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Effect of Hexadecyltrimethylammonium (HDTMA) Loading on Organo-Silver-Kaolinite on Antibacterial Activity against *Cutibacterium acnes*

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Abstract

Acne vulgaris is brought on by *Cutibacterium acnes*. In order to find out how varied HDTMA concentrations added to silver kaolinite affect its ability to combat *C. acnes* bacteria, this investigation was carried out. Silver-kaolinite (AgKaol) was treated with HDTMA in the following proportions: one part AgKaol to 0.001, 0.005, 0.01, 0.02, and 0.05 parts HDTMA. Fourier Transform Infrared (FTIR), Scanning Electron Microscope (SEM) with Energy Dispersive X-ray Spectroscopy (EDX), X-ray PowderDiffraction (XRD), and Transmission Electron Microscopy (TEM) were used to characterise the produced materials. n-Hexane and dH2O were mixed in a bilayer to study the dispersion behaviour. Disk Diffusion Test (DDT) and Minimum Inhibitory Concentration (MIC) tests were carried out for the antibacterial assay. The characterization results revealed that Ag^+ and HDTMA attached successfully to kaolinite. As the concentrations of HDTMA rose, the hydrophilicity of the organo-silver-kaolinite gradually changed to hydrophobic. According to the results of the antibacterial assays, HDTMA at a concentration of 0.01 on AgKaol is the best concentration for preventing the growth of *C. acnes* and performs best under dH2O conditions. By targeting *C. acnes*, organo-silver-kaolinite is an antibacterialagent that has been shown to have a synergistic effect that boosts its antibacterial activity in comparison to silver-kaolinite without the addition of HDTMA.

Keywords: HDTMA; Organo-Silver-Kaolinite; *C. acnes*; Antibacterial Activity

Introduction

Acne is a common problem for teens, young adults, and even adults. Acne vulgaris may be brought onby *Cutibacterium acnes*, which prefers anaerobic conditions for growth and lives deep within the skin follicle and sebaceous gland. Our skin may have an immune reaction from their extracellular products (Zouboulis, 2004). As a result of the extensive use of oral and topical antibiotics to treat acne and otherchronic skin conditions, which *C. acnes* did not involve, strains of *C. acnes* resistant to tetracyclines, macrolides, and lincosamides arose (McLaughlin et al., 2019).

Therefore, it's critical to design a new antibacterial product that is vulnerable to *C. acnes* in orderto combat acne issues across a range of age groups. While kaolinite is a suitable carrier to carry the antibacterial chemicals to the targeted bacterium, silver ion (Ag^+ is known to have antibacterial characteristics. This is due to the negatively charged surface of kaolinite, which can be

replaced by cations like Ag^+ (Isah et al., 2020). The bioavailability of Ag^+ determines how effective silver is against bacteria. Ag^+ can readily interact with proteins, cell membranes, and microbiological cell walls (Edwards-Jones, 2009). In response to Ag^+, hydroxyl radicals that denature proteins bacteria and harmtheir DNA and lipids, ultimately causing cell death (Gordon, 2010). Ag^+ can also produce sensitization, however this is rarely reported. Because silver affects numerous target locations within the bacterial cell, the formation of bacterial resistance through mutation is improbable. In a prior investigation, HDTMA was found to boost the antibacterial activity of modified silver-kaolinite. Furthermore, Gram- positive bacteria were more susceptible to silver-kaolinite modified with HDTMA than Gram-negative bacteria (Saad et al., 2016). As a result, it may have the potential to become a susceptible antibacterialagent in the fight against *C. acnes*. Silver-based samples have been found to be a good antibacterial agent; nevertheless, the low concentration of $AgNO_3$ utilised due to high concentration restrictions canpromote bacterial resistance. Because $AgNO_3$ is very soluble in water and can provide a large amount of Ag^+ (Salim & Malek, 2017), it is commonly employed in the creation of antibacterial products. As a result, the addition of HDTMA to silver-based samples can provide a synergistic effect that boosts antibacterial activity without utilising a high concentration of AgNO3 and prevents the production of insoluble precipitates (Asraf et al., 2020)

Nonetheless, because HDTMA can be skin-damaging to humans, the quantity of HDTMA loadingin silver-based samples should be examined to discover the optimum concentration that can provide the best antibacterial action without creating additional skin issues (Hrenovic & Ivankovic, 2007). *C. acnes* was used to assess the antibacterial activity of organo-silver-kaolinite. If organo-silver-kaolinite is shown to be successful in inhibiting the growth of *C. acnes*, this may imply that organo-silver-kaolinite in antibacterial treatments can inhibit the formation of acne vulgaris in humans.

Materials and methods

For this study, raw kaolinite (Kaol KM88C) was used, and silver-kaolinite (AgKaol) was prepared beforeadding the various concentrations of HDTMA. In 2000 ml of distilled water (dH_2O), 0.35 g of AgNO3 wasadded. The mixture was then stirred overnight with 50 g of Kaol KM88C. The AgKaol sample was then used to make organo-silver-kaolinite with varying concentrations of HDTMA. HDTMA was added to silver-kaolinite (AgKaol) in the following proportions: one part AgKaol to 0.001, 0.005, 0.010, 0.02, and 0.05part HDTMA.

The chemical bonds in each prepared sample, including unmodified kaolinite (Kaol), were identified using Perkin Elmer Fourier Transform Infrared (FTIR) Spectrometer. A potassium bromide (KBr) pellet for each sample was prepared with the ratio of sample to KBr was 1:100. The surface morphology of the prepared samples was examined using a Scanning Electron Microscope (SEM) withEnergy Dispersive X-ray (EDX) Spectroscopy at Universiti Tun Hussein Onn Malaysia (UTHM), Pagoh.The sample was coated with gold (Au). Before transferring the sample into the instrument, the samplewas fixed on a carbon conductive tape.

The X-ray Powder Diffraction (XRD) technique was also used to identify the phases of the synthesized sample by using a Bruker AXS GmH (German) machine. The diffractogram obtained fromunmodified kaolinite, AgKaol, and HDTMA-Ag-Kaol were stacked and compared. Transmission Electron Microscopy (TEM) was utilised to get the microscopic images of samples' internal frameworks. Its focused electron beam allows electrons to pass through the sample's thickness. Before imaging, thesamples were coated with Au using a Leica EM ACE200 vacuum coater. The dispersion behaviour of the prepared sample was studied by adding 5 mg of each sample in the mixture of 2 ml n-hexane and 2 ml dH_2O. The position of each sample particle was observed immediately after adding to the mixture,after shaking for 30 minutes, and after being left in static condition for 24 hours.

Cultivation of *C. acnes* ATCC® 6919™ was conducted according to the instruction provided by the product KWIK-STIK™ by Microbiologics®. The saturated *C. acnes* swab was transferred to

the blood agar plates. Four primary culture plates were inoculated by gently rolling the swab over one-thirdof the plates. Streaking was done with a sterile loop to achieve single colonies. The primary culture plates were placed upside down into the Oxoid anaerobic jar, supplemented with Anaero GenTM 2.5L and anaerobic indicator, and incubated in a 37°C incubator.

The antibacterial activity of HDTMA-modified silver-kaolinite was determined by conducting a Disk Diffusion Test (DDT) and Minimum Inhibitory Concentration (MIC) test. DDT was conducted according to Kirby-Bauer Disk Diffusion Susceptibility Test Protocol, due to the anaerobic requirementand media preference of *C. acnes*, reinforced clostridial agar was used instead of Mueller Hinton (MH)agar plate. The antibacterial disks were placed on the surface of the agar and incubated as mentionedin the above paragraph. Meanwhile, for the MIC test, a dropped plate technique was conducted in twoconditions which were 0.9% saline solution and dH_20. The different weights of each prepared sample and information on positive and negative controls are given in Table 1.

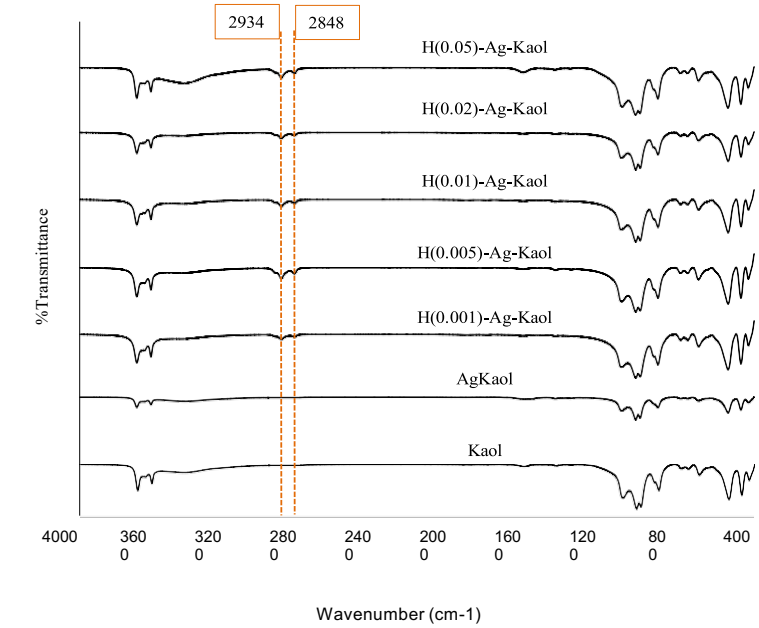
Table 1: Weights of prepared samples for MIC test.

Control	**Concentration**	
Kanamycin sulphate (positive control)	50 mg/ml soaked into filter paper disk	
Kaol KM88C (negative control)	0.050 g	
Samples	**Concentration**	
	Low weight (g)	**Increased weight (g)**
AgKaol, H(0.001)-Ag-Kaol, H(0.005)-Ag-Kaol, H(0.01)-Ag-Kaol, H(0.02)-Ag-Kaol, and H(0.05)-Ag-Kaol	i. 0.005	vi. 0.030
	ii. 0.010	vii. 0.035
	iii. 0.015	viii. 0.040
	iv. 0.020	ix. 0.040
	v. 0.025	x. 0.050

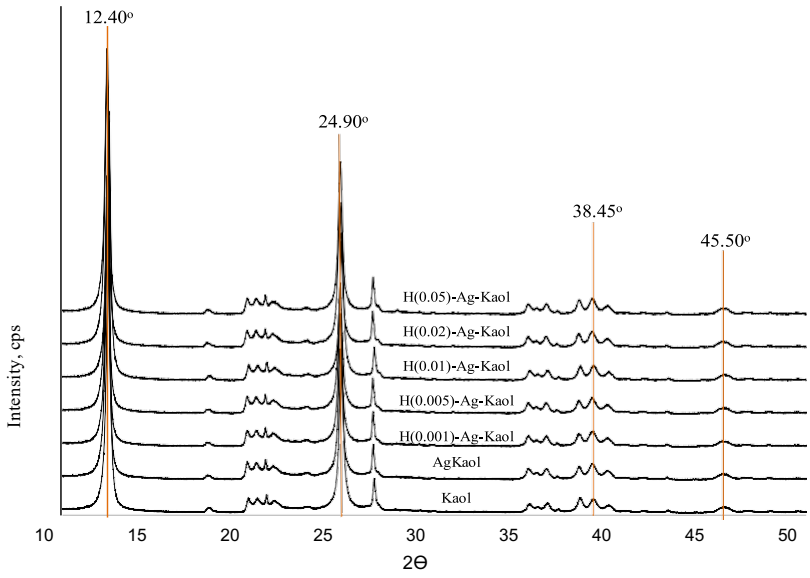
Results and discussion

Based on FTIR spectra in Figure 1 (a), bands ranging from 1200 cm^{-1} to 400 cm^{-1} denote the fingerprint region of kaolinite, indicating the existence of Si-O-Si, Si-O-Al, hydroxyl groups, and aluminium-oxygenlinkages in all prepared samples (Malek et al., 2019). Thus, the same FTIR bands in the fingerprint areafor all samples demonstrate that the modification of kaolinite with Ag^+ and HDTMA did not disrupt the structure of the kaolinite since there was no noticeable difference in the FTIR spectra of modified kaolinite compared to unmodified kaolinite (Malek et al., 2019). The presence of bands at 2934 cm^{-1} and 2848 cm^{-1} were expected for samples with the addition of HDTMA, due to C-H bonds in the alkyl group of HDTMA (Asraf & Malek, 2020). Based on XRD patterns in Figure 1 (b), the presence of significant peaks at 12.40°, 24.90°, 38.45°, and 45.40° indicate the structure of kaolinite (Asraf & Malek,2020). This shows that the original structure of the kaolinite did not affect by the addition of Ag^+ and HDTMA since there is no significant change in the XRD patterns of AgKaol and HDTMA-Ag-Kaol fromthe unmodified kaolinite.

The SEM image of a sample H(0.005)-Ag-Kaol in Figure 2 (a) demonstrates the morpohology ofkaolinite and also when added with HDTMA. Unmodified kaolinite is more defined and has neat intercalating layers. An HDTMA-modified silver-kaolinite looked more rough, disordered and jagged. This is because the surface of the samples modified with HDTMA has small-size broken edges to facilitate HDTMA adsorption (Asraf & Malek, 2020). Meanwhile, the TEM image in Figure 2 (b) shows the area of a sample where the silver compound was detected (shown as blackened dots). The detected silver compounds can be silver metal (Ag) or Ag_2O (Sadasivam & Rao, 2016). Moreover, the increase in the percentage of carbon atomic mass was successfully detected by the EDX



(a)



(b)

Figure 1 FTIR spectra (a) and X-ray diffractogram (b) of prepared samples.

according to the HDTMA increasing concentration as shown in Table 2. HDTMA is an organic compound, rich in carbonbackbone which justify the carbon elements found in EDX.

Furthermore, for the dispersion behaviour of the prepared samples (image not shown), sample Kaol, AgKaol, and H(0.001)-Ag-Kaol appeared to be dispersed at both layers (n-hexane and dH_2O), forming a cloudy suspension immediately after addition to the mixture. However, the samples are resting at the top layer (n-hexane) as they progress from H(0.005)-Ag-Kaol to H(0.05)-Ag-Kaol, indicating that the amount of HDTMA added is now sufficient to change the sample's dispersion property from hydrophilic to hydrophobic (Isah et al., 2020). The particles of Kaol, AgKaol, and H(0.001)-Ag-Kaolsettled at the bottom layer after 30 minutes of shaking. The kaolinite particles separated and settled to their lowest possible locations (Gunatilake & Bandara, 2017). Meanwhile,

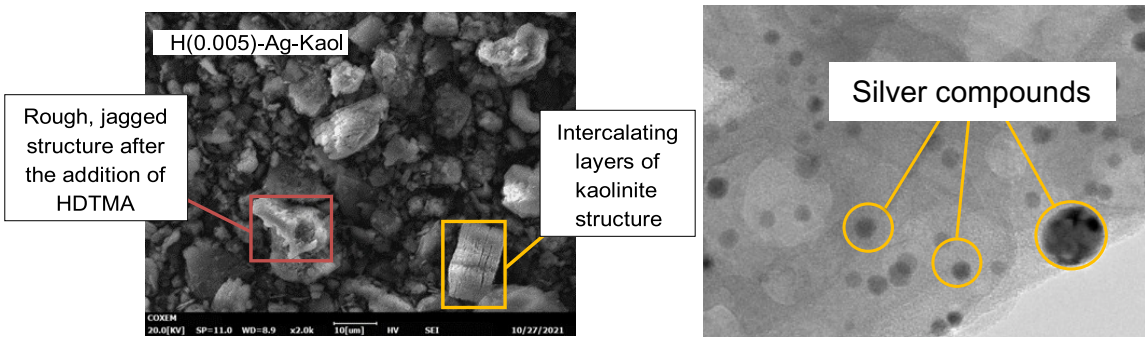


Figure 2 SEM image (a) and TEM image (b) of sample H(0.005)-Ag-Kaol.

Table 2: EDX result for carbon atomic mass in HDTMA-Ag-Kaol.

Sample	Carbon atomic mass (%)
Kaol	-
AgKaol	-
H(0.001)-Ag-Kaol	ND
H(0.005)-Ag-Kaol	20.62 ±6.72
H(0.01)-Ag-Kaol	25.11
H(0.02)-Ag-Kaol	26.39 ±2.19
H(0.05)-Ag-Kaol	26.74 ±2.14

sample H(0.005)-Ag-Kaol, which was previously located at the top layer, is now located at the bottom layer, with a few particles remaining at the top layer. This is because the HDTMA concentration is insufficient to keep the samplehydrophobic during agitation. Samples H(0.01)-Ag-Kaol until H(0.05)-Ag-Kaol, on the other hand, remained unchanged. Except for H(0.005)-Ag-Kaol, all samples remained after 24 hours in the static condition. Because of the gravitational force acting on the particles, it appeared that all of its particles had been sedimented at the bottom layer (Gunatilake & Bandara, 2017). When the concentration of HDTMA was increased, the hydrophilicity of kaolinite and AgKaol gradually changed to become more hydrophobic.

Furthermore, the AgKaol sample without HDTMA could not demonstrate antibacterial activity in the DDT antibacterial assay. This could be because the release of Ag+ from kaolinite caused by the solid-to-solid interaction was ineffective at delivering antibacterial agents to the target bacteria or attracting bacteria to adhere to the sample (Fomina & Skorochod, 2020). The inhibition zone size was significantly increased when the lowest HDTMA concentration, H(0.001)-Ag-Kaol, was added. This demonstrates that adding HDTMA to AgKaol boosts its antibacterial activity. Furthermore, increasing the HDTMA concentration to 0.02 and 0.05 parts in 1 part AgKaol resulted in no significant enlargement of the inhibition zone. As shown in Figure 3 and Table 3, the most significant increase in inhibition zone diameter occurred at an HDTMA concentration of 0.01 part in 1 part AgKaol.

The Minimum Inhibitory Concentration (MIC) test was conducted to test the susceptibility of the prepared samples against *C. acnes* in an aqueous form. Therefore, two conditions which were dH_2O and 0.9% saline solution, were used to determine the samples' efficacy. MIC values were determined when the *C. acnes* colonies started to show a slight decrease in their colonies. According to Table 4, the MIC values of susceptible samples are lower (better) in dH_2O conditions compared to 0.9% saline solution. This is due to chloride ion (Cl^-) in the saline solution; insoluble precipitates like AgCl can form, and reduce the antibacterial action of Ag^+ (Asraf et al., 2020). Besides, the AgKaol sample and low

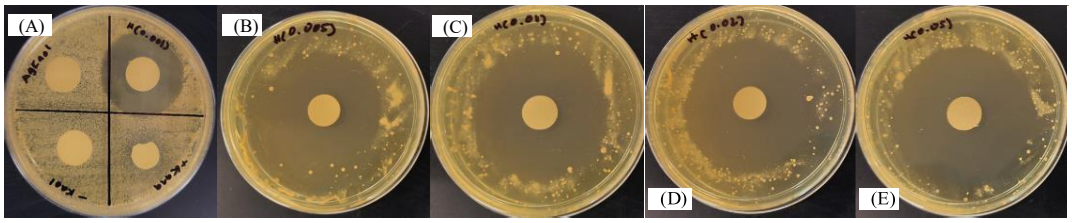


Figure 3 *C. acnes* inhibition zones by HDTMA-Ag-Kaol with different HDTMA concentrations.

Table 3: Diameter of *C. acnes* inhibition zones by HDTMA-Ag-Kaol with different HDTMA concentrations.

Samples	Inhibition zones (cm)
Kanamycin sulphate 50 mg/ml (Positive control)	0.0
Kaol KM88C (Negative control)	0.0
AgKaol	0.0
H(0.001)-Ag-Kaol	3.5
H(0.005)-Ag-Kaol	5.0
H(0.01)-Ag-Kaol	5.6
H(0.02)-Ag-Kaol	5.7
H(0.05)-Ag-Kaol	5.9

Table 4: MIC values of unmodified Kaol, AgKaol, and HDTMA-Ag-Kaol against *C. acnes*.

Samples	MIC values (g)	
	dH_2O condition	**0.9% saline solution condition**
Kanamycin sulphate 50 mg/ml (Positive control)	Not susceptible	Not susceptible
Kaol KM88C (Negative control)	Not susceptible	Not susceptible
AgKaol	> 0.050	> 0.050
H(0.001)-Ag-Kaol	> 0.050	> 0.050
H(0.005)-Ag-Kaol	> 0.050	> 0.050
H(0.01)-Ag-Kaol	0.025	0.035
H(0.02)-Ag-Kaol	0.015	0.025
H(0.05)-Ag-Kaol	0.015	0.025

concentration of HDTMA in H(0.001)-Ag-Kaol and H(0.005)-Ag-Kaol were unable to show MIC values even in the highest weight tested in this research. This means a higher weight beyond 0.05 g is required for these samples. Moreover, similar to DDT, the increased concentration of HDTMA in the sample of H(0.02)-Ag-Kaol and H(0.05)-Ag-Kaol did not show significantly better results where MIC values for both concentrations were 0.015 g in dH_2O and 0.025 g in saline solution. In a summary, the MIC value was shown by H(0.01)-Ag-Kaol, in which the bacterial colonies started to reduce in numbers for both dH_2O and saline conditions.

Conclusion

Based on the characterization results, the appearance of new peaks in the FTIR spectra of HDTMA-modified samples indicates the attachment of HDTMA on kaolinite, while TEM images prove the appearance of silver compound attached on kaolinite. The XRD and SEM-EDX results however are not

significant in showing any changes in the kaolinite framework after adding Ag^+ and HDTMA. This indicates that the addition of Ag^+ and HDTMA did not alter the kaolinite's original framework, and the attachment of Ag+ and HDTMA on kaolinite can be declared successful. Besides, when the concentration of HDTMA was increased, the hydrophilicity of kaolinite and AgKaol progressively became more hydrophobic. When HDTMA-modified silver-kaolinite samples were tested for antibacterial assays, the HDTMA concentration of 0.01 part in 1 part AgKaol seems to be the optimum concentration to inhibit the growth of *C. acnes*. Thus, the HDTMA-modified silver-kaolinite is a susceptible antibacterial agent in inhibiting the growth of *C. acnes* and has proven to have a synergistic effect that increases its antibacterial activity compared to silver-kaolinite without the addition of HDTMA. Moreover, when the HDTMA concentration increased the diameter of inhibition zones also increased while the MIC values decreased.

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