

ANTIMICROBIAL ACTIVITY OF COPPER KAOLINITE AND SURFACTANT MODIFIED COPPER KAOLINITE AGAINST GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

Nor Syafawani Sarah Md Saad, Nik Ahmad Nizam Nik Malek*, Chun Shiong Chong

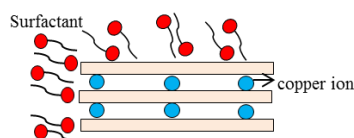
Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Malaysia.

Article history

Received
10 February 2015
Received in revised form
13 August 2015
Accepted
15 August 2015

*Corresponding author
niknizam@utm.my

Graphical abstract



Abstract

The aim of this research was to determine the antimicrobial activity of kaolinite modified with antimicrobial compounds against Gram positive and Gram negative bacteria. Copper kaolinite (Cu-kaolinite) was prepared by loading raw kaolinite with copper nitrate trihydrate (CuNO_3) while surfactant modified Cu-kaolinite (SM-Cu-kaolinite) was prepared by adding cationic surfactants hexadecyltrimethyl ammonium (HDTMA) on Cu-Kaolinite. Samples was characterized by Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) analyzer. The antimicrobial activity of the samples was tested against Gram negative bacteria (*Escherichia coli* ATCC 11229 and *Pseudomonas aeruginosa* ATCC 15442), and Gram positive bacteria (*Staphylococcus aureus* ATCC 6538 and *Enterococcus faecalis* ATCC 29212) through disc diffusion technique (DDT) and minimum inhibition concentration (MIC). The results showed that the antimicrobial activity of Cu-kaolinite increased after modified with HDTMA due to the synergistic effects of Cu ions and HDTMA molecules on the kaolinite. The antimicrobial activity for surfactant modified Cu-kaolinite was greater for Gram positive bacteria compared to Gram negative bacteria. In conclusion, the attachment of HDTMA on Cu-kaolinite contributed to the enhanced antimicrobial activity against wide spectrum of bacteria (Gram positive and Gram negative bacteria).

Keywords: Kaolinite; copper; HDTMA; antimicrobial activity

Abstrak

Tujuan kajian ini adalah untuk menentukan aktiviti antimikrob kaolinit yang telah diubahsuai dengan agen antimikrob terhadap bakteria Gram negatif dan Gram positif. Tembaga-kaolinit (Cu-kaolinit) telah dihasilkan melalui kemasukan kaolinit oleh tembaga nitrat trihidrat (CuNO_3) manakala surfaktan-tembaga-kaolinit (SM-Cu-kaolinit) telah dihasilkan dengan menambahkan surfaktan kationik heksadesiltrimetil ammonium (HDTMA) pada Cu-kaolinit. Sampel telah dicirikan melalui kaedah spektroskopi inframerah (FTIR), penyerakan sinar-X (XRD), mikroskopi imbasan elektron (SEM) dan penganalisis serakan tenaga sinar-X (EDX). Aktiviti anti-mikrob bagi sampel telah diuji dengan menggunakan bakteria Gram negatif (*Escherichia coli* ATCC 11229 dan *Pseudomonas aeruginosa* ATCC 15442), dan bakteria Gram positif (*Staphylococcus aureus* ATCC 6538 dan *Enterococcus faecalis* ATCC 29212) melalui kaedah cakera resapan (DDT) dan perencatan kepekatan minimum (MIC). Hasil kajian menunjukkan bahawa aktiviti antimikrob Cu-kaolinit telah meningkat selepas diubahsuai dengan HDTMA disebabkan oleh kesan sinergi oleh ion-ion Cu dan molekul-molekul HDTMA pada kaolinit. Aktiviti antimikrob surfaktan yang telah diubahsuai dengan Cu-kaolinit lebih berkesan terhadap bakteria Gram positif berbanding dengan bakteria Gram negatif. Kesimpulannya, HDTMA yang diubahsuai bersama Cu-kaolinit meningkatkan kesan penghalang yang tinggi terhadap pelbagai jenis bakteria (bakteria Gram positif dan negatif).

Kata kunci: Kaolinit; tembaga; HDTMA; aktiviti antimikrob

1.0 INTRODUCTION

Antimicrobial agents are used to prevent contamination or in the sterilization of contaminated surfaces to avoid the growth of pathogenic microorganisms. Nowadays, most of the household products contain antimicrobial agents that can inhibit bacterial growth or kill bacteria or other microorganism. Some of the antimicrobial agents lose their effectiveness due to the antimicrobial resistance problems [1]. Thus, the developments of new antimicrobial materials have been of great interest in recent years.

In recent decades, many researches have been done to increase the effectiveness of antimicrobial agents such as by loading antibacterial agents on a carrier system for example kaolinite [2-5]. Kaolinite is a clay mineral with the chemical composition of $\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$. The basic unit of kaolinite is tetrahedral sheet of SiO_4 and octahedral sheet with Al^{3+} as the octahedral cation [6]. This property allows kaolinite to adsorb and retain antibacterial agents and, therefore becoming suitable carrier system for the antibacterial agents. Furthermore, it has high ion-exchange capacity, high surface area, sorption capacity, negatively charge, low in toxicity and chemical inertness [7].

Modification of montmorillonite with copper ion showed the antibacterial effect of the clay that act as single antimicrobial carrier [8]. Metallic ions such as Ag^+ and Cu^{2+} are loaded into the ceramic matrix by ion-exchange [7]. The adsorption capacity varies from metal to metal and it depends on the type of clay and modified clay that is being used [6]. From the previous studies, copper have the ability to disrupt the cell function. Copper ions will absorbed to the bacteria cell surface and will penetrate through the cell membrane and inhibit the bacteria replication [9].

The combination of the two antimicrobial agents will enhance the antimicrobial activity when both of the ions are loaded into the same carrier [5]. Kaolinite and bentonite have a capability as a support system for copper ions and cetyltrimethyl ammonium bromide (CTAB) and used as antibacterial agents [5]. Thus, in this study the usage of natural kaolinite and two antimicrobial agents which were heavy metal (Cu) and cationic surfactant has been investigated for its antibacterial activity.

2.0 EXPERIMENTAL

2.1 Chemicals and Materials

Kaolinite KM40 was supplied from Kaolin (M) Sdn. Bhd. from Perak, Malaysia. Copper nitrate trihydrate ($\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$) and cationic surfactant hexadecyltrimethyl ammonium bromide (HDTMA) were purchased from Qrec and Sigma Aldrich, respectively. Luria Bertani (LB) broth and Muller Hinton (MH) agar from Bacto Difco (BD) were used as a medium and agar, respectively in antimicrobial assay.

2.2 Preparation of Materials

The preparation of copper modified kaolinite (Cu-kaolinite) was basically done to allow Cu^{2+} intercalated in the kaolinite framework. The procedure for the preparation of Cu-kaolinite followed method described elsewhere [10] where the loading capacity was based on 50% of CEC of kaolinite. About 0.2341 g of $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ (Sigma Aldrich) was weighed and dissolved in 1000 mL of distilled deionized water. The Cu solution was then added with 20 g of raw kaolinite. The mixture was stirred for 16 hours and then, the homogenized mixture was then filtered by using pump vacuum and dried in an oven at 80°C overnight. The dried solid product was crushed into powder using mortar and pestle and sieved for further application. Based on the suggested method by the patent document [10], the initial concentration of surfactant (HDTMA) that need to be loaded on kaolinite was in excess and more than the critical micelle concentration (CMC) of the HDTMA. Thus, in the preparation of surfactant modified kaolinite (SM-kaolinite) and surfactant modified Cu-kaolinite (SM-Cu-kaolinite), 0.3532 g of HDTMA that has been dissolved in 1000 mL of distilled water was mixed with the raw kaolinite and Cu-kaolinite, respectively. The rest procedure followed the procedure for the preparation of Cu-kaolinite.

2.3 Characterization of Materials

All samples were characterized using Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD), and energy dispersive X-ray (EDX) analyzer. Thermo Scientific Nicolet iS10 with Smart iTR Diamond crystal FT-IR spectrophotometer equipped with Attenuated Total Reflectance (ATR) was used for the structural analysis. About 1-2 mg of sample was placed on a sample area. The FTIR spectrum was then recorded using OMNIC software in the range of 4000 to 650 cm^{-1} . The samples also were characterized by XRD (X-ray diffraction) instrument (Bruker, D8 advance). The XRD patterns were recorded with a CuK α radiation at $\lambda = 1.5406\text{ \AA}$ at 40 kV

and 20 mA in the range of $2\theta = 5^\circ$ to 35° with a scanning speed of 0.05° per second. The morphology of kaolinite and modified kaolinite was taken by CARL ZEISS 35 VP Supra Field Emission Scanning Electron Microscopy (FESEM).

2.4 Antimicrobial Assay

2.4.1 Disc Diffusion Technique

The antimicrobial activity of Cu-kaolinite and SM-Cu-kaolinite were evaluated by disc diffusion technique (DDT). The antimicrobial activity of the samples was tested against Gram negative bacteria (*Escherichia coli* ATCC 11229 and *Pseudomonas aeruginosa* ATCC 15442) and Gram positive bacteria (*Staphylococcus aureus* ATCC 6538 and *Enterococcus faecalis* ATCC 29212). The bacteria stock cultures were purchased from American Type Culture Collection (ATCC). Bacteria culture was prepared from streaks on a nutrient agar plate. Bacteria colonies were suspended in 0.9% saline solution. The inoculum turbidity was adjusted equivalent to 0.5 McFarland standard. The plate was covered entirely with bacteria by streaking the bacteria on plate. 3 mg of disc samples that were prepared by using hydraulic pressure were placed on the Mueller-Hinton plate. All plates were incubated overnight at an incubation temperature of 37°C .

2.4.2 Minimum Inhibition Concentration

Antimicrobial activity of the samples was studied by determination of minimum inhibition concentration (MIC) against the same bacteria as in DDT. 10% of bacteria culture was transferred in 90 mL of fresh LB and shaken at 200 rpm at 37°C until reached $\text{OD}_{550\text{nm}}$ at the range 0.6-0.8. Bacteria solution was prepared using distilled water and 0.9% saline solution. 5 mL of bacteria solution was added in the flask containing different range of concentration (0.3, 0.6, 0.9, 3, 6, 9, 12 g/L). All samples were incubated about 30 minutes. After 30 minutes, 10 μL of bacteria solution was dropped on MH agar and the lowest concentration that can kill the entire bacteria was determined.

3.0 RESULTS AND DISCUSSION

3.1 Characterization of the Cu-kaolinite and SM-Cu-kaolinite

3.1.1 Fourier Transform Infrared (FTIR) Spectroscopy

Figure 1 shows the FTIR spectra of unmodified and modified kaolinite. The general O-H stretching absorption bands for kaolinite are shown at 3695, 3669 and 3652 cm^{-1} [11]. The Si-O-Si vibration of the tetrahedral sheet and Si-O-Al stretching vibration of the octahedral sheet shows band below 1000 cm^{-1} [2]. For the detection of the presence of the cationic surfactant molecules on kaolinite, the onset in Figure 1

shows the FTIR spectra of the cationic surfactant, HDTMA. The FTIR spectra of kaolinite treated with surfactant shows additional absorptions peaks at 2850 and 2920 cm^{-1} [2-3]. The former was attributed to symmetric and the latter for asymmetric stretching vibrations of C-H in the alkyl chain of HDTMA [2-3]. These peaks are evidences of the modification of kaolinite by HDTMA.

Based FTIR spectra, it is proved that the kaolinite was successfully modified with surfactant. The FTIR spectra at the range of $1200\text{-}500\text{ cm}^{-1}$ are obviously shown that there is no difference between FTIR spectra of kaolinite and Cu-kaolinite. Therefore, it shows that the copper ions intercalated into the kaolinite by the ion exchange process without changing their structure.

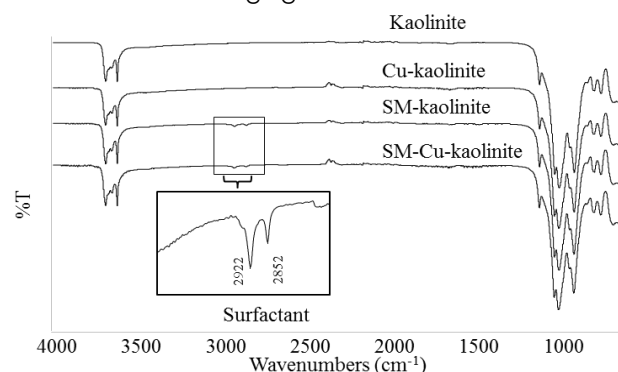


Figure 1 Infrared spectra of kaolinite, Cu-kaolinite, SM-kaolinite and FTIR

3.1.2 X-Ray Diffraction (XRD)

Figure 2 shows XRD diffractograms of modified kaolinite. Based on Figure 2, XRD patterns between Cu-kaolinite and SM-Cu-kaolinite show the same patterns between parent clay even though the kaolinite has been modified with copper ion and surfactant. The structure remains the same after modification because the cations are adsorbed onto the kaolinite via ion-exchange reaction and will not affect the structure of kaolinite. In XRD patterns from previous works, the XRD of surfactant modified kaolinite did not show much changes compared to that of unmodified kaolinite [2,5].

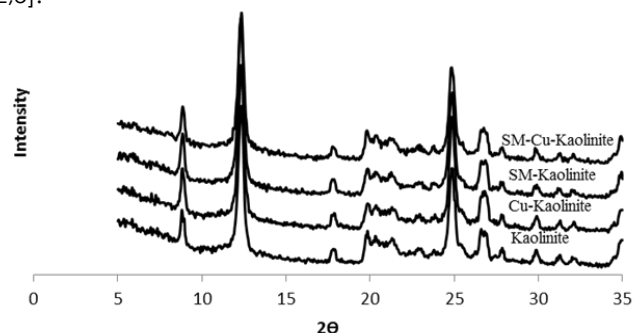


Figure 2 XRD patterns of kaolinite, Cu-kaolinite, SM-kaolinite and SM-Cu-kaolinite

3.1.3 Energy Dispersive X-Ray (EDX) Analyzer

The presence of Cu ions in the studied materials was detected by Energy Dispersive X-Ray (EDX). Figures 3 and 4 show EDX spectra of the studied samples. The common elements exist in kaolinite are oxygen (O), aluminium (Al), and silica (Si). In Figures 3 and 4, the EDX spectra have shown the presence of Cu element and thus, Cu-kaolinite and SM-Cu-kaolinite samples has been confirmed to contain Cu ions. EDX spectra were collected randomly from the selected points on the surface of the modified kaolinite. From the EDX spectra, the presence of 0.67% of Cu^{2+} for Cu-kaolinite and 0.19% for SM-Cu-kaolinite were detected.

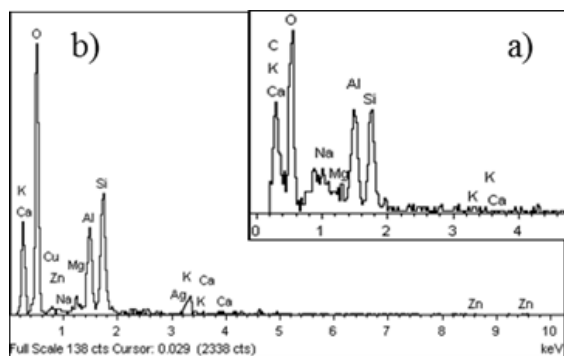


Figure 3 EDX spectra of a) Kaolinite b) Cu-kaolinite

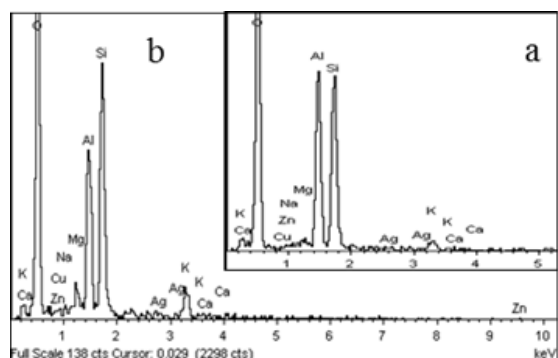


Figure 4 EDX spectra of a) SM-kaolinite b) SM-Cu-kaolinite

3.1.4 Scanning Electron Microscopy (SEM)

Figures 5 and 6 show SEM micrograph of Cu-kaolinite and SM-Cu-kaolinite. The morphological characteristics of the raw and modified kaolinite is plate-like and has many layers. There are no changes in term of the morphology of modified kaolinite with that of raw kaolinite. This was due to the preparation process of modified kaolinite which only involves the ion exchange process. The copper ions and cationic surfactant molecules are attached on kaolinite via ion exchange [5] without distorting the main framework of kaolinite, thus the modification process does not affects the morphology of kaolinite. This result also supports the results from FTIR spectroscopy and XRD.

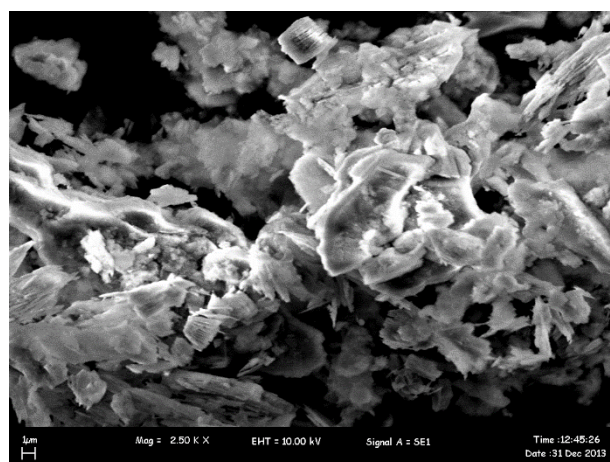


Figure 5 SEM micrographs of Cu-kaolinite magnified at 2500 times

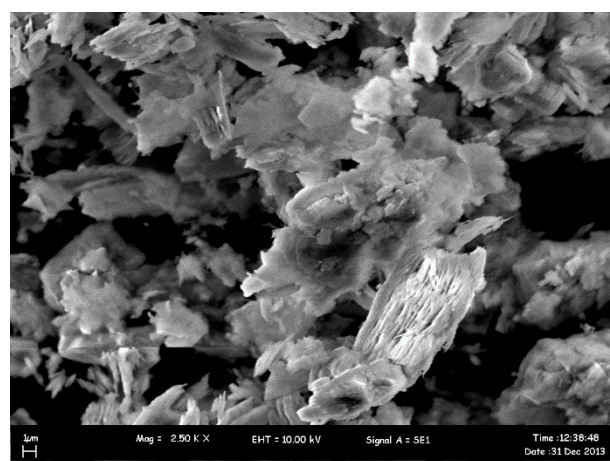


Figure 6 SEM micrographs of SM-Cu-kaolinite magnified at 2500 times

3.2 Antimicrobial Activity

3.2.1 Disc Diffusion Technique

Results from Table 1 show an absence of antimicrobial effects for all samples of Cu-kaolinite and SM-Cu-kaolinite against Gram negative bacteria. Gram negative bacteria are not susceptible to any studied materials compare to Gram positive bacteria since the structure of the cell membrane of Gram negative bacteria is more complex than Gram positive ones [5].

Therefore, high concentration of the copper ion is required to penetrate the membrane wall of Gram negative bacteria and eventually, kill or inhibit the bacteria [12]. In Table 1, Cu-kaolinite showed no antimicrobial activity against *E. faecalis* and *S. aureus*, but when the Cu-kaolinite is modified with cationic surfactant, it shows the antimicrobial activity.

Table 1 Inhibition zone values from DDT

Strain	Inhibition zone(mm)			
	Kao	S-Kao	Cu-Kaolinite	SM-Cu-Kaolinite
Gram (-ve) <i>E. coli</i>	-	-	-	-
<i>P. aeruginosa</i>	-	-	-	-
Gram (+ve) <i>E. faecalis</i>	-	19.16	14.16	19.16
<i>S. aureus</i>	-	17.5	-	17.5

Notes: Initial diameter: 13.5mm; -; - no inhibition

The bacteria were killed by electrostatic forces. The electrostatic forces occur when the negatively charged bacteria membrane is attracted to the positively charged copper ion that kills or inhibits the bacteria growth [13]. The structure of the cell wall of the bacteria will be destroyed and become uneven after treated with copper-montmorillonite [14].

3.2.2 Minimum Inhibition Concentration

The antimicrobial activity of Cu-kaolinite and SM-Cu-kaolinite was determined by the determination of MIC against Gram negative and Gram positive bacteria in saline solution and distilled water as shown in Table 2. The investigation was performed in distilled water and saline solution in order to observe the effect of electrolyte (saline solution) on the antibacterial activity of the studied sample.

Table 2 MIC values of Cu-kaolinite and SM-Cu-kaolinite against Gram negative and Gram positive bacteria

Strain	Cu-kaolinite (g/L)		SM-Cu-kaolinite (g/L)	
	Saline	Distilled water	Saline	Distilled water
Gram (-ve) <i>E. coli</i>	>12	>12	>12	3
<i>P. aeruginosa</i>	>12	>12	>12	6
Gram (+ve) <i>E. faecalis</i>	>12	>12	0.9	3
<i>S. aureus</i>	>12	>12	0.9	3

All samples show the absence of the antimicrobial effect for Cu-kaolinite for both saline solution and distilled water. However, the combination of both antimicrobial agents show positive results for SM-Cu-kaolinite in saline solution and distilled water. This might be due to the anion exchange process where the cationic surfactant can adsorb the bacteria cells on the surface of kaolinite and eventually, kill the bacteria. Cationic surfactant and copper ions attached on the clay framework structure will further enhance the antibacterial activity because of the synergistic effect of the antibacterial agents [5]. The antibacterial activity of SM-Cu-kaolinite was higher in distilled water for the Gram positive bacteria and vice versa for the Gram negative bacteria. This showed that the electrolyte in the bacteria solution affects the

antibacterial activity of SM-Cu-kaolinite. Further studies are needed to explain this effect.

4.0 CONCLUSION

Modification of Cu-kaolinite with HDTMA enhanced the antimicrobial activity of Cu-kaolinite against Gram positive and Gram negative bacteria. Therefore, the modified kaolinite with two antibacterial agents could become an alternative inorganic antibacterial agents.

Acknowledgement

Authors would like to acknowledge Ministry of Education (MoE), Malaysia for financial support under Prototype Research Grant Scheme (PRGS) (Vot No: 4L619) and Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia.

References

- [1] Giuliano, C. A. and Rybak, M. J. 2015. Efficacy of Triclosan as an Antimicrobial Hand Soap and Its Potential Impact on Antimicrobial Resistance: A Focused Review. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*. 35(3): 328-336.
- [2] Malek, N. A. N. N. and Ramli, N. I. A. 2015. Characterization and Antibacterial Activity of Cetylpyridinium Bromide (CPB) Immobilized on Kaolinite with Different CPB Loadings. *Applied Clay Science*. 109: 8-14.
- [3] Li Z. and Gallus, L. 2005. Surface Configuration of Sorbed Hexadecyltrimethylammonium on Kaolinite as Indicated by Surface and Counterion Sorption, Cation Desorption, and FTIR. *Colloid and Surfaces A: Physicochemical and Engineering Aspects*. 264(1): 61-67.
- [4] Holešová, S., Valášková, M., Hlaváč, D., Madejová, J., Samilková, M., Tokarský, J. and Pazdziora, E. 2014. Antibacterial Kaolinite/urea/chlorhexidine Nanocomposites: Experiment and Molecular Modelling. *Applied Surface Science*. 305: 783-791.
- [5] Malek, N. A. N. N., Ishak, S. A. and Kadir, M. R. A. 2013. Antibacterial Activity of Copper and CTAB Modified Clays against *Pseudomonas aeruginosa*. *Advanced Materials Research*. 626: 178-182.
- [6] Bhattacharyya, K. G. and Gupta, S. S. 2008. Adsorption of a Few Heavy Metals on Natural and Modified Kaolinite and Montmorillonite: a Review. *Advances in Colloid and Interface Science*. 140: 114-131.
- [7] Magana, S. M., Quintana, P., Aguilar, D. H., Toledo, J. A., Angeles-Chavez, C., Cortes, M. A., Leon, L., Freile-Pelegri, Y. and Sánchez, R. M. T. 2008. Antibacterial Activity of Montmorillonites Modified with Silver. *Journal of Molecular Catalysis A: Chemical*. 281(1): 192-199.
- [8] Özdemir, G., Limoncu, M. H. and Yapar, S. 2010. The Antibacterial Effect of Heavy Metal and Cetylpyridinium-Exchanged Montmorillonites. *Applied Clay Science*. 48(3): 319-323.
- [9] Raffi, M., Mehrwan, S., Bhatti, T. M., Akhter, J. I., Hameed, A., Yawar, W. and ul Hasan, M. M. 2010. Investigations into the Antibacterial Behavior of Copper Nanoparticles against *Escherichia coli*. *Annals of Microbiology*. 60(1): 75-80.
- [10] Malek, N. A. N. N. and Ramli, N. I. A. 2013. Antibacterial agent. Malaysia Patent. PI 2013700023.
- [11] Tunney, J. J. and Detellier, C. 1994. Preparation and Characterization of Two Distinct Ethylene Glycol Derivatives of Kaolinite. *Clay and Clay Minerals*. 42(5): 552-560.

- [12] Malachova, K., Praus, P., Rybkova Z. and Kozak, O. 2011. Antibacterial and Antifungal Activities of Silver, Copper and Zinc Montmorillonites. *Applied Clay Science*. 53(4): 642-645.
- [13] Hu, C.H. and Xia, M. S. 2006. Adsorption and Antibacterial Effect of Copper-Exchanged Montmorillonite on *Escherichia Coli*, K₈₈. *Applied Clay Science*. 31(3): 180-184.
- [14] Tong, G., Yulong, Ma., Peng, G. and Zirang, Xu. 2005. Antibacterial Effects of the Cu(II)-Exchanged Montmorillonite on *Escherichia Coli* K₈₈ And *Salmonella Choleraesuis*. *Veterinary Microbiology*. 105(2): 113-122.