Effectiveness Of Extracted Antibacterial Compound From Hydroid *Aglaophenia Cupressina* Lamoureoux Against Bacterial Cell Of *Escherichia Coli*

Eva Johannes, Elly Ishak, Hanapi Usman, Mariyati Bilang

Abstract: - Pollution by pathogenic microorganisms in food materials is capable of resulting in various diseases and increased resistance against various types of antimicrobial. Therefore, new more effective antibiotics are needed to overcome the multi-drug resistant bacteria. This study was aimed to examine the effectiveness of extracted antibacterial compound from hydroid *Aglaophenia cupressina* Lamoureoux in inhibiting and killing the *Escherichia coli*, an organism that frequently pollute food material. This study was an experimental study with the following treatment steps: isolation and characterization of compounds from hydroin *A. cupressina* Lamoureoux by chromatography, elucidation of the isolate molecular structure by UV, IR, and NMR. Two isolates obtained were hexadecanoic acid and agalo E.Unhas, both of them were tested against *E. coli* at various concentrations (10 ppm, 20 ppm, and 30 ppm). The damage to the *E. coli* were examined by Electron Microscopic Scanning. Study results indicated that hexadecanoic acid at concentration of 10 ppm and 20 ppm and Aglao E. Unhas at concentration of 10 ppm, 20 ppm, and 30 ppm, was bacteriostatic against *E. coli*, whereas the hexadecanoic acid at concentration of 30 ppm was bactericidal against *E. coli*.

Keywords: Effectiveness, extracted compound, antibacterial, hydroid *Aglaophenia cupressina* Lamoureoux, *Escherichia coli*.

INTRODUCTION

Resistance against various types of drug is a major problem in treating infectious diseases caused by pathogenic microorganisms. According to Suwandi [1], a new effective antimicrobial is needed to fight against the resistant pathogenic bacteria. Therefore, studies on novel and more effective antibiotic sources in overcoming the resistant pathogenic bacteria are very important to address the multi-drug resistant bacteria. The relatively high infectious case rate from the polluted food materials by pathogenic microbes has made the use of antibiotics increases [2,3]. This has been the important focus in the new bioactive compound studies. According to Murniasih [5], development of new medicines from marine biota is the current trend in many studies because of the high marine biodiversity and the uniqueness of the secondary metabolites structures they have. Joana et al, [6] suggested that this can be implemented by isolating bioactive compounds from marine natural material, with safe properties to health while capable of inactivating bacteria polluting food materials. Most of the marine biocompounds accumulate in marine invertebrates such as sponges, tunicate, soft coral and mollusca, and Cnidaria [7,8].

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Hydroid *Aglaophenia cupressina* Lamoureoux is an invertebrate from phylum cnidaria, living from attarching to sponges. In addition to alkaloid compounds, diterpene, tridentatol A, and prostaglandin, this hydroid also contains histamine, histamine liberator, and protein at its nematocyst [9]. Suada and Ni Wayan Suniti [10] also provided a proof that crude extract from *Aglaophenia sp* (0,05%) is capable of inhibiting the growth of *Fusarium oxysporum f. sp. vanillae*. It is estimated that there are still many other bioactive compounds from secondary metabolites of hydroid *A. cupressina* L, which can be utilized in chemical and pharmaceutical industries. Johannes [11], through isolation and characterization of secondary metabolites from hydroid *Aglaophenia cupressina* Lamoureoux, discovered several compounds including (1) carboxylate acid compound, namely hexadecanoic acid, (2) alkaloid group considered as a new compound and temporarily named as aglao E. Unhas, and (3) steroid class, β-sitosterol. Hexadecanoic acid and aglao E. Unhas were known having antimicrobial activity against *Staphylococcus aureus*, *Salmonella thypi*, *Candida albicans*, and *Malassezia furfur*. However, activity test against *Escherichia coli* has not been performed, whereas *E. coli* is pathogenic bacterium polluting food materials, particularly fresh fruits and vegetables. According to Marriot, in Winarti C., and Miskiyah [12], several strains of *E. coli* are capable of causing diseases in human and animals by producing enterotoxin, and can survive for a long time in its host cell. Examples of microbe *Escherichia coli* contamination in food crops are in tomatoes (2.0×10^5 cells to 2.6×10^6 cells/g), carrot (1.8×10^6 cells to 1.2×10^8 cells/g), lettuce (3.63×10^4 cells to 2.09×10^7 cells/g) [13]. The mechanism and the extent of inhibition activity of these compounds against the pathogenic bacteria *Escherichia coli* are needed to be further explored, to examine the effectiveness of these compounds. This study was conducted by reisolating the compounds from hydroind *A. cupressina* Lamoureoux, followed by antibacterial test against *E. coli* to determine the activity of the test in inhibiting or killing the bacteria, so they can be

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used as one of the natural microbial sources.

MATERIALS AND METHODS

This study was conducted in Microbiological Laboratory and Organic Chemistry Laboratory of Hasanuddin University during February 2012 to June 2012, by using an experimental method. Hexadecanoic acid, agalao E. Unhas, pure culture (*Escherichia coli*), dimethyl sulphoxide (DMSO) (E. Merck), chloramphenicol (PT Alpharma), physiologic NaCl 0.9%, nutrient glucose medium agar, nutrient agar medium, nutrient broth medium, and Muller Hinton Agar medium (oxoid).

Extraction, Partition, and Isolation

The dried samples were mashed and then macerated with methanol for 1 x 24 hours at room temperature. The obtained extract was then vaporized with rotafavor until a thick macerates were obtained, and the was liquid-liquid partitioned using n-hexane solvent. The thick extract of n-hexane was then analyzed using thin-layer chromatography. The products of partition were fractionated using vacuum chromatography column into several fractions and then were monitored using thin-layer chromatography. These fractions were then flash/press column chromatographed using appropriate eluent. This proses was implemented repeatedly until a pure isolate was obtained.

Structural elucidation

The structure of the compounds were elucidated using spectroscopy including UV and IR spectra and NMR.

Antibacterial test using agar diffusion method

The medium of 20 mL sterile Muller Hinton Agar (MHA) was poured aseptically into petri disk and left until solid at part of bottom layer. After that, suspension of test bacteria, 1 mL each, was entered into 10 mL of the medium above the bottom layer and left until semi-solid as seeding layer. Six proposers with diameter of 5 mm, outer diameter of 8 mm, height of 100 mm, were placed aseptically with sterile pincer at medium surface with interval between proposers was 2-3 cm from petri disk edges, and kept in room temperature. Each of the proposer was filled with 0.5 mL hexadecanoic acid and aglao E. Unhas from Hydroid *A. cupressina* Lamoureoux at concentration of 10 ppm, 20 ppm, and 30 ppm. Accordingly, the chloramphenicol solution as positive control and DMSO as negative control, 0.25 mL each, was then incubated at 37°C for 24 hours and 48 hours, respectively. Observations were performed by measuring the diameter of bacterial growth inhibition zone around the proposer by using caliper, to observe ability of the compound in inhibiting the growth of the test bacteria. Measurement of inhibition activity at 24 and 48 hours were tabulated and analyzed by Scanning Electron Microscope to determine the morphological damage suffered by the *E. coli*.

RESULTS AND DISCUSSION

Physiochemical analysis of the isolates

1. Structural analysis of the compound (1)

Yellow-white crystals weighted 23 mg, melting point of 43° to 44°C, fluorescent under UV and had a blue color, but was invisible with thin-layer chromatography. UV spectrum (CH_3OH) indicated maximum absorption at λ_{maks} 212 (164) nm; IR spectrum (KBr) V_{max} 3472 cm^{-1} (OH), 2921, 2855 cm^{-1}

(C-H aliphatic), 2672 cm^{-1} (C-H aliphatic), 1707 cm^{-1} (C-O), 1466 cm^{-1} and 1415 cm^{-1} (CH_2 and CH_3), 1299 cm^{-1} (C-O). Analysis by NMR spectrometer including ^{1}H-NMR, ^{13}C-NMR, and HMBC indicated results as shown in Figure 1 and Table 1.
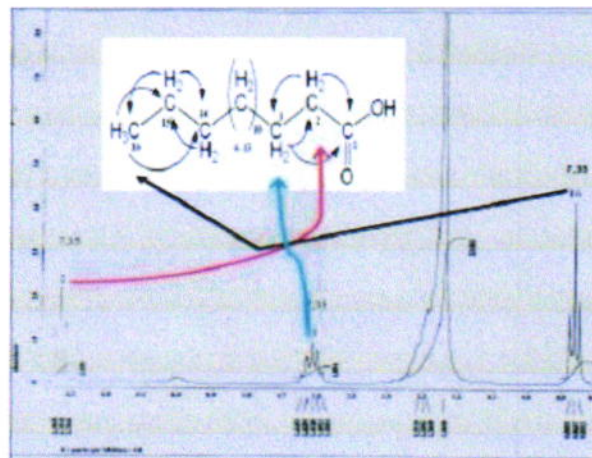

Figure 1. ^{1}H-NMR and ^{13}C-NMR spectra of the compound (1)

Table 1. ^{1}H-NMR and ^{13}C-NMR data of Compound (1)

No	^{13}C-NMR δ_C (ppm)	^{1}H-NMR δ_H: ppm (multiplicty, J in Hz)	HMBC
1	180.64	-	-
2	34.3	2.33 (2H, *t* J=7.35)	1.3
3	24.85	1,62 (2H, *m*, J=7,35)	1.2
4-13	29.89; 29.79; 29.56; 29.44; 29.25	1.24-1.28 (20 H, m)	-
14	32.12	1,28-1.29 (2H, m)	15
15	22.88	1.31-1.33 (2H, m)	14, 16
16	14.31	0.87 (3H, *t*, J=6.70)	14, 15
OH	-	3.75 (1H, s)	

Analysis of ^{13}C-NMR spectra indicated that compound (1) had 16 carbons, identified by signals appearing as follow: δ_C 180.4 signal with furthest chemical displacement, indicating carob at carboxil group, signal at δ_C 34.30 and 32.12 indicating C-2 and C-14, respectively. There were 6 carobs in symmetric position, namely C-7, C-8, C-9, C-10, and C-11 produced one signal with high intensity, that is δ_C 29.89. In addition, there were signals appeared very closely at δ_C 9, 89;29,79;29,56;29,44;29,25, indicating C-4, C-5, C-13, C-6 and C-12, respectively. Signals at δ_C 24,85, 22,88 and 14,31 indicating C-13, C-15, and C-16, respectively.

Analysis of ^{1}H-NMR spectra indicated signals as follow: δ_H 2,33 (2H,*t*,J= 7,35) indicated 2 protons at C-2, signal at δ_H 1,62 (2H,*m*,J=7,35) indicated 2 protons at C-3, signal at δ_H 1,24-1,28 (20H,*m*) indicated 20 hydrogens at C-4, C-5, C-6, C-7, C-8, C-9, C-10, C-11, C-12, and C-13, signal at δ_H 1,28-1,29 (2H, *m*) indicated 2 protons at C-14, signal at δ_H = 1,31-

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1,33 (2H, *m*) indicated 2 protons at C-15, signal at δ_H = 0,87 (3H, *t*, J=6,70) indicated 3 protons at C-19, signal at 3,75 (1H, *s*) indicated proton at hydroxyl group, the number of hydrogens at the compound were 32.

Elucidation of the compound structures was confirmed by HMBC spectrum, indicating far distance correlation between proton signals and carbon signals as follow: proton signal δ_H 2,33 (H-2) was distantly correlated with carob signals δ_C 180,64 (C-1) and 24,85 (C-3), proton signal δ_H 1,31-1,33 (H-15) was correlated to carbon signal δ_C 32,12 (C-14) and δ_C 14,31 (C=16) and the correlation between proton signal δ_H 0,87 (H-16) and carbon signals δ_C 32,12 (C-14) and δ_C 32,12 (C-14) and δ_C 22, 88 (C-15).
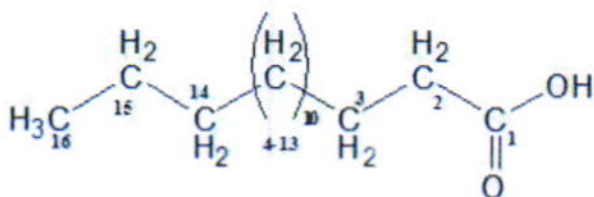

Figure 2. Hexadecanoic Acid

2. Structural analysis of the compound (2)

White crystals weighted 22.4 mg, melting point of 55° to 56°C, flourescent under UV, but was invisible with thin-layer chromatography. UV spectrum (CH_3OH) indicated maximum absorption at λ_{maks} 292 (455) nm and λ_{max} 220,8 (142) nm ; IR spectrum (KBr), V_{max} 3327, 3396, 3249 cm^{-1} (secondary N-H), 2920 cm^{-1} and 2828 cm^{-1} (CH_2 and CH_3), 1735 cm^{-1} and 1670 cm^{-1} (C-N), 1462 cm^{-1} (C-H), 1128 cm^{-1} and 1049 cm^{-1} (C-O-C). Analysis by NMR spectrometer including ^{1}H-NMR, ^{13}C-NMR, and HMBC indicated results as shown in Figure 6 and Table 2.
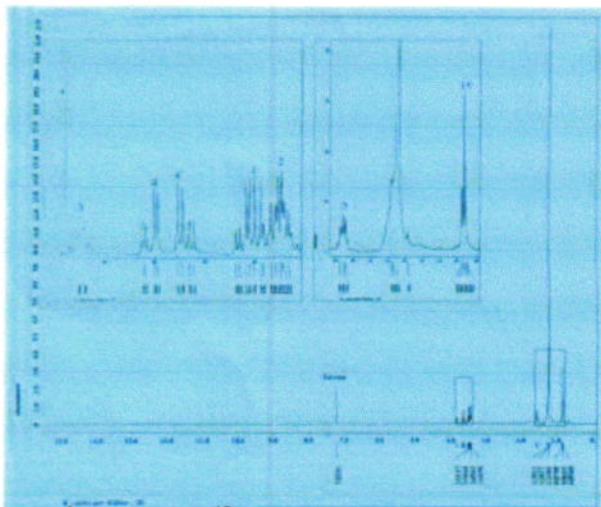

Figure 3. ^{1}H-NMR and ^{13}C-NMR spectra of the compound (2)

Analysis of ^{13}C-NMR spectra indicated the presence of two carbon groups; the group with signal appearing at the range of 64.6 to 72.69 with more distant chemical displacement, and the group with signal appearing at the range of 14.23 to 32.10 with smaller chemical displacement. Four signals in the first group were δ_C 72.69, 72.03. 70.57 and 64.46 indicating four carbons in heterocyclic ring: C-1, C-2, C-3, and C-4. Six signals in the second group represented fifteen carbons, in

which five signals were δ_C 32.10, 29.54, 26.25, 22.87 and 14.32 indicating C-13, C-2, C-1, C-14 and C-15, respectively. One signal with high intensity at chemical displacement δ_C 29,87; 29,84; 29,79; 29,76 indicated ten symmetric carbons: C-3, C-4, C-5,C-6, C-7, C-8, C-9,C-10' C-11'' and C-12'.

Table 2. ^{1}H-NMR and ^{13}C-NMR data of Compound (2)

No	^{13}C-NMR δ_C (ppm)	^{1}H-NMR δ_H: ppm (multiplicty, J in Hz)	HMBC
1	72.69	3.52 (1H, *dd*, J=9.75; 3.65) 3.48 (1H, *dd*, J=9.80; 6.15)	2.4
2	72.03	3.45 (2H, *m*, J=4.30)	1',2',3
3	70.57	3.84 (1H, *m*, J=4.25)	4
4	64.46	3.72 (1H, *dd*, J=11.6; 3.65) 3.69 (1H, *dd*, J=11.6; 3.65)	2
4'	-	3.62-3.64 (1H, *dd*, J=11.6; 4.85)	4
1'	29.54	1.56 (2H, *m*, J=7.35)	2'
2'	26.63	1.24-1.34 (2H, *m*)	3'
3'-12'	29.87; 29.84; 29.79; 29.76	1.24-1.34 (20H, *m*)	-
13'	32.1	1.24-1.34 (2H, *m*)	12'
14'	22.87	1.24-1.31 (2H, *m*)	13'
15'	14.32	0.87 (3H, *t*, J=7.30)	13', 14'

Analysis of ^{1}H-NMR spectra indicated similar profile with ^{13}C-NMR, which indicated also two signal groups at hydrogen spectrum: δ_H 3.45-3,84 and δ_H 0,87-1,56 Hz groups. Characteristic of proton in the first group was identified at δ_H 3, 84 (1H, *m*,J= 4,25), indicating one proton at C-3, signal at 3,69-3,72 (2H, *dd*,J= 11,6; 3,65) indicated two proton at C-4, signal at δ_H 3,62; 3,64 (1H,*dd*, J = 11,6; 4,85) indicated one proton bound to hetero-nitrogen atom as secondary amine, signal at δ_H 3.48 and 3,52 (2H, *dd*, J = 9,80; 6,15 and 9.75; 3,65) indicated two protons C-1, signal at δ_H 3,45 (2H,*m*, J = 4,30) indicated proton at C-2. Therefore, there were 8 hydrogen were identified at the group. Several signals in the second group were also identified as 31 hydrogens with chemical displacement as follow: signal at δ_H1,56 (2H,*m*,J = 7,35) indicated proton at C-1, signal appearing at δ_H 1,24-1,34 (2H,*m*) indicated C-2, signal appearing at δ_H 1,18-1,34 (2H,*m*) indicating C3-12. Based on the analysis above, it can be deduced that the compound had 39 hydrogens.

Elucidation of the compound structure was confirmed by HMBC spectrum, indicating a distant correlation between proton signal and carbon signal as follow: proton signal δ_H 3,52 (H-1) and carbon signal δ_C 72,03 (C-2) and 64,46 (C-4),

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proton signal δ_H 3,45 (H-2) and carbon signal δ_C 29,54 (C-1), 26,63 (C-2) dan 70,60 (C-3), proton signal δ_H 3,84 (H-3) and carbon signal δ_C 64,5 (C-4), and correlation between proton δ_H 3,71 (H-4) and carbon signal δ_C 26,63 (C-2'). There were also a distant correlation between proton signal δ_H 1,56 (H-1') and carbon signal 26,63 (C-2'), between proton signal δ_H 1,24 (H-2') and carbon signal δ_C 29,87 (C-3') and correlation between proton signal δ_H 0,87 (H-15') and carbon signal δ_C 22,9 (C-14') and 32,1 (C-13').
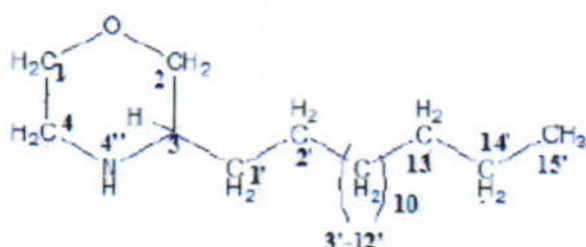

Figure 4. Aglao E. Unhas

Antibacterial test of the compound (1)

against Escherichia coli

Antibacterial test of hexadecanoic acid at concentration of 10 ppm against *E. coli* for 24 hours showed inhibition zone diameter of 18 mm, which was reduced to 14.50 mm after 48 hours. At the concentration of 20 ppm, the hexadecanoic acid was observed bacteriostatic because at 48 hours the inhibition zone diameter was reduced when compared to that of 24 hours (Figure 1). At the concentration of 30 ppm, the inhibition zone diameter at 24 hours was 13.00 mm and increased to 16.50 mm at 48 hours.
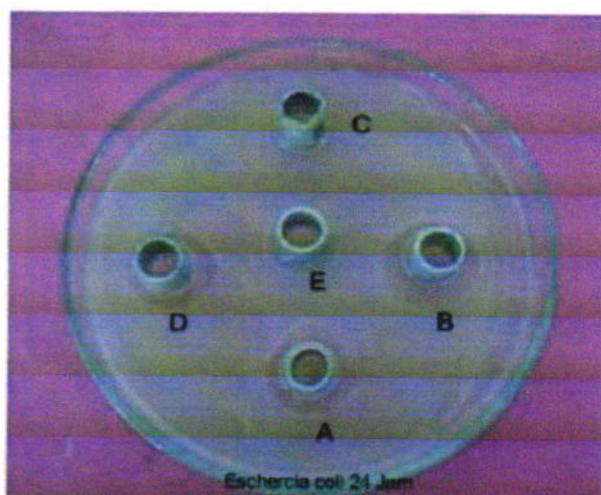

Figure 4. *E. coli* at 24 hours incubation

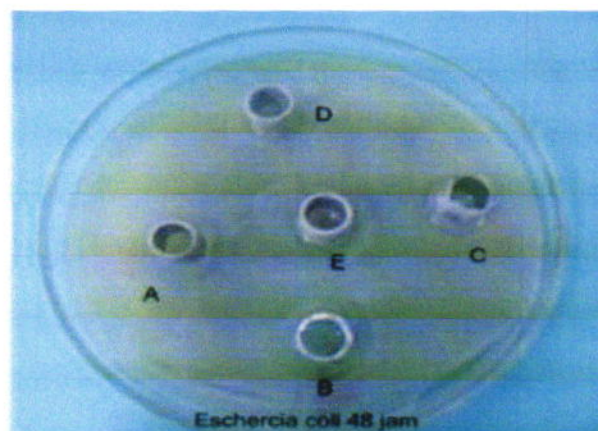| Treatment	Inhibition Zone Diameter (mm)
A = hexadecanoic acid 10 ppm	18.00
B = hexadecanoic acid 20 ppm	19.00
C = hexadecanoic acid 30 ppm	13.00
D = negative control (DMSO)	0.00
E = positive control (chloramphenicol)	15.00

Figure 5. *E. coli* at 48 hours incubation.

Treatment	Inhibition Zone Diameter (mm)
A = hexadecanoic acid 10 ppm	14.50
B = hexadecanoic acid 20 ppm	17.00
C = hexadecanoic acid 30 ppm	16.50
D = negative control (DMSO)	0.00
E = positive control (chloramphenicol)	14.00

At the concentration of 10ppm-20ppm, hexadecanoic acid could only inhibit the growth of *E. coli* but didn't kill the bacteria, because within 24 hours, the diameter of inhibition zone at 10ppm and 20ppm was reduced at 48 hours. For the concentration of 30 ppm, hexadecanoic acid was bactericidal against *E. coli*, as indicated by the inhibition zone diameter formed at 24 hours (13.00 mm) and reduced to 16.50 mm at 48 hours. At the concentration of 30 ppm, besides inhibiting the growth of *E. coli*, hexadecanoic acid also killed the bacteria. Madigan et. al. [14] suggested that the influence of antibacterial component against bacterial cell could result in cell damage which in turn killed the bacteria. The resulting cell damage by the antibacterial component can be microcidal (irreversible damage) or microstatic (reversible damage). A componen will become microcidal or microstatic depending on concentration of the component and microbe culture used [15]. This was also confirmed from scanning electron microscopic results, which indicated cell membrane damage of the *E. coli* by forming bubble.
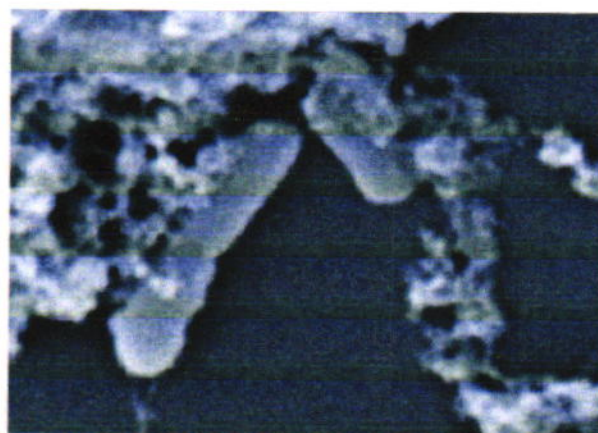

Figure 6. SEM photograph of *E. coli* treated by hexadecanoic acid.

Antibacterial test of the compound (2) against *Escherichia coli*

Antibacterial test of aglao E. Unhas at concentration of 10 ppm against *E. coli* at 24 hours showed inhibition zone diameter of 15.50 mm, which was increased to 16.00 mm at 48 hours. At the concentration of 20 ppm, aglao E. Unhas was observed bacteriostatic because at 48 hours the inhibition zone diameter was not changed when compared to that of 24 hours (17.00 mm) (Figure 4). At the concentration of 30 ppm, the inhibition zone diameter at 24 hours was 16.00 mm and reduced to 15.50 mm at 48 hours.
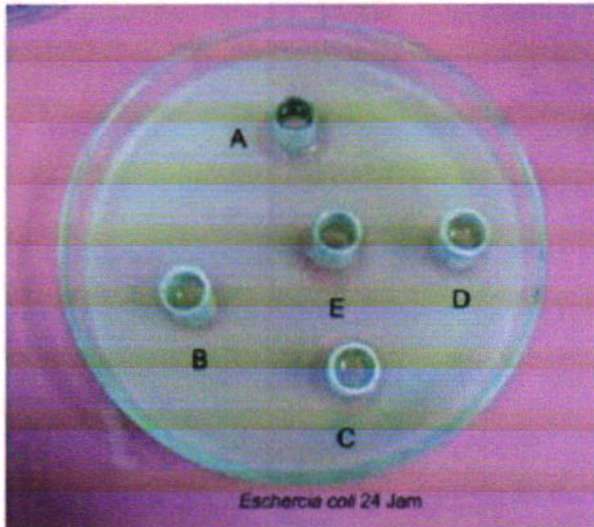
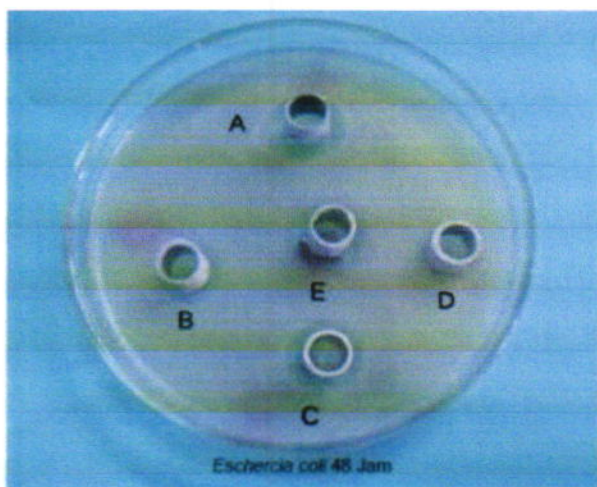| Treatment	Inhibition Zone Diameter (mm)
A = Aglao E. Unhas 10 ppm	16.00
B = Aglao E. Unha 20 ppm	17.00
C = Aglao E. Unha 30 ppm	15.50
D = negative control (DMSO)	0.00
E = positive control (chloramphenicol)	15.00

Aglao E. Unhas at the concentration of 10 ppm, 20 ppm, and 30 ppm was bacteriostatic against *E. coli* bacteria, even though at the concentration of 10 ppm and 20 ppm it indicated a relatively bactericidal properties, but not significantly different with those of 30 ppm which is bacteriostatic. According to Kanazawa et. al [16], the inhibition process antimicrobe was due to antimicrobial compound adherence to microbial cell surface or the compound could be diffused into the microbial cell. This was evidenced from scanning electron microscope, which was showing damaged cell wall of the *E. coli*, so the cell membrane was bubbled.
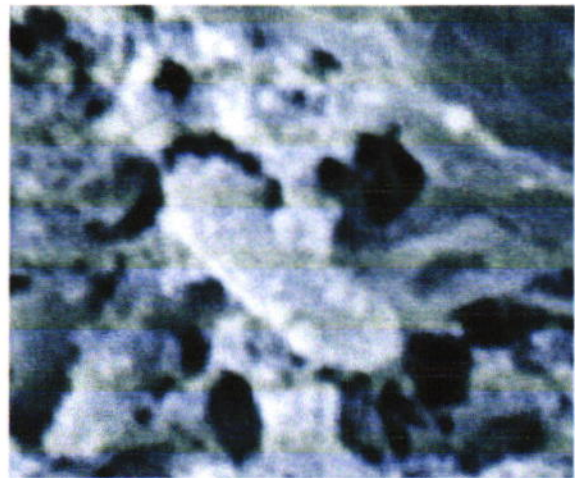

Figure 7. *E. coli* at 24 hours incubation.

Treatment	Inhibition Zone Diameter (mm)
A = Aglao E. Unhas 10 ppm	15.50
B = Aglao E. Unha 20 ppm	17.00
C = Aglao E. Unha 30 ppm	16.00
D = negative control (DMSO)	0.00
E = positive control (chloramphenicol)	15.50

Figure 9. *E. coli* treated by aglao E. Unhas.

Figure 8. *E. coli* at 48 hours incubation.

Figure 10. Normal cell of *E. coli*.

CONCLUSION

Hexadecanoic acid and Aglao E. Unhas have bioactivity as antibacterial in *E. coli*. In their use as antibacterial against *E. coli*, hexadecanoic acid and Aglao E. Unhas are relatively more effective compared to chloramphenicol in term of inhibition zone.

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