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The Exploration of C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene as Antibacterial Agent

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Abstract

The research has been conducted to determine the ability of C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene as antibacterial agent. The investigation has also studied the increasing its effectiveness due to the presence of Ag(1) in the calix complex. The antibacterial activity assay of the C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene and C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene-Ag(I) complex against *Staphylococcus aureus* and *Escherichia coli* was carried out by measuring the inhibition zone diameter using the paper disc diffusion method. The result showed that the C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene its self does not have antibacterial activity toward *Escherichia coli* in various calix concentration of 10%, 15%, 20%, 25%, and 30%. However, the compound has moderate antibacterial activity against *Staphylococcus aureus.* The existence of Ag(I) metal in calix compound complex cause the increasing in antibacterial activity for both of gram-positive bacteria, i.e. *Escherichia coli*, and gram-negative bacteria of *Staphylococcus aureus.* The complex calix-Ag(I) compound indicated strong response for both *Staphylococcus aureus* and *Escherichia coli.*

Keywords: *C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene; Ag(I) complex; antibacterial activity; paper disc diffusion; strong response*

1. Introduction

The interaction between humans and their environment can cause contact with bacteria, viruses, fungi and various other forms of parasitic life. One result of the interaction between humans and microbes can cause infectious diseases. Infectious disease is a problem in the health sector that develops over time and can be transmitted from one person to another or from animals to humans [1,2]. Infectious diseases can occur due to the entry of microorganisms, which in general, consisting of bacteria, fungi and protozoa, enter the body and will reproduce so that they can cause various kinds of diseases [3]. Infections often endanger human life. Lots of pathogenic microorganisms, the cause of infection such as bacteria, viruses, parasites or fungi, one of which is bacteria [4]. The pathogenic bacteria that most often cause infections include Staphylococcus aureus and Escherichia coli. Various methods have been done to prevent or treat infectious diseases caused by bacteria, this treatment can be overcome with the use of antibiotics/ antibacterial compounds. Many effective chemicals act as antibacterial agents and have been used as antibiotics. Increasing resistance to existing medicines has mankind looking for a new, more effective drug with safety profile. In recent years, the medical chemistry of macrocyclic compounds has been actively developing [5,6,7]. Macrocyclic compounds, especially for calixarenes, can interact with biological targets [8,9] through spatially dispersed interaction. This phenomena is due to the large size, structure, cavity, and the possibility of simple modification by reseptor fragments [10,11]. During the past decades, macrocyclic compounds such as cyclodextrins, cucurbituril, porphyrin, macrocyclic peptides, calyxarene have been used in drug discovery and design, as part of a biosensor, for bioimaging, targeted drug delivery and other biomedical applications [12,13] The ability of calix as a host molecule for guests makes it widely used as a stationary phase for HPLC, as an antidote, as well as for the development of medicinal compounds. The most recent development in the field of macromolecular chemistry is the use of calixarene compounds as antibacterial agents. Based on the research of Utomo et al., [6] the compound C-4-methoxyphenylcalix[4] modified resorcinarene hexadecyl trimethyl ammonium-bromide has antibacterial activity against *Staphylococcus aureus* bacteria. Research on the use of calixarenes as an antibacterial is growing rapidly. Our recent research indicates that the compound C-4-methoxyphenylcalix[4]resorcinene has fairly good antibacterial activity. Even with the modification via the addition of the quaternary ammonium group was shown to increase its activity [6]. This is also in line with the results of the study by Padnya et. Al [14] who reported that the calixarene compound will increase its ability as an antibacterial agent with the addition of quaternary ammonium group to the p-tert-butylthiacalix[4]arene compound. However, the synthesis procedure requires more than three reaction steps. Therefore, it is necessary to innovate the modification of the parent compound of calixarene, in this case calix[4]resorcinarene, which is simple and inexpensive procces but can increase the ability of the parent compound itself. Thus, in this work, we report on the test for the antibacterial activity of the new calix[4]resorcinarene derivative compound, i.e C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene, and its enhancement by modification through complexation with Ag metal.

2. Experimental

2.1. Materials

The materials were used in this work involve *p*-anisaldehyde, 2,6-dihydroxybenzoic acid, absolute ethanol, HCl 37%, methanol, n-hexane, sodium hydroxide, dichloromethane, DMSO, nitric acid, silver nitrate, chloramphenicol, nutrient agar media, Mc.Farland standard solution, sodium chloride, *S. aureus* ATCC, *E. coli* ATCC, Muller Hinton Agar media, Brain Heart Infusion media and distilled water. All chemicals were directly used after purchase from Merck without prior purification. The Atomic Absorption Spectrophotometer AA-6650-F Shimadzu, FT-IR

Spectrophotometer Shimadzu Prestige-21, Bruker AC300F 400 MHz NMR, electro thermal 9100, autoclave, laminar air flow, and incubator (Thermo Fisher Scientific) were used for instrumental measurements.

2.2. Synthesis C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene

The synthesis was carried out according to the preceding procedure [15] with slight modifications. 1.54 g (10 mmol) of 2,6-dihydroxybenzoic acid was reacted with 1.215 mL (10 mmol) p-anisaldehyde under reflux condition in the presence of HCl as the catalyst. Reflux process was maintained until all the reactants have reacted completely. The mixture was then cooled and the solvent was evaporated. The residue obtained was purified with a mixture of distilled water and methanol (1:1). The crystals formed were washed with n-hexane and dried.

2.3 Synthesis C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene-Ag(I) Complex

As much as of 0.5 grams of calix[4]resorcinarene product was stirred with a metal solution of Ag (I) (40 ppm) for 18 hours at a pH of 4-5 and a temperature of 25 ^{o}C. The mixture was filtered then the metal concentration in the filtrate and in the blank were determined by the atomic absorption spectrometry (AAS) method.

2.4 Preparation of the Concentration Series of samples

Preparation of various concentrations of C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene and C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene-Ag(I) complex are made in 5 concentration series, i.e 10%, 15%, 20%, 25%, and 30% (w/v). The solutions were maintained by using DMSO as the sovent.

2.5 Antibacterial Activity Test for C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene and C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene-Ag(I) complex

Antibacterial activity test was carried out by using the agar diffusion method. In the initial stage, Muller Hinton Agar (MHA) media and bacteria culture were made according to the previous procedure [6]. One ose of bacterial colonies from slanted Nutrient Agar (NA) media was diluted using a sterile 0.9% of NaCl solution until it had turbidity according to the Mc. Farland standard (107 – 108 CFU/mL). A Sterile cotton swab was inserted into the tube containing the bacterial suspension, then evenly rubbed on the MHA media. A total of 5 µl of the test solution (both C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene and C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene-Ag(I) complex) were injected on a blank disc paper using a micropipette. After the solution was completely absorbed, the paper disc containing the sample was placed on MHA media that contains the targeted bacteria, then incubated at 37 °C for 24 hours. A clear zone that forms around the disk indicates that the sample can inhibit bacterial growth and can determine its diameter.

3. Results and discussion

Synthesis C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene-Ag(I) Complex

In this research, Calixresocinarene compounds can be formed through a continuous aromatic substitution reaction between 2,6-dihydroxybenzoic acid and p-anisaldehyde. The reaction can take place under acidic conditions by adding concentrated HCl to the reactor system. The obtained product, after going through the purification stage, was found in the form of a brownish red crystalline solid with a melting point of 330 ^{0}C (at decomposed). C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene is water insoluble, slightly soluble in acetone and dichloromethane, but completely soluble in DMSO and DMF. Due to its small solubility in water, it is strongly suspected that this compound will be able to take up cationic metals in aqueous systems. Based on the H-NMR spectra (Figure 1), there is

a strong undeniable evidence of a successful synthesis process of the C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene product. The evidence is the appearance of absorption in the 4.34 ppm region which indicates the proton resonance of the methine group (-CH-). This peak is a specific peak of the compound calix[4]resorcinarene, which is not possessed by the reactants present, neither 4-methoxybenzaldehyde nor 2,6-dihydroxybenzoic acid. This means that methine bridge (-CH-), connecting the aromatic ring of *p*-anisaldehyde residues and 2,6-dihydroxybenzoic acid, has been formed resulting in a cyclic polymer of C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene.

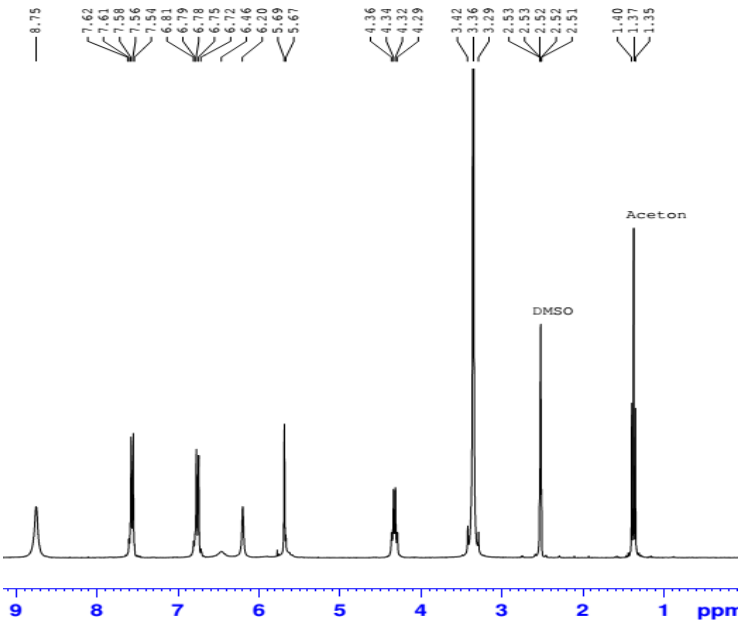


Figure 1. [1]H-NMR Spectra of C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene

The next step is the complexing process of C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene with Ag(I). This was done through the process of contacting the product calix[4]resorcinarene with a metal solution of Ag (I) (40 ppm) for 18 hours at a temperature of 25 ^{o}C. This condition was chosen because based on previous research [15] stated that the maximum adsorption of Ag(I) metal occurred at a metal concentration of 40 ppm in the experimental range of 10-50 ppm. In addition, the pH of the solution was maintained in the pH range of 4-5, because if at a high acidity level (pH < 4), it will result the adsorption cannot take place properly because there will be competition for H^+ ions with metal ions. This competition results in at least metal ions being adsorbed by resorcinnarene [16] and causes the adsorbent to be protonated. In other words, the active side tends to be positively charged, resulting in acidic conditions allowing for electrostatic repulsion between the adsorbent and the adsorbate (Ag metal) because they have the same properties, i.e positive charge. If the pH that used at a low acidity level (pH > 6), the solubility of the adsorbent in water increases and the adsorbate begins to precipitate. The formation of the complex C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene-Ag(I) was confirmed conclusively by the atomic absorption spectrometry and FTIR data. AAS data was based on a review of the concentration of Ag(I) metal in solution, while FTIR was based on a review of changes or shifts in the absorption of a different functional group in the complexation process of resorcinarene with Ag(I). Based on data processing correspond to the AAS indicate that the host-guest complex has a maximum adsorption capacity of 38.05 mg (Ag)/g C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene. Meanwhile, based on the results of FTIR, the biggest difference in the absorption shift lies in the absorption of the O-H functional group with a wavelength of 3417.04 cm^{-1} for resorcinarene without

adsorption and at a wavelength of 3407.40 cm^{-1} for resorcinarene with adsorption. According to Permanasari's research [17], it was stated that a shift in the wave number value occurred after the adsorption process, so in this study, it indicated that there was an interaction between the adsorbent, especially O-H group, with Ag metal as the adsorbate. In the presence of active site hydroxyl group on the compound, a model for the interaction that occurs with Ag metal cations can be proposed, i.e as a chemical interaction. According to research by Utomo [16], the interaction between the active site and metal ions can be in the form of coordinating covalent bonds or hydrogen bonds with hydrated metal ions. The proposed model for the interaction of Ag^+ metal ions with resorcinarene compounds can be seen in Figure 2(a). This interaction model was also supported by analysis using Hyperchem calculation. The results of the optimization of these compounds indicate that the metal ion of Ag^+ was more easily bound to the active group of -OH with the most stable energy obtained -0.522344 kcal/mol. For more details the interaction of the resorcinarene compound to Ag^+ metal ions after optimization, it can be seen in Figure 2(b).

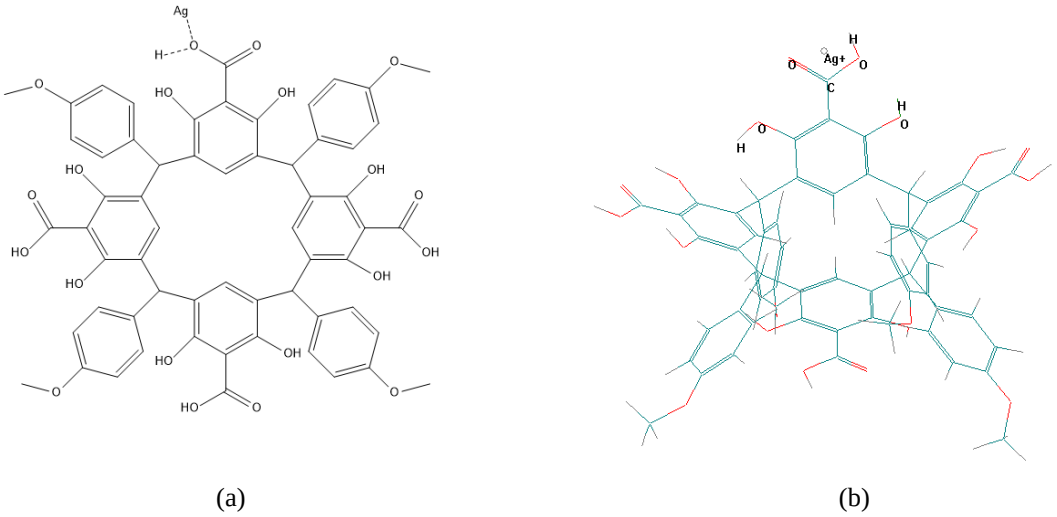
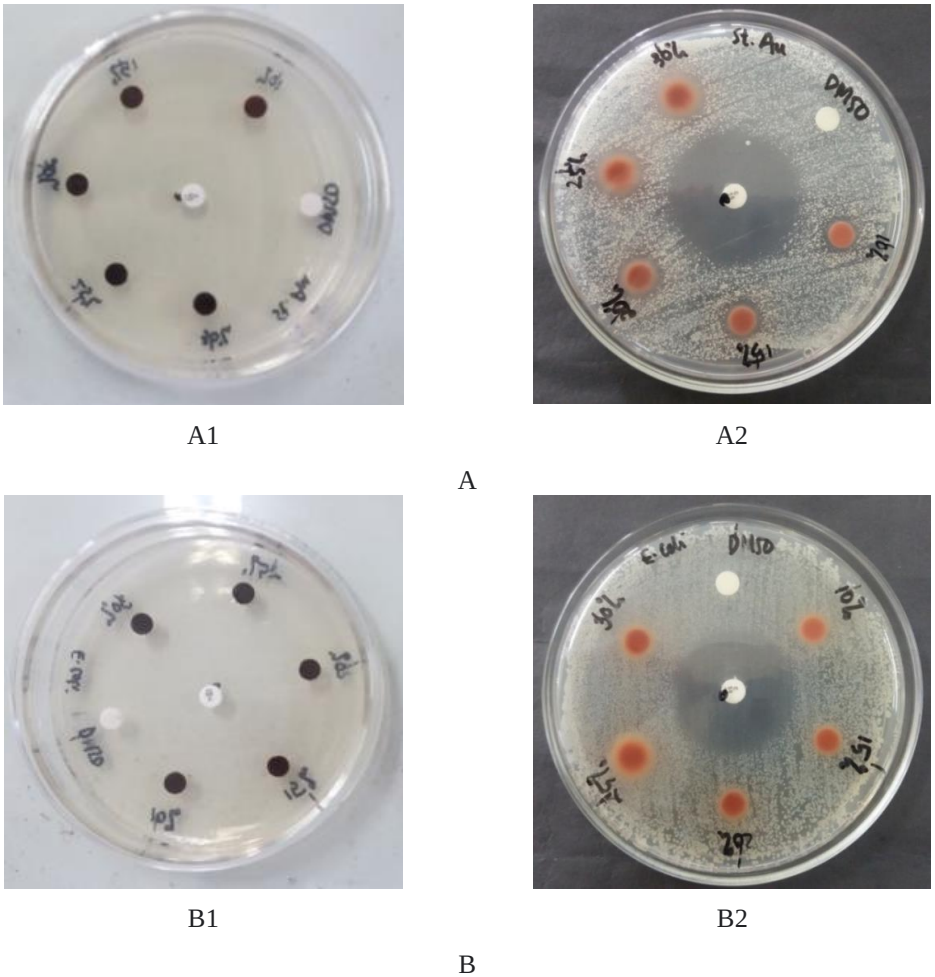


Figure 2. The proposed model for the interaction of Ag^+ metal ions with resorcinarene compounds

Antibacterial activity of C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene-Ag(I)

The antibacterial activity of the C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene and its Ag(I) complex compounds were tested by agar diffusion method. After incubation for 24 hours, it can be seen whether or not there is a clear zone around the solution disk from the assay. The size of the clear zone formed, shows the inhibitory power of the resorcinarene compound. The wider of the clear zone indicate the stronger inhibitory power of the compound against bacterial growth. Figure 3 provides some examples of the inhibitition ability of C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene, negative control (DMSO as solvent), and chloramphenicol as positive control. Based on Figure 3, it can be seen that for the test results against *S. aureus* bacteria there is a clear zone formed around the disk containing the test solution. Meanwhile, the test results on *E.coli* bacteria did not show a clear zone, except for the chloramphenicol disk. The clear zone formed correspond to the measured antibacterial activity (inhibition zone) of each test solution. The next step is to determine the inhibition zone of the bacteria by measuring the diameter of the clear zone. The magnitude of the inhibition zone of C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene and its Ag(I) complex compounds against *S. aureus* and *E. coli* bacteria is shown in Table 1. The measurement result of the diameter of the inhibition zone in Table 1

shows that the parent compound, C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene , has antibacterial activity against *S. aureus* bacteria but not active to *E.coli* bacteria. Its indicate that the *S. aureus* bacteria more sensitive to the resorcinarene compound than that of to *E.coli* bacteria. Natheer et al. [18] explained that the cell wall layer of *E. coli* is more complex than that of *S. aureus*. Gram-positive bacteria (*S. aureus*) cell walls are only composed of peptidoglycan and a single plasma membrane. Meanwhile, gram-negative bacteria (*E. coli*) are composed of an outer plasma membrane, an inner plasma membrane and peptidoglycan. The outer plasma membrane in the cell wall can protect the bacteria by blocking the entry of antibiotics and also the host defense system. Therefore, *E. coli* has a stronger resistance toward C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene compound. Based on Table 1, both the parent resorcinarene compound and the resorcinarene-Ag complex compound showed that the higher the resorcinarene concentration, the larger of the inhibition zone diameter. This indicates that the concentration of an antibacterial compound is one of the determining factors for the compound's ability to inhibit the growth of the tested bacteria. However, at a certain concentration the increasing in concentration is not always followed by an increasing in the diameter of the inhibition zone consistently. This possibility occurs due to the differences in diffusion rate of antibacterial compounds on the agar medium. If the concentration of antibacterial compounds is too large, the rate of diffusion in the medium will decrease.



A1 A2

A

B1 B2

B

Figure 3. Antibacterial activity test of C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene at various concentrations against *S. aureus* (A) and *E. coli* (B) bacteria. (A1 and B1: Before incubation; A2 and B2: After incubation for 24 hours).

The results showed that the C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene compound has antibacterial activity against *S. aureus* at a concentration of 10%; 15%; 20%; 25%; and 30% with an inhibition zone respectively of 7 mm; 8.1 mm; 9 mm; 10 mm; and 10 mm. On the other side, the complex compound of C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene-Ag at the same concentration variation, the inhibition zones were 13.5 mm; 13.7mm; 14.6 mm; 15 mm; and 16 mm. According to Davis and Stout [19], the criteria for antibacterial strength were: inhibition zone diameter <5 mm was categorized as weak, 5-10 mm was categorized as moderate, 11-20 mm was categorized as strong, and > 20 mm was categorized as very strong. Thus, C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene has moderate response antibacterial activity against *S. aureus*. Meanwhile, the complex compound of C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene-Ag is indicated as a strong antibacterial agent toward *S. aureus* bacteria.

Table 1. Clear zone diameter of C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene and its Ag(I) complex compounds at various concentrations

Concentration	***S.aureus* Bacteria**		***E.coli* Bacteria**	
of sample	**A (mm)**	**B(mm)**	**A(mm)**	**B(mm)**
DMSO	0	0	0	0
10 %	7	13,5	0	12
15 %	8,1	13,7	0	12
20 %	9	14,6	0	13,5
25 %	10	15	0	14,5
30 %	10	16	0	15
chloramphenicol	33	28	33	30

Note :

A : Clear zone diameter of C-tetracarboxyl-calix[4]resorcinarene

B : Clear zone diameter of C-tetracarboxyl-calix[4]resorcinarene-Ag complex

Although C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene is not active against *E. coli* bacteria, but with a slight modification through the formation of complexes with Ag metal, the complex compound become a strong antibacterial agent (12-15 mm) against *E. coli* bacteria. The results showed that at a concentration variation of 10%; 15%; 20%; 25%; and 30%, the complex compound of C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene-Ag has inhibition zones of 12 mm; 12 mm; 13.5 mm; 14.5 mm and 15 mm, respectively.

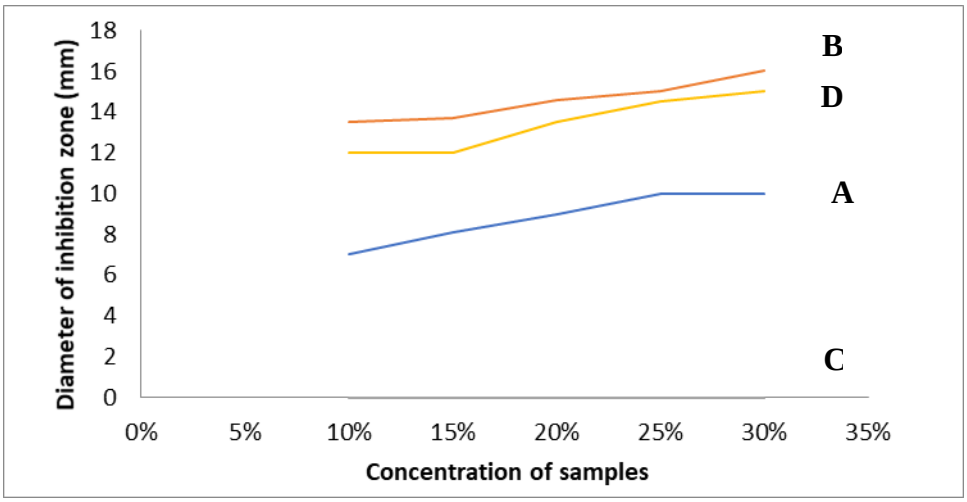


Figure 4. Comparison of Antibacterial Activities of C-tetracarboxyl-calix[4]resorcinarene and Its Ag-complex

Note: A = C-tetracarboxyl-calix[4]resorcinarene for *S.aureus*

B = C-tetracarboxyl-calix[4]resorcinarene-Ag complex for *S.aureus*

C = C-tetracarboxyl-calix[4]resorcinarene for *E.coli*

D = C-tetracarboxyl-calix[4]resorcinarene-Ag complex for *E.coli*

From Table 1 above, a comparison graph of the inhibition zones of *S. aureus* and *E. coli* bacteria then can be made before and after C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene complexed with Ag metal (Figure 4). Figure 4 shows more clearly the significant differences in diameter of inhibition zone between C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene its self and its complex compound with Ag. The inhibitory zone of the complex compound of C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene-Ag was higher than that of the parent resorcinarene compound. This occurs in all variations of the concentration tested. This phenomenon shows that the presence of Ag metal contributes positively to the antibacterial activity of C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene. This is due to the metallic properties of silver itself, which also has antibacterial activity [20]. In its ionic form, Ag is a strong antibacterial and is also toxic to cells. Silver metal has the ability to damage bacterial cell walls, disrupt cell metabolism and inhibit bacterial cell synthesis. The presence of silver ions also causes the diffusion process into the cell membrane to take place faster. It was found that treating the bacteria with Ag^+ ions led to faster diffusion of Histone-like nucleoid-structuring proteins in live bacteria [21].

4. Conclusion

This study has proven that the compound of C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene has antibacterial activity in the moderate category against *Staphylococcus aureus* bacteria, for in concentration range of 10%-30%. However, the compound is inactive toward gram-negative bacteria (*E. coli*) due to the difficulties on the diffusion process of the compound into the complex membrane cell of the bacteria. By modifying the formation of complexes with Silver metal, the complex compound of C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene-Ag is able to enhance its antibacterial ability both against gram-positive bacteria of *S. aureus* and gram -negative bacteria of *E. coli*. C-4-methoxyphenyl-5,11,17,23-tetracarboxyl-calix[4]resorcinarene-Ag complex is a strong antibacterial agent with inhibition zone diameters of 13.5-16 mm for *S. aureus* and 12-15 mm for *E. coli*.

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