried weight.

Qualitative phytochemical screening

The preliminary phytochemical analysis of the extracts was carried out using standard procedures to identify the various constituents described by Bargah (2015), Siddiqui and Ali (1997) and Harborne (1973).

Test for Alkaloids

Three mL of extract was stirred with 3 mL of 1% hydrocholic acid (RCI Labscan, Bangkok, Thailand) in Dry bath incubator and then stirred with 1 mL of Mayer's reagent. The appearance of buff colored precipitate was indication of presence of alkaloids (Bargah, 2015).

Test for Tannins

Two mL of the extract was stirred with 50 µL of 5 % of ferric chloride (Univar, Ajax Finechem, Australia) solution were added. Formation of bluish black or greenish black precipitate was indication of presence of tannins (Siddiqui and Ali, 1997). The extracts were also confirmed using HCl-vanillin (Merck, Damstadt, Germany) reagent. The development of a red color indicated the presence of condensed tannins (Falcão et al., 2011).

Test for Terpenoids

Five mL of chloroform (RCI Labscan, Bangkok, Thailand) was added in 2 mL of the extract and evaporated to dryness. Two mL of conc. H_2SO_4 (J.T. Baker, Phillipsburg, NJ, USA) was then added and heated for 2 minutes. Development of reddish brown or grayish color indicates the presence of terpenoids (Harborne, 1973)

Test for Saponins

Five mL of extract was shaken vigorously with 5 ml of distilled water in a test tube and warmed for 10 minutes. The formation of stable foam was indication of the presence of saponins (Bargah, 2015).

Bacteria isolation and Antimicrobial disk susceptibility test

Bacteria from internal organs of *Hoplobatrachus rugulosus* were isolated and identified following the standard procedures of Veterinary Diagnostic Laboratory, Animal Health Service Center, Faculty of Veterinary medicine, Chiang Mai University, Thailand. In vitro susceptibility test interpretive criteria were established by the Clinical Laboratory Standards Institute (CLSI, 2018). The following antibiotics against pathogens were used: amoxicillin (AML; 10 µg), chloramphenicol (C; 30 µg), ciprofloxacin (CIP; 5 µg), cephalexin (CL; 30 µg), gentamicin (CN; 10 µg), norfloxacin (NOR; 10 µg), oxytetracycline (OT; 30 µg), sulfamethoxazole (SXT; 25 µg). Interpretation of the test were sensitivity (S), intermediate sensitive (I) and resistant (R) as CLSI criteria.

Green synthesis of Cu-nanoparticles

The green synthesis of CuNPs was initiated with dissolving $CuSO_4.5H_2O$ (Univar, Ajax Finechem, Australia) in Deionized water (DI) water to obtain a blue solution of 0.05 M copper sulphate which was minimum toxic concentration on examined bacteria. Five hundred µL of copper sulphate solution was mixed with 200 µL of extract (10 mg/mL) and 300 µL of DI water.

The mixtures were refluxed at 80 °C, 30 min in dry bath 20-blocks incubator (Major Science, Orlando, Florida, USA) for activating reduction of the metallic salts. The color of dispersion gradually changed from clear transparent into yellow and then orange indicated the formation of fine nanoscales as indicated by Lv (et al., 2012). The solution was allowed to cool to the ambient temperature and was used for further characterizations.

Characterization of copper nanoparticles

The formation of CuNPs was monitored on V-1200 spectrophotometer with UV-Professional analysis software. UV-Visible spectra of CuNPs was measured in 1.5 mL disposable PMMA cuvette (BRAND GMBH + CO KG, Wertheim, Germany) in the wavelength region 320 to 1,000 nm operated at a resolution of 5 nm. The extracts were also monitored at the same concentration in DI water.

Determination of size, size distribution, and zeta potential of CuNPs

Size and size distribution (PDI) of CuNPs was determined using Zetasizer NanoZs (Malvern Instruments Ltd., UK) working on principle of photon correlation spectroscopy (PCS) as described by Sassa-deepaeng et. al., (2016) with some modified. Briefly, one milliliter of CuNPs dispersed in water was transfer into quartz cuvette (Malvern Instruments Ltd., UK) prior exposing to laser light diffraction. The intensity of peak which highest population of CuNPs was recorded. Zeta potentials of CuNPs was also measured using the same instrument in a folded capillary zeta potential cell (DTS 1060, Malvern Instruments Ltd., UK). The average results were automatically calculated by instrument.

Evaluation of antibacterial activity

Antibacterial activity was screened by modified blood agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India) well diffusion method (Omara, 2017) against five pathogen bacteria, including *Aeromonas sorbia*, *Edwardsiella tarda*, *Enterobacter* spp., *Klebsiella pneumoniae*, and *Pseudomonas* spp. The wells on agar plate created using sterile 2.5-mm diameter bead were prepared. The bacteria grew in log phase was swabbed using sterile cotton bud to make completely cover on plate. Each well was filled up by various concentrations of CuNPs to assess the activity compared to aqueous extract at the same concentration and plates were incubated at 37 °C for 24 hours. The inhibition zone was measured as mm and the mean values ± standard deviation were reported. MIC was the minimum inhibitory concentration of agent that can prevent bacteria from obvious growth after incubation on blood agar plate at 37 °C for 24 h. CuNPs at concentration of $5.00x10^{-6}$, $3.75x10^{-6}$, $2.50x10^{-6}$, and $1.25x10^{-6}$ M in aqueous were used to determine MIC. All experiments were carried on in triplicate.

Statistical analysis

Obtained data were analyzed using Microsoft Excel 2016 for Windows. The data were initially analyzed by one-way ANOVA and Duncan's mean

comparison test at the 5% significance level. All experiments were done in triplicated and presented as mean ± SD.

RESULTS

Bacteria isolation and antibiotic susceptibility testing

It was founded that the most of bacteria were resistant to traditional antibacterial drugs such as amoxicillin, chloramphenicol, oxytetracycline, sulfa-trimethoprim as indicated in Table 1.

Table 1 Drug susceptibility of bacteria isolated from bullfrog.

	Antibacterial susceptibility							
	AML	**C**	**CIP**	**CL**	**CN**	**NOR**	**OT**	**SXT**
***Aeromonas sorbia* (n=5)**	R	R	S	S	S	S	R	R
***Edwardsiella tarda* (n=5)**	I	S	R	R	S	R	R	R
***Enterobacter* spp. (n=5)**	R	S	S	R	S	S	I	S
***Klebsiella pneumonia* (n=5)**	R	R	S	S	S	S	R	R
***Pseudomonas* spp.(n=5)**	R	R	R	R	R	R	R	R

R = resistant, I = intermediate, S = sensitive, AML = amoxicillin, C = chloramphenicol, CIP = ciprofloxacin, CL = cephalexin, CN = gentamicin, NOR = norfloxacin, OT = oxytetracycline, SXT = sulfamethoxazole

Antioxidant and phytochemical properties

Antioxidant activity, total phenolic content (TPC) into phytochemical constituents and phytochemical properties of the extracts were shown in Table 2. DPPH assay for assessing antioxidant activity of *G. mangostana* revealed IC_{50} of 230 ±10 µg/mL directly related to high content of TPC value of 37.58±2.29 µg GAE/mg DW. As a result of Alsultan et al. (2017), the highest amount of flavonoids and tannins classified as phenolic compounds should be major factors in mangosteen leaf extract which were contributed to the best antioxidative activity significantly. In addition, tannins were known to have antibacterial meanwhile, the co-occurrence of alkaloids and saponins in system could be significantly reduced antioxidant activity too (Milugo et al, 2013). The correlation between TPC and antioxidant activity was also significant in *C. sinensis* leaf extract. The great DPPH IC_{50} value of 400±30 µg/mL related to high content of TPC value of 16.18±1.03 µg GAE/mg DW and related to high flavonoids content of 32.83±0.25 µg QE/mg DW. Additionally, high content of tannins and terpenoids which possessed high antioxidant properties (Selvan et al., 2018; Zengin and Baysal, 2014) were also found in *C. sinensis* leaf extract. Interestingly, the strong correlation between RP antioxidant (mg of gallic acid equivalent per mg plant dry weight) and DPPH radical scavenging activity (%) was found in all plant extracts as indicated in figure 1. The formation of CuNPs by using these materials was further investigated by using UV-Vis Spectroscopy technique.

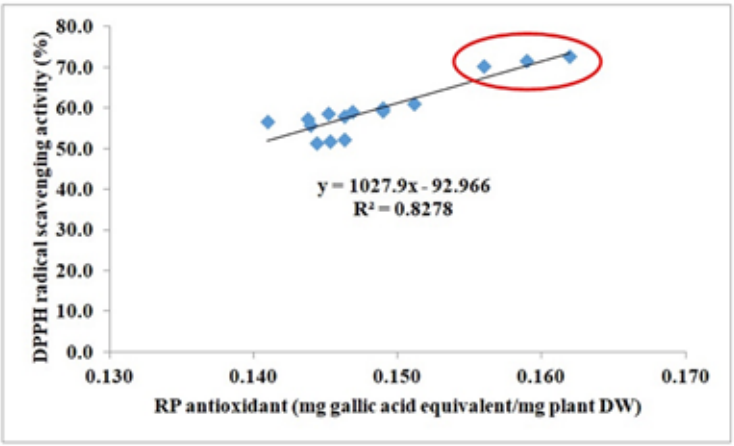

Figure 1 Correlation between RP antioxidant (mg of gallic acid equivalent per mg plant dry weight) and DPPH radical scavenging activity (%). The correlation of *G. mangostana* was revealed in circle.

Table 2 Phytochemical properties of the extracts

Plant name	IC_{50} of DPPH (µg/mL)	TPC (µgGAE/ mgDW)	Flavonoids (µgQE/ mgDW)	Alkaloids	Tannins	Terpenoids	Saponins
C. sinensis	400 ± 30^{b}	16.18±1.03^{ab}	32.83±0.25^{c}	+	++	+	+
G. mangostana	230 ± 10^{a}	37.58±2.29^{c}	82.94±6.49^{d}	-	+++	+	+
P. amarus	450±10^{b}	16.55±0.69^{ab}	27.97±3.43^{ab}	+	+	+	-
P. urinaria	430 ±10^{b}	14.95±1.51^{a}	23.94±1.16^{a}	+	+	-	-
P. virgatus	490 ±10^{c}	18.64±1.66^{b}	29.83±0.41^{b}	+	+	+	-

The data were presented as mean value ± standard deviation of triplicate analyses. Different letters in the same column indicate statistically significant values ($P < 0.05$). Mean ± SD +++ = strongly positive, ++ = positive, + = mildly positive, - = negative

UV-Vis Spectroscopy analysis

The photograph in figure 2 showed that the blue solution of $CuSO_4$ turned to green (Rajesh et al., 2018), yellow (Shikha et al., 2015), or brownish yellow color (Sua´rez-Cerda et al., 2017) according to the effect of the surface plasmon resonance (SPR) which was sensitive to size and shape of the particles (Yadav et al., 2017). Consequently, reduction of copper ions to CuNPs could be monitored by color change. From the results, the absorption spectra between 320 to 1,000 nm in UV-Vis spectroscopy of each solution showed SPR band with maximum absorbance at 340 nm as indicated by Ashtaputrey et al., (2017).

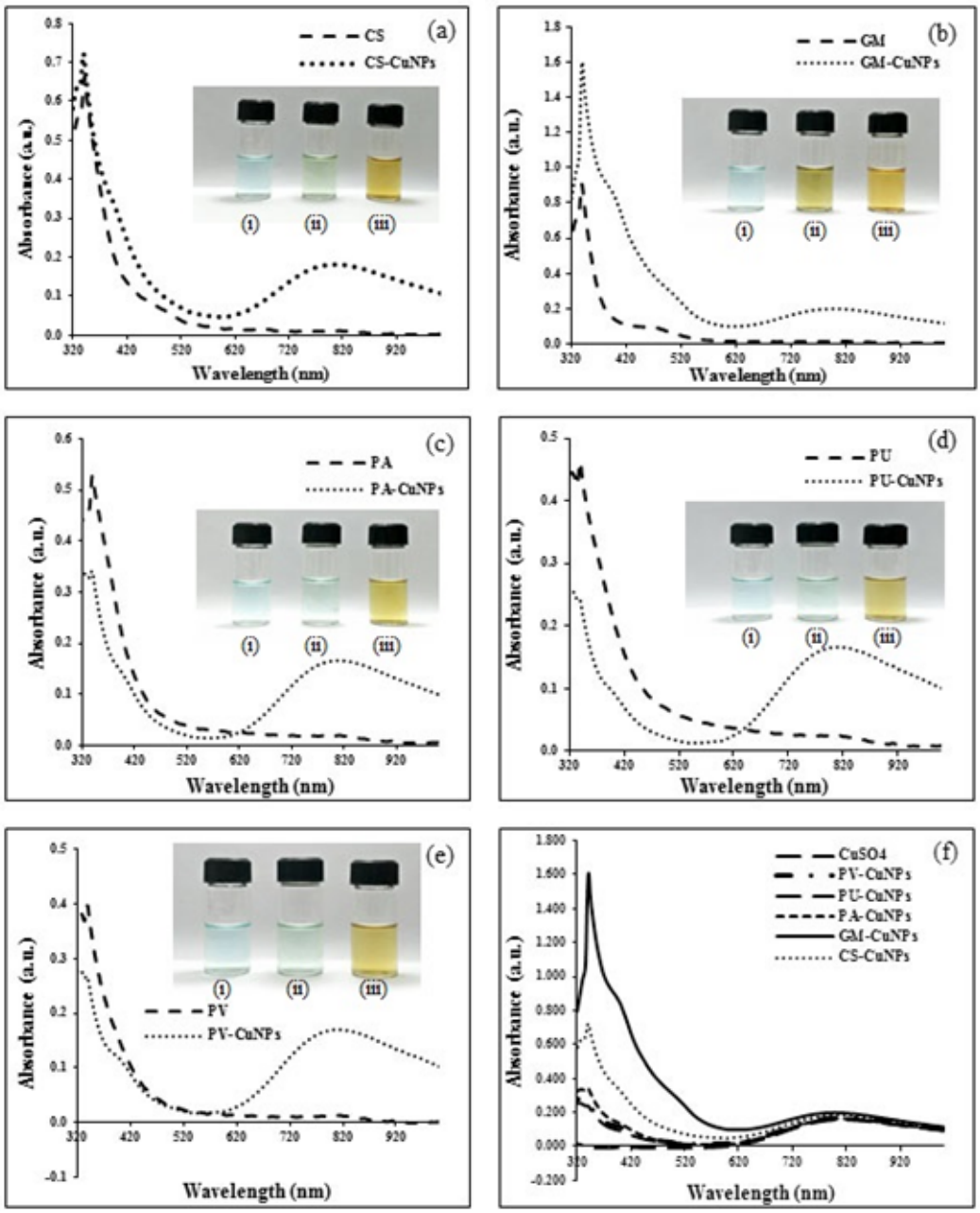

Figure 2 Ultraviolet-Visible absorption spectrum of (a) CS and CS-CuNPs (b) GM and GM-CuNPs (c) PA and PA-CuNPs (d) PU and PU-CuNPs (e) PV and PV-CuNPs and (f) $CuSO_4$ and all CuNPs intensity. (i) = $CuSO_4$, (ii) = $CuSO_4$ + extract, and (iii) = extract.

Antibacterial activity analysis

The 5 MDR bacteria isolated from bullfrog were tested at various concentrations of CuNPs to determine the antibacterial properties by blood agar well diffusion method. From the results, CuNPs possessed good antibacterial activity against gram-negative bacteria as indicated in Table 3. The great results of antibacterial activity of *G. mangostana* (GM-) and *C. sinensis* (CS-) mediated CuNPs against all 5 pathogens were obtained. Maximum clear zone of using GM-CuNPs and CS-CuNPs were achieved in *A. sorbia* with diameter of 26.0±3.5 mm and 24.7±2.3 mm, respectively and minimum clear zone were

achieved in bacteria *E. tarda* with diameter of 2.7±1.2 mm and 4.0±0.2 mm, respectively. Interestingly, only GM-CuNPs and CS-CuNPs could inhibited the growth of MDR *Pseudomonas* spp. and *A. sorbia* meanwhile PA-CuNPs, PU-CuNPs and PV-CuNPs could not be done. In general, CuNPs can generate reactive oxygen species inside bacteria cell resulted cell death. The thickness of cell wall and peptidoglycan content are also key factors on this mechanism. In addition, CuNPs can bind peptidoglycan in cell wall and interact with other membrane protein causing perforation and resulted in releasing of intracellular matrix of bacteria (Lutsenko et al., 2007). The changed environment was also indirect effect which impacted on effectiveness of CuNPs against bacteria (Naika et al., 2015). Therefore, it might be suggested that GM-CuNPs possessed strongest antibacterial activity against all MDR bacteria due to these mechanisms.

The minimum inhibitory concentration (MIC) of green synthesized CuNPs against these MDR gram negative (-ve) bacteria were determined and were indicated in Table 4. MIC values of GM-CuNPs revealed the greatest inhibitory effect at concentration of $1.25x10^{-6}$ M for *Aeromonase sorbia*, *Enterobacter* spp., and *Pseudomonas* spp., $2.50x10^{-6}$ M for *Edwardsiella tarda* and *Klebsiella pneumoniae*, similar to MIC values of CS-CuNPs which inhibited *Klebsiella pneumoniae* at concentration of $3.75x10^{-6}$ M. Unfortunately, MIC values of PA-CuNPs, PU-CuNPs and PV-CuNPs were not available for *Aeromonase sorbia* and *Pseudomonas* spp.

Table 3 Zone of inhibition (mm) of Extracts, Extract+CuNPs against 5 pathogens

Plant name	Samples	*Aeromonas sorbia*	*Edwardsiella tarda*	*Enterobacter* spp.	*Klebsiella pneumoniae*	*Pseudomonas* spp.
C. sinensis	Ext	-	-	-	-	-
	Ext+CuNPs	25±2	$4±2^{ab}$	$13±3^{abc}$	$21±1^{bc}$	22±0
G. mangostana	Ext	-	-	$8±2^{a}$	-	-
	Ext+CuNPs	26±4	$3±1^{a}$	$12±2^{ab}$	$23±1^{c}$	19±1
P. amarus	Ext	-	-	-	-	-
	Ext+CuNPs	-	$6±1^{b}$	$17±2^{c}$	$19±1^{b}$	-
P. urinaria	Ext	-	-	$20±4^{c}$	-	-
	Ext+CuNPs	-	$4±0^{ab}$	$19±3^{c}$	$16±2^{a}$	-
P. virgatus	Ext	-	-	-	-	-
		-	$5±1^{ab}$	$17±2^{c}$	$19±1^{b}$	-

Ext = Extract, CuNPs = Copper nanoparticles. Different letters in the same column indicate statistically significant values ($p < 0.05$).

Table 4 Minimum inhibitory concentration (Molar) of Extract+CuNPs against 5 pathogens

Samples	*Aeromonas sorbia*	*Edwardsiella tarda*	*Enterobacter* spp.	*Klebsiella pneumoniae*	*Pseudomonas* spp.
CS-CuNPs	$1.25x10^{-6}$	$2.50x10^{-6}$	$1.25x10^{-6}$	$3.75x10^{-6}$	$1.25x10^{-6}$
GM-CuNPs	$1.25x10^{-6}$	$2.50x10^{-6}$	$1.25x10^{-6}$	$2.50x10^{-6}$	$1.25x10^{-6}$
PA-CuNPs	-	$5.00x10^{-6}$	$2.50x10^{-6}$	$2.50x10^{-6}$	-
PU-CuNPs	-	$5.00x10^{-6}$	$2.50x10^{-6}$	$2.50x10^{-6}$	-
PV-CuNPs	-	$5.00x10^{-6}$	$2.50x10^{-6}$	$2.50x10^{-6}$	-

Dynamic Light Scattering (DLS) analysis

DLS was occupied to examined the sized distribution and the polydispersities of colloidal particles of GM-CuNPs. The single size distribution peak was obtained as shown in figure 3. It was found that size of GM-CuNPs was 254±144.9 nm with polydispersity index (PDI) of ~0.2. The dimeter of GM-CuNPs was slightly larger than reported by Yadav et al. (2017).

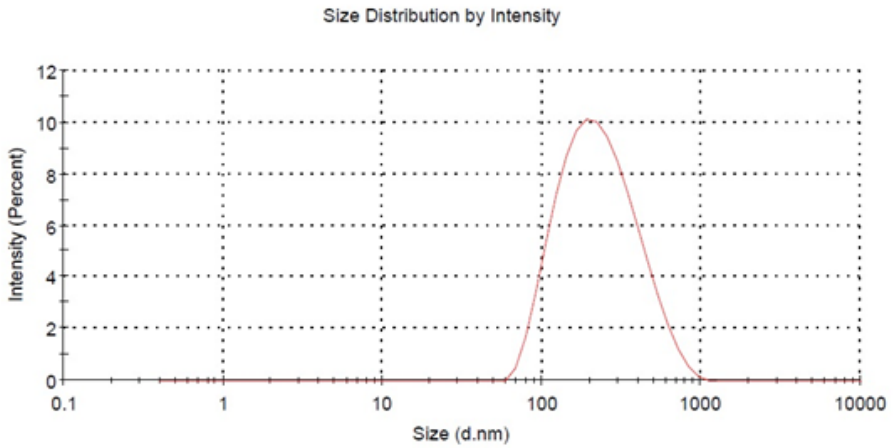

Figure 3 The size distribution by intensity of GM-CuNPs. The mean size of GM-CuNPs was 254±144.9 nm.

Zeta potential

The zeta (ζ) potential is resulted by the net electrical charge contained within the region bounded by the slipping plane of nanoparticles in colloidal dispersions. In general, the ζ potential is a parameter commonly used to assesses the stability of metal nanoparticles in solution. Particles which have a large positive or negative ζ potential are repel each other resulted are relatively stable. Unfortunately, The ζ potential of GM-CuNPs measurement elicited the appearance of maximum peak at -0.37±11.3 mV (Figure 4) indicating that the developed CuNPs by mangosteen might be less stable and have possibility for aggregation.

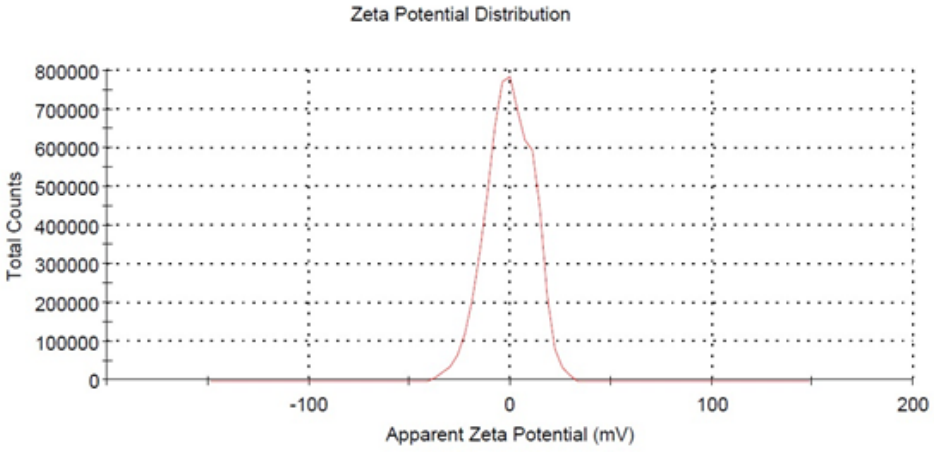

Figure 4 Zeta potential graph of GM-CuNPs. The appearance of maximum peak elicited at -0.37±11.3 mV..

DISCUSSION

All isolates from bullfrog exhibited resistance to AML which was β-lactamase inhibitor. It can be suggested that all them were seen to carry the bla_{TEM} gene as indicated by Buranasinsup et al., (2018). Moreover, most of them (except *Enterobacter* spp.) were also revealed resistance to sulfamethoxazole which was folate pathway inhibitor. Therefore, it should be assumed that the *sulII* gene was also found among them. Interestingly, the phenomena were also shown in oxytetracyclin test. Thus, it was indicated that most of them also contain *tet*(S) gene as reported in fish by Hedayatianfard et al., (2014). However, most isolates (except *Pseudomonas* spp.) were sensitive to aminoglycoside, gentamycin. Interestingly, it was also found that all bacteria were gram negative strains which were typically found in environment. Previous clinical reports of diseased frogs had implied *Aeromonas hydrophila* (Gibbs et al., 1966), *Edwardsiella tarda* (Green et al., 1999), *Pseudomonas* spp. (Glorioso et al., 1974) as potential pathogens. However, only *A. hydrophila* and *E. tarda* were reported as MDR strains in Bullfrog but not *Pseudomonas* spp.

To develop antibacterial agents in nano-size range, the highest antioxidant property was investigated in aqueous plant extracts. it was found that flavonoids, tannins and terpenoids should be important phenolic compounds which were affected on its antioxidative activity in *G. mangostana* and *C. sinensis*. The IC_{50} of antioxidant activities of *P. amarus*, *P. urinaria*, and *P. virgatus* extracts were found in lower and revealed in similar values in the range of 430.5 - 487.3 µg/mL with were positively related to high content of TPC values. These results were supported the finding of Navarro et al. (2017) and Singh et al. (2014) that flavonoids, tannins, and terpenoids were major components which were contributed to antioxidant activity. Furthermore, the result of RP antioxidant also confirmed that these extracts possessed high ferric reducing power activities positively related to radical scavenging activities as indicated in figure 1 ($R^2 = 0.8278$). After CuNPs contribution processes, The SPR band of CuNPs possessed maximum absorbance at 340 nm because of the collective oscillation of free conduction band electrons which were excited by incident electromagenetic radiation. GM-CuNPs exhibited the highest absorbance intensity indicated a high conversion of Cu^{2+} to Cu as nanoparticles and leaded to more effective capping capacity of *G. mangsostana* following *C. sinensis*, *Phyllanthus amarus*, *Phyllanthus urinaria*, and *Phyllanthus virgatus*, respectively. It was found that GM-CuNPs and CS-CuNPs had high antibacterial activity against all 5-pathogens. However, these effects might be attributed to phytoconstituents of *G. mangostana* leaf extract or *C. sinensis* leaf extract and CuNPs led to synergism of antibacterial activity.

Base on the effective dose, GM-CuNPs and CS-CuNPs possessed antibacterial properties in the range of 1-4 mM (Table 4) but antibiotics revealed this property in higher usage amount, such as amoxicillin; 27 mM, chloramphenicol; 93 mM, ciprofloxacin; 15 mM.; cephalexin; 86 mM, gentamicin; 21 mM; norfloxacin; 31 mM; oxytetracycline; 65 mM, and sulfamethoxazole; 99 mM (CLSI, 2018). Therefore, it can be concluded that the antibacterial property of CuNPs is greater than these antibacterial agents at the same concentration. Furthermore, CuNPs also exhibited in broad-spectrum bactericidal properties.

The dimeter of GM-CuNPs was slightly larger than reported by Yadav et al. (2017) who synthesized CuNPs ranging from 58.78 – 190.14 nm by using $NaBH_4$ as reducer and also was larger than reported by Chung et al. (2017) who received CuNPs ranging from 28 – 105 nm by using cupric acetate as starter. Therefore, it can be suggested that reducer and starter were important factors to produce CuNPs. However, GM-CuNPs was produced in acceptable nano-sized range. The ζ potential of GM-CuNPs measurement elicited the appearance of maximum peak at -0.37±11.3 mV (Figure 4) indicating that the developed CuNPs by mangosteen might be less stable and have possibility for aggregation. Therefore, to improve the stability of CuNPs, some researcher employed the polymer such as cyclodextrin (Mandava et al., 2017) or surfactants such as poloxamer (Chitra et al., 2015) as stabilizer in the system.

CONCLUSION

The copper nanoparticles produced by plant materials was an economical product according to the use of local plant materials as reducer and the use of commonly chemicals available in general chemistry laboratory as starter. It also possessed efficient antibacterial activity and eco-friendly due to the use of non-toxic chemicals in all process of synthesis. The results of DPPH and RP antioxidant assay indicated that *G. mangostana* had highest reducing power and had highest potential in using as reducer for CuNPs formation. UV–vis spectrophotometer has confirmed the reduction of copper(II)sulphate to copper nanoparticles. The zones of inhibition were formed in the antimicrobial screening test indicated that the GM-CuNPs synthesized has the efficient antimicrobial activity against 5-MDR pathogenic bacteria. The biologically synthesized copper nanoparticles could be of immerse use in a medical field for their efficient antimicrobial function. The present findings suggest that GM-CuNPs might be developed into promising antibacterial agent candidates for MDR bacteria in bullfrog farm.

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CONFLICT of INTEREST

The authors declare that they have no conflict of interest.

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