Synthesis of ZnO-Ag with Clove Oil Using Ultrasonication Method and Its Antibiofilm Activity Against Klebsiella pneumoniae

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Submission date: 30-Mar-2021 10:45AM (UTC+0800)
Submission ID: 1546006860
File name: Nisyak_2020_IOP_Conf._Ser.__Mater._Sci._Eng._833_012081_2.pdf (644.55K)
Word count: 3856
Character count: 20661

Synthesis of ZnO-Ag with Clove Oil Using Ultrasonication Method and Its Antibiofilm Activity Against *Klebsiella pneumoniae*

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Abstract. ZnO-Ag nanocomposite synthesis has been successfully carried out by ultrasonication using clove oil as a reducing agent. Synthesis reactions were carried out in 30, 60, 90, 120, 240, and 300 minutes time variations. ZnO-Ag crystallinity was analyzed using X-Ray Diffractometer (XRD) and the average particle size was calculated using Scherrer equation. The morphology of ZnO-Ag which has the smallest particle size was analyzed by Scanning Electron Microscope - Energy dispersive X-Ray Spectroscopy (SEM-EDX). ZnO-Ag antibiofilm activity against *Klebsiella pneumoniae* was determined by microtiter plate test. Based on research that has been done, the best time for synthesizing ZnO-Ag with clove oil through ultrasonication is 90 minutes, the resulting particle size of 21.46 nm with the composition of 51.4% Ag (cubic), 20% wulfingite (orthorhombic), 22.9% $Zn(OH)_2$ (orthorhombic), and 5.7% ZnO (cubic). ZnO-Ag has strong antibacterial activity against *K. pneumoniae* and can inhibit the growth of *K. pneumoniae* biofilms.

1. Introduction

Biofilms are bacterial cell colonies that attach to inert materials, metals, plastics, and even to human body tissues by synthesizing extracellular polysaccharides, called Extracellular Polymeric Substances (EPS) [1]. Biofilm-forming bacteria can live in conditions of nutrient deficiencies, able to prevent bacterial phagocytosis, inhibit antibody responses, and resistant to antibiotics and disinfectants. The occurrence of infections is related to biofilm forming bacteria, making it difficult in terms of therapy and often causing resistances that lead to death [2]. *Klebsiella pneumoniae* is one of the Gram negative bacteria. It can forms biofilms that cause nosocomial infections which are difficult to cure because they are related to the presence of antibiotic resistance [3]. Biofilms from *K. pneumoniae* cause infections in patients who have immune disorders [4].

The main source of the transmission of *K. pneumoniae* infection is through medical equipment which is less sterile [5]. Several studies have reported the disease outbreaks in hospitals due to *K. pneumoniae* isolates that are resistant to third generation cephalosporins, aminoglycosides, and quinolones [6]. The emergence of various diseases caused by biofilm forming bacteria has encouraged the continued exploration of active materials that can be developed into antibiofilm agents, one of which is nanomaterial from metal composites.

Nowadays the development of materials used to deal with the problem of nosocomial infections in public health services is directed at nanoscale functional materials, one of which is nanocomposite. Nano-sized material has a high surface area and specific reactivity so that it is suitable to be used as a therapeutic agent for a disease in the health field [7]. A nanocomposite is a solid material composed of two or more materials which have different properties from one another and produce different characteristics and properties from the constituent material [8]. Nanocomposites in the health sector can be functionalize as antimicrobials [9]. The material that has been widely studied and has the potential as an antimicrobial is ZnO and silver. ZnO has been shown to have active antimicrobial activity against Gram-positive, Gram-negative, and fungal pathogens [10]. ZnO has unique properties, biocompatible, and low toxicity [11]. Silver shaped nanoparticles have a high surface area and stronger antibacterial activity in damaging bacterial cell walls, inhibiting cell synthesis, as well as disrupting bacterial cell metabolism [12]. Technological innovations to form ZnO-Ag nanocomposites can be functionalized as antibiofilm agents that can inhibit the formation of biofilms, prevent the attachment of biofilms to material, and destroy biofilms.

Development of green synthesis methods for the manufacturing of nano materials using natural product compounds as bioreductors and stabilizing agents continues to be explored. The green synthesis method is considered to be environmentally friendly, effective, and better yield produced [13]. The main requirements for chemical compounds from natural products that can be used as reducing agents are compounds containing electron-rich functional groups such as hydroxyl groups (-OH), carboxyl groups (-COOH), alkene groups, and benzene, for example in flavonoid group compounds [14]. The use of major chemical compounds in essential oils as reducing agents in the synthesis of nanoscale materials has not been widely reported. Research from Azizi et al (2016) reports zerumbone compounds in ginger oil can be used as reducing agents and stabilizers in ZnO-Ag synthesis [15].

In this research, the reducing agent used for ZnO-Ag synthesis is clove oil. Clove oil is one of the typical essential oils from Indonesia which contains eugenol compounds (Figure 1), phenylpropanoid compounds that have benzene rings, methoxy groups, hydroxyl groups, and double bonds. The presence of electron-rich functional groups in eugenol can be a reducing and stabilizing agent in ZnO-Ag synthesis. Important aspects in the green synthesis method are the efficient use of energy, reagents that are not excessive, and short reaction times to produce the best synthesis products, one of which is to utilize ultrasonic waves [16]. Utilization of ultrasonic waves can cause acoustic cavitation, increase product purity, modify the selectivity of starting materials, and break particles to the smallest size [17].

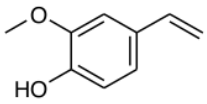

Figure 1. Eugenol from clove oil [18].

Synthesis of ZnO-Ag with clove oil is carried out using the one-pot synthesis method. The synthesis process is carried out in one container with a continuous stage using ultrasonic waves. One of the strengths of the pot synthesis method is that it is economical, efficient, and applicable to industrial scale. The ZnO-Ag produced was characterized by its particle size, crystallinity, and morphology. ZnO-Ag antibiofilm activity against *K. pneumoniae* was tested by microtiter plate test. ZnO-Ag Antibiofilm activities included prevention tests for biofilm adhesion, inhibition of biofilm formation, and biofilm degradation. The profile of ZnO-Ag antibiofilm activity can be the basis for developing safe and effective nanocomposite-based health equipment.

2. Experimental Section

2.1. Material and Instrumentation

The materials used in this research were silver nitrate (Merck), zinc acetate dihydrate (Merck, clove-leaf oil (CV. Nusaroma Depok), aquadest (Brataco Chemical), ethanol (Merck), ammonia (Merck), *K. pneumoniae* isolate from Tropical Disease Center of Airlangga University (ATCC 700603), nutrient broth (Merck), Muller Hinton agar (Merck), and crystal violet (Merck). The instrumentations used in this research were ultrasonicator (40 kHz, 120 W), oven (DGG 9053A), analytical balance (Ohaus px224/E), X-Ray Diffractometer (Panalytical X'Pert Pro, XRD), scanning electron microscope – energy dispersive X-Ray spectroscopy (SEM-EDX, Hitachi FLEXSEM 1000), gas chromatography-mass spectrophotometer (Shimadzu, GC-MS QP2010), and microplate reader.

2.2. Synthesis ZnO-Ag with clove oil

ZnO-Ag was synthesized using modification method according to previous report [19]. A 15 mL of clove-leaf oil was added to a flask containing 50 mL ethanol and sonicated for 30 minutes. Then 0.003 M of zinc acetate dihydrate was added dropwise to the mixture. Ammonia was added until the pH of solution was 9 and sonicated for 3 hours. ZnO-Ag formation begins by adding 0.001 M silver nitrate solution dropwise and sonicated for 30 minutes. The resulting reaction product is centrifugated and the precipitate obtained was washed with ethanol and dried at 110 °C for 2 hours. The reaction with ultrasonication after the addition of silver nitrate was carried out in the time variations of 30, 60, 90, 120, and 240 minutes.

2.3. Characterization of reaction product

ZnO-Ag crystallographic analysis was performed with XRD and morphological analysis with SEM-EDX. The diffractogram obtained was identified by its shape form using Match software version 3.8.3.151 and Origin Pro. The average crystallite size was calculated by using Scherrer's equation as following:

$$I = (0{,}9\lambda) / (\beta\cos\theta) \quad (1)$$

where I stand for grain size (nm), λ for wavelength of the XRD, β for FWHM, and θ for Bragg's angle. The degree of crystallinity was calculated by plotting crystalline area with total area of diffractogram as

$$\text{Degree of crystallinity} = (\text{Area of crystalline} / \text{Total area}) \times 100\% \quad (2)$$

2.4. Antibiofilm Test

A total of 200 µL of ZnO-Ag solution was put into the microplate, then the microplate was closed and incubated at 37 °C for 1 hour. The contents of the microplate were removed and inserted by 200 µL of the bacterial suspension. 200 µL of nutrient broth were put into the microplate closed and incubated at 37 ºC for 24, 48, 72, and 96 hours. After incubation, the microplate is removed and washed with distilled water for three times. The microplate was given a 200 µL of 1% violet crystal and incubated for 15 minutes. Violet crystal dye was washed and rinsed with distilled water for three times. Subsequently 200 µL of ethanol were added to the microplate and incubated for 15 minutes. The absorbance of microplate was measured using microplate reader at λ of 595 nm. The negative control used was DMSO 9.8% solution and positive control used was meropenem as antibiotics. The test was carried out in triplo. The percentage of biofilm inhibition was calculated using this formula:

$$\%\ \text{biofilm inhibition} = ((\text{OD negative control} - \text{OD sample}) / \text{OD negative control}) \times 100\% \quad (3)$$

where OD is optical density from microplate reader.

3. Result and Discussion

3.1. Synthesis ZnO-Ag with clove oil using ultrasonication method

ZnO-Ag synthesis with clove oil consists of two reaction stages, namely the reaction of ZnO formation continued with the addition of silver nitrate solution to form ZnO-Ag. The whole reaction process is carried out in one container so it can be called as one pot synthesis. The content of clove-leaf oil was analyzed by GC-MS to determine its eugenol levels. Based on chromatogram in Figure 2, clove-leaf oil contains three main compounds namely eugenol 71.41%, trans-caryophyllene 25.96%, and alpha-humulene 2.63%. The presence of high levels of eugenol in clove-leaf oil, acts as a bioreductor and stabilizer of the ZnO-Ag synthesis reaction.

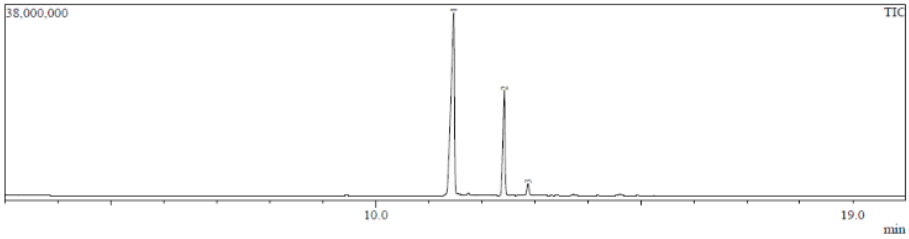

Figure 2. Total Ionic Chromatogram (TIC) of clove-leaf oil.

The use of ultrasonic waves in synthesis reactions encourages physical and chemical changes in the liquid medium and cause acoustic cavitation. The effect of ultrasonic waves in the material synthesis reaction process can produce magnetic particles and break down large crystal aggregates into small sizes to the nanoscale. ZnO formation is done by adding zinc acetate solution to a mixture of ethanol and clove-leaf oil. The formation of ZnO is strongly influenced by the alkaline condition so it needs to be conditioned by the pH of alkaline solution with ammonia. Ammonia and pH play an important role in the formation of ZnO [20]. The formation of ZnO-Ag is done by adding silver to the ZnO that has been formed. The variation of the time reaction is used to determine the effect of time reaction to phase crystal and its particle size.

3.2. Phase identification and crystallinity of ZnO-Ag

ZnO-Ag obtained from ultrasonication reaction was analyzed for type compound, phase identification, particle size, and crystallinity using XRD. In Figure 3, the diffractogram of the all times reactions variations is analyzed for the difference in peaks. At an angle 2θ of 38º and 44º, the difference at each reaction time can be analyzed. The diffraction peaks at 2θ of 38º and 44º, identify the existence of cubic structure Ag according to JCPDS No. 04-0783. At the reaction time of 90 minutes and 300 minutes, the peak at 2θ of 38º is higher than the other reaction times, which shows the high level of Ag in ZnO-Ag.

ZnO-Ag phase identification was done using Match! software from the diffractogram to obtain the results which are shown in Figure 4. Match! software shows that there are differences in the composition of ZnO-Ag elements at each reaction time variation. ZnO-Ag nanocomposites consists of five phases that is hexagonal zinc oxide, orthorhombic $Zn(OH)_2$ (wuelfingite), trigonal (hexagonal axes) $Zn(OH)_2$, hexagonal Ag and cubic Ag. Based on the results of the phase identification, ZnO-Ag with the most AgNP composition were obtained at the reaction time of 90 minutes (51.4%). Whereas, ZnO-Ag with the most ZnO-NP composition was obtained during the reaction time of 30 minutes (33.5%). The side product of this synthesis was $Zn(OH)_2$ which is produced most during the reaction time of 30 minutes. It was consisted of 14.9% trigonal (hexagonal axes) $Zn(OH)_2$ and 17.6% orthorhombic $Zn(OH)_2$ (Wuelfingite). Based on these results, the composition of $Zn(OH)_2$ was inversely proportional to the composition of ZnO, so there was a possibility of an equilibrium reaction between ZnO and Zn $(OH)_2$ during the synthesis process. The best composition of ZnO-Ag is shown at the reaction time of 90

minutes which consists Ag of 51.4% (cubic), wulfingite of 20% (orthorhombic), $Zn(OH)_2$ of 22.9% (orthorhombic), and ZnO of 5.7% (cubic).

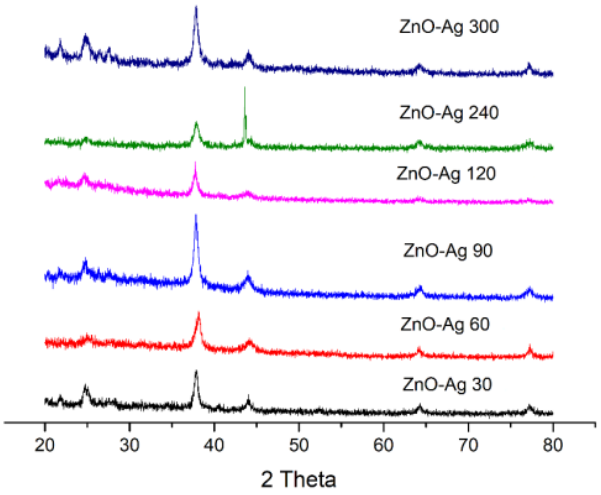

Figure 3. Diffractogram of ZnO-Ag.

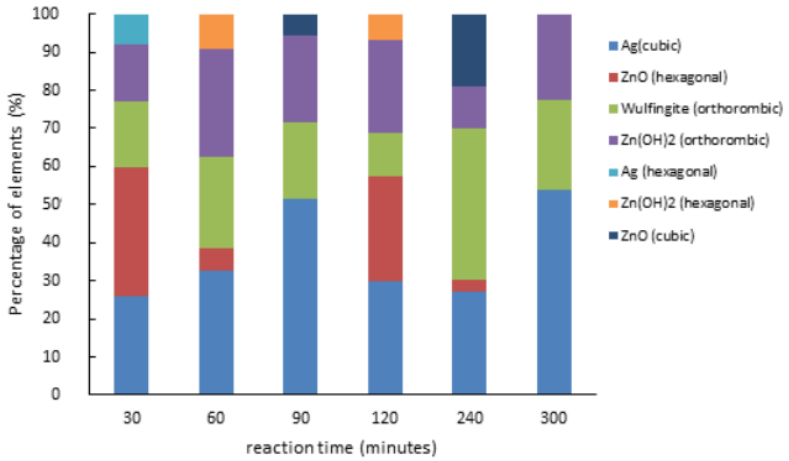

Figure 4. Composition of ZnO-Ag.

The average particle size was determined using the Scherrer equation and degree of crystallinity (%) was calculated using the Origin Pro and Microsoft Excell software. The results of the calculations are shown in Table 1. ZnO-Ag with the smallest average particle size and the highest degree of crystallinity were obtained at a reaction time of 90 minutes, which was 21.46 nm and 24% of degree crystallinity. Based on the data in Table 1, the degree of crystallinity of all ZnO-Ag was categorized to low, because there is no calcination process in the synthesis ZnO-Ag, so not all $Zn(OH)_2$ is converted to ZnO. The

size range of ZnO-Ag was from 21.46 nm to 44.67 nm in diameter according to Debye-Scherer's equation.

Table 1. Average particle size and degree crystallinity of ZnO-Ag.

No	Reaction time (minutes)	Average Particle Size (nm)	Crystallinity (%)
1	30	24.98	18
2	60	42.45	18
3	90	21.46	24
4	120	25.54	17
5	240	44.67	19
6	300	22.38	23

3.3. Morphology of ZnO-Ag

The morphology of the ZnO-Ag nanocomposite form was analyzed by SEM to observe the position of Ag and ZnO in the nanocomposite. The morphology of ZnO-Ag synthesized with a reaction time of 90 minutes is shown in Figure 5, in which Ag particles attached to ZnO particles. ZnO looks like fiber, while silver is cuboid. Based on the EDX spectrum in ZnO-Ag, there are Zn (38%), Ag (38%) and O (24%) elements. The presence of ultrasonic waves in the process of formation reaction can act as a source of reaction energy and vibrations that can split particles into small sizes.

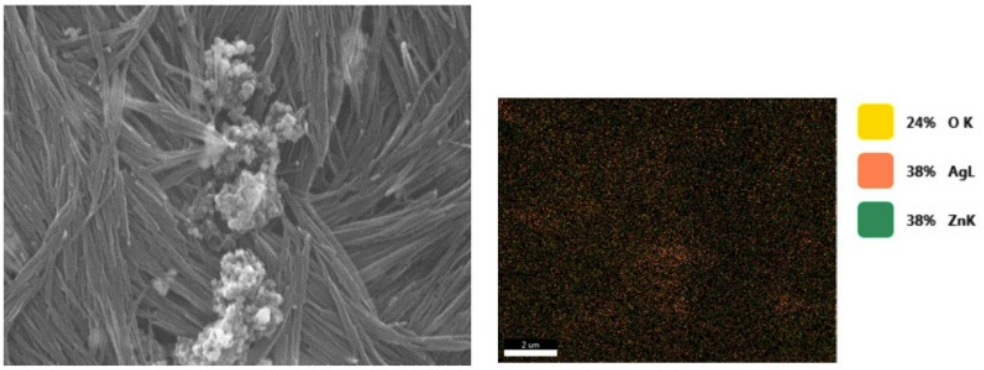

Figure 5. Micrographs SEM of ZnO-Ag with a magnification of 20,000x.

Based on the data analysis using diffractogram of XRD and micrograph of SEM-EDX, ZnO-Ag can be formed properly. Eugenol acts as reducing agent in the formation of ZnO-Ag, the functional group in eugenol can reduce Ag^+ ions to Ag particles. The mechanism for the reaction of ZnO-Ag formation is shown in Figure 6. $Zn(OH)_2$ complexes formed in nanoscopic metabolites occur through electron transfer from hydroxyl groups to the coordinates of transition metal bonds to form coordinating covalent bonds. Complex compounds formed and contained electrons in their allyl groups can give their free electron pairs to Ag^+ ions, so that Ag^+ ions can be reduced and form Ag nanoparticles. In addition, trans-caryophillene and alpha-humulent compounds in clove oil are also thought to reduce Ag^+ ions to form Ag nanoparticles. Furthermore, the formed $Zn(OH)_2$ complex can become ZnO nanoparticles through a thermal decomposition process using ultrasonic waves.

3.4. Antibiofilm activity of ZnO-Ag to K. pneumoniae biofilm

ZnO-Ag antibiofilm activity against *K. pneumoniae* was performed by microtiter plate assay method. ZnO-Ag was made in series of concentrations of 10 – 100 ppm and reacted with biofilm *K.*

pneumoniae that has been formed. Interactions between ZnO-Ag and *K. pneumoniae* biofilms were observed at incubation times of 24, 48, 72, and 96 hours. ZnO-Ag antibiofilm activity at 24 hours incubation was determined as antibacterial activity, 48 hours incubation time as inhibitor of biofilm attachment, 72 hours incubation time as an effort to prevent biofilm maturation, and 96 hours incubation time as biofilm degradation. The results of ZnO-Ag antibiofilm activity against *K. pneumoniae* are shown in Figure 7.

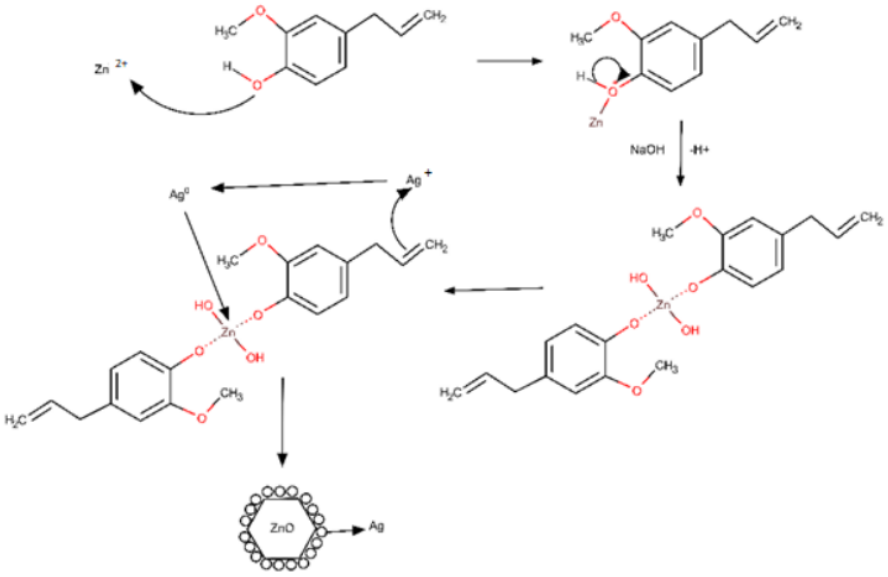

Figure 6. Reaction mechanism of Zn-Ag nanocomposite formation with clove oil.

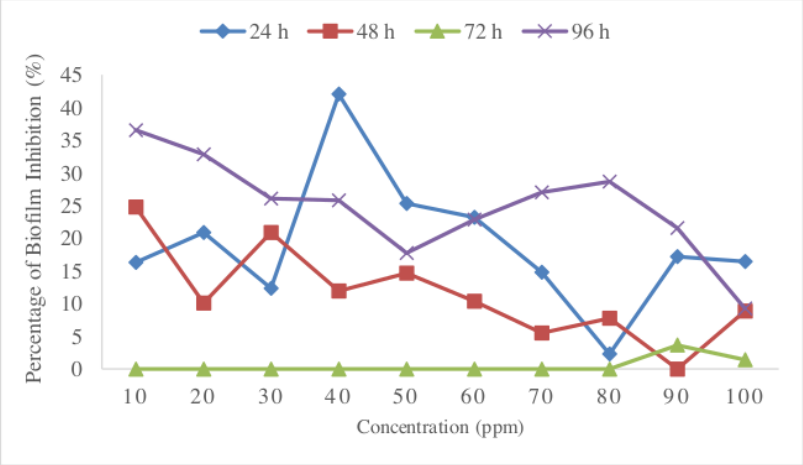

Figure 7. Antibiofilm Activity of ZnO-Ag to *K. pneumoniae* biofilm.

Based on the graph shown in Figure 7, the observation of ZnO-Ag antibiofilm activity against *K. pneumoniae* at 24 hours incubation showed that the concentration of 40 ppm gave the greatest inhibitory value of 42.02%. This value indicates that ZnO-Ag is an antibacterial agent against *K. pneumoniae*. On the observation at 48 hours incubation time, ZnO-Ag showed the best activity at a concentration of 10 ppm which could inhibit the attachment of biofilms to the surface of material by 24.76%. ZnO-Ag can destroy *K. pneumoniae* biofilms at a concentration of 10 ppm with a biofilm inhibition value of 36.54%. The results obtained show that ZnO-Ag concentration is not proportional to its antibiofilm activity. These results can be influenced by the condition of the *K. pneumoniae* biofilm and external influences in the optical density measurement process.

ZnO-Ag nanocomposite antibiofilm activity is influenced by the crystal shape, particle size, and surface area. There are four interactions in ZnO-Ag antibiofilm mechanism that cause bacterial cell death and prevent biofilm attachment. The first interaction is the interaction of ZnO-Ag with bacterial cell membranes by releasing Ag^{+} and Zn^{2+} ions on the bacterial cell membrane, then the formation of reactive oxygen species (ROS) which causes damage to the bacterial cell membrane. The interaction of Ag^{+} ions with bacterial cell ribosomes inhibits bacterial protein synthesis. ZnO-Ag interacts intracellularly with bacterial cell membranes and causes bacterial cell death [14]. The combination of these interactions is thought the inhibition of biofilms formation and prevention of biofilms attachment to the material. This is because bacterial cells undergo lysis due to interactions with ZnO-Ag.

4. Conclusions

ZnO-Ag has been synthesized via a facile green method using essential oil of clove through ultrasonication method. The main component in clove oil can play a role as a reducing agent in the formation of ZnO-Ag. The best time for synthesis of ZnO-Ag with clove oil through ultrasonication method is 90 minutes. The resulting particle size is 21.46 nm with the composition Ag of 51.4% (cubic), wulfingite of 20% (orthorhombic), $Zn(OH)_2$ of 22.9% (orthorhombic), and ZnO of 5.7% (cubic). ZnO-Ag has strong antibacterial activity against *K. pneumoniae* and can inhibit the growth of *K. pneumoniae* biofilms. For further research, it is recommended to perform calcination procedures to form ZnO so that the crystallinity is good.

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Acknowledgements
The author thanks to the Ministry of Research, Technology and Higher Education who has provided research funding 2019 through *Penelitian Kerjasama Perguruan Tinggi* with contract number 113/SP2H/LT/DRPM/2019. The author also thanks Dr. Elvina Dhiaul Iftitah, M.Sc. and Dr. Arie Srihardyastutie, M.Kes. from Universitas Brawijaya who provided guidances and directions in this research.

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