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RESEARCH ARTICLE

Antibacterial Activity of Bis (2 - Ethylhexyl) Phthalate Leaves Fraction of *Colocacia esculenta L* against Enteric Gram Negative Bacteria

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Abstract

This study is aimed at testing the antibacterial activity of the compound Bis (2-Ethylhexyl) phthalate leaves fraction of *Colocacia esculenta* L against enteric gram negative bacteria. The search for bioactive compounds employs the vacuum liquid chromatography method used for fractionation and purification, spectroscopic analysis (infrared, proton and carbon core magnetic resonance, chromatography-mass spectroscopy) gas, Minimal Inhibitory Concentration (MIC), Minimal Bactericidal Concentration (MBC), Agar diffusion and TLC-Bioautography for activity testing against Enteric Gram Negative Bacteria. The results of the fractionation shows that based on agar diffusion, the B fraction obtains the MIC value is 0.1%, and MBC value is 1%. Spectroscopic analysis of the secondary metabolites of the leaves fraction of *Colocacia esculenta* L obtains the chemical structure of Bis (2-Ethylhexyl) phthalate with the molecular formula $C_{24}H_{38}O_4$ and molecular weight 390.5. The test on the compound Bis (2-Ethylhexyl) phthalate from the leaves fraction of *Colocacia esculenta L* by TLC-Bio autography obtains an active Rf value of 0.7 against bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella thypi*, *Streptococcus mutans*, and *Vibriocholerae*, by diffusion Agar method, the largest inhibition zone in *Salmonellathypi* bacteria. Based on the results, the identification of secondary metabolites from the leaves fraction of *Colocacia esculenta* L obtains the chemical compound Bis (2-Ethylhexyl) phthalate and based on the results of the antibacterial activity test of the chemical compound Bis (2-Ethylhexyl) phthalate leaves of *Colocacia Esculenta* L is potential for the treatment of enteric gram negative bacteria infections.

Keywords: *Colocacia esculenta L, Bis (2-Ethylhexyl) phthalate, Antibacterial.*

Introduction

Humankind has long used chemical compounds in plants for medication [1]. Added to this, the new, safe, and efficient alternative treatments have long been developed from antibacterial, antioxidant, antineoplastic, antimicrobial activities derived from secondary metabolites of a plant [2]. In the line with this, one of the plants that its chemical content can be used as an antibacterial is *Colocacia esculenta* L. It is a food plant originating from Asia (Japan, Indonesia, China, and the like) growing in tropical and sub-tropical regions [3]. In traditional medication, it is used as an expectorant, astringent, inflammation, diarrhea [4], antitumoral/anti-metastatic [5-6], anti-hyperlipidemic /anti-hypercholesterolemic [7], anxiolytic [8], wound healing [9], anti-melanogenic [10], anti-inflammatory [11], probiotic [12], anti-hypertensive [13], antioxidant [14], anti-microbial [15], anthelmintic [16], insecticidal [17] and antiviral [18]. Moreover, the chemical components contained in this plant include organic acids, phenolic compounds, anthocyanins, tannins, sterols, phytocystatin, alkaloids, saponins, terpenes [19-21]. Furthermore, the use of *Colocacia esculenta* L against infectious diseases such as infections in the digestive tract with chemical

contents, are saponins, tannins, flavonoids, cardiac glycosides, and alkaloids [22]. Based on the results of the phytochemical analysis of *Colocacia esculenta* L, it shows that ethanol extract is effective in having an effect on *Salmonella thypi* with chemical flavonoids [23]. Besides, based on the bioactive compounds contained in *Colocacia esculenta* L, the chemical structure of medicinal compounds is used to handle infectious diseases, especially bacterial infections to make sure it is safe to use by society.

Material and Methods

Materials

The materials used are leaves of *Colocacia esculenta* L from South Sulawesi province of Indonesia and some bacteria: *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella thypi*, *Streptococcus mutans*, and *Vibrio cholerae* strains. The test bacteria used are Enteric Gram Negative Bacteria derived from clinical culture Laboratory of Microbiology, Faculty of Pharmacy, Universitas Muslim Indonesia

Methods

Sample Extraction

Leaves Extraction of *Colocacia esculenta* L is conducted by Maceration Method. The leaves sample of *Colocacia Esculenta* L, with a weight of 3250 grams, is dried until it weighted 1150 grams of Simplicia. Then, it is put into a maceration vessel. After that, the samples are processed by powdering or coarsely grinding put into a maceration container in the form of a closed vessel using 96% ethanol. Then, it is stored at room temperature for three days. After that, it is stirred repeatedly until all the samples dissolved in the solvent used. Moreover, the filtered liquid is sorted to obtain liquid extract and residue. Finally, the liquid extract is dried or evaporated until it produces a dry extraction.

Fractionation of Bioactive Compounds Using Vacuum Liquid Chromatography

The extract obtains from the leaves of *Colocacia esculenta* L using ethanol is fractioned using the Liquid Vacuum Chromatography method (using silica gel G60 GF254 as stationary phase and n-hexane - ethyl acetate eluting fluid). Before that, a sample of 10 g of dry ethanol extract is impregnated with 20 g silica gel (size 50-100 mesh). Then, the dry ethanol extract and silica gel are mixed until they are homogeneous and dry. This stage is carried out with elusive liquid with different levels of polarity with elusive liquid, namely n-hexane (2 times), n-hexane: ethyl acetate (10: 1, 1: 1, 1:10), ethyl acetate (2) times), ethyl acetate: ethanol (10: 1, 1; 1, 1:10), ethanol 100 mL (3 times) each with a volume of 100 mL. The results of vacuum liquid chromatography are collected in a container according to the mobile phase used until obtaining the fractions.

Then, they are analyzed by TLC (stationary phase of silica GF254) with n-hexane: ethyl acetate (10: 1) elusive fluid then identified under UV light 254 and 366 nm. The stains on the chromatogram with the same Rf value are combined to obtain non-polar, polar, and semi-polar fractions. Finally, the liquid fraction is dried using a rotary evaporator at a temperature of ± 40 ° C to obtain the dry fraction.

Antibacterial Activity of the Leaves Fraction of *Colocacia esculenta L* with Agar Dilution Method

Minimum Inhibitory Concentration (MIC) Testing. The Agar dilution technique with medium-Nutrient Agar is conducted by diluted the leaves fraction of *Colocacia esculenta* L with a concentration of 0.1%, 0.5%, 1% and antibiotics using 30mg of tetracycline, 5mg of Ciprofloxacin, 15mg of Erythromycin, and 30mg of Chloramphenicol using Escherichia *coli*, *Pseudomonas aeruginosa*, *Salmonella thypi*, *Streptococcus mutans*, and *Vibrio cholerae*. Determination of the Minimum Inhibitory Concentration (MIC) is considered by planting the bacteria in the liquid seeds used for MIC inside.

Therefore, the incubation takes out for 1 x 24 hours at 37°C. Minimum Bactericidal Concentration (MBC) testing in the Agar dilution technique with medium Nutrient Agar is conducted by diluted the leaves fraction of *Colocacia esculenta* L with a concentration of 0.1%, 0.5%, 1% and antibiotics using 30mg of tetracycline, 5mg of Ciprofloxacin, 15mg of Erythromycin, and 30mg of Chloramphenicol using *Escherichia Coli*, *Pseudomonas Aeruginosa*, *Salmonella Thypi*, *Streptococcus Mutans*, and *Vibrio Cholerae*. Consideration of the value of the minimum kill concentration (MBC) is

determined by the clear zone or the absence of bacterial growth around the test sample on the surface of the medium agar. Then, the incubation is carried out (incubation time 1 x 24 hours, with an incubation temperature of 37^{o}C).

Isolation of the active Compound Leaves Fraction of *Colocacia esculenta* L by TLC-Preparative and Compound Purification Analysis by TLC-Two Dimensional and Multi-Eluent System

The active compound from the leaves fraction of *Colocacia esculenta*is isolated by Preparative Thin Layer Chromatography (TLC-Preparative) with a glass plate-sized of 20x20 cm using a stationary phase of silica Gel PF254 which is activated by heating at 1100C for one (1) hour. Fraction B (active fraction) dissolved with chloroform: methanol (1: 1 v / v) is spotted longitudinally on the TLC-Preparative plate. Then, eluted with n-hexane: ethyl acetate (10: 1) elution solution.

The TLC-Preparative plate is dried and observed with UV rays of 254 nm and 366 nm. The TLC-preparative compound, which forms the bands, is dredged. Then, each band is purified by dissolving it using methanol: chloroform (1: 1, v / v). After that, they are dried. Furthermore, the single compound purification is analyzed by TLC-Two Dimensions in 1 (one) direction using n-hexane: ethyl acetate (10: 1) and *aran* 2 (two) eluting liquid using n-hexane: ethyl acetate (1: 1) eluting fluid and the elution analysis of the multi-eluent system using variations of elusive fluid, namely n-hexane: ethyl acetate (10: 1 and 15: 1), n-hexane: chloroform (10: 1).

Analysis of Compounds by Spectrophotometry

The isolates obtained from the leaves fraction of *Colocacia esculenta* L are analyzed based on data from infrared spectroscopy (determining functional groups), nuclear magnetic resonance spectroscopy (determining the number of protons and carbon), and chromatography-mass spectroscopy gas (the molecular weight of bioactive compounds).

Antibacterial Activity of Bis (2-Ethylhexyl) phthalate Leaves Fraction of *Colocacia esculenta* L employs the Agar diffusion method

10 mL of sterile medium Nutrient Agar is put in a petri dish until the medium solidifies. Then as much as 5 mL of sterile medium Nutrient Agar a suspension of enteric gram negative bacteria is added, *Escherichia coli, Pseudomonas aeruginosa, Salmonella thypi, Streptococcus mutans* and *Vibrio cholerae* as much as 20 µl until homogeneous and put into a petri dish until the medium solidifies.

The test samples are the compound Bis (2-Ethylhexyl) phthalate from the leaves fraction of *Colocacia esculenta*at L a concentration of 0.1%, 0.5%, 1%, and antibiotics using tetracycline 30mg, Ciprofloxacin 5mg, Erythromicyn 15mg, Chloramphenicol 30mg placed on the surface of the medium on the Petri plate. Then, the incubation is carried out for 1x24 hours with a temperature of 37^{0}C. After that the measurement is conducted based on the diameter of the limpid area around the test sample and antibiotics.

Antibacterial Activity of Bis (2-Ethylhexyl) phthalate Leaves Fraction *Colocacia esculenta* L employing TLC-Bioautography

10 mL of sterile medium Nutrient Agar adds a suspension of tested bacteria 20 µL and is put in a petri dish with aseptic processing until the medium solidified. The leaves fraction of *Colocacia esculenta* L is placed on the TLC plate and eluted using n-hexane: ethyl acetate (10: 1) solvent. The chromatogram plate is placed on the solidified medium surface for 30 minutes. Then, it is removed. After that, the Petri dishes are incubated for 1 x 24 hours with an incubation temperature of 37^{0}C. Then, the pellucid area is observed and calculated by the RF value on the chromatogram.

Results

The result of the fraction of the ethanol extract of the leaves of *Colocacia esculenta* L using the liquid vacuum chromatography method with elusive fluid based on different polarity, namely n-hexane (2 times), n-hexane: ethyl acetate (10: 1, 1: 1, 1:10), ethyl acetate (2 times), ethyl acetate: methanol (10: 1, 1; 1, 1:10).

Ethanol 100 mL (3 times) produced 13 fractions with a combined fraction, 5 fractions, namely fraction A: 1-2; Fraction B: 3-7; Fraction C: 8-10; Fraction D: 11-13.

The results obtained from the extraction of *Colocacia esculenta* L leaves can be seen in the Table 1.

Table 1: Ethanol extract fractionation of *Colocacia esculenta* L leaves using vacuum liquid chromatography

Sample	Fractionation by Vacuum Liquid Chromatography		
	Sample weight (gram)	**Fraction**	**Combined Fraction**
Ethanol Extract of Colocacia esculenta L. Leaves	10	13 Fraction	Fraction A = 1-2 Fraction B = 3-7 Fraction C = 8-10 Fraction D = 11-13

Determination of the minimum inhibitory concentration employs several concentration variations, with a concentration 0.1%, 0.5%, and 1%, which give activity against *Escherichia coli, Pseudomonas aeruginosa, Salmonella thypi, Streptococcus mutans, and Vibrio cholerae*.

It takes 1 x 24 hours of incubation time using temperature 370C. The MIC value obtained is at a concentration of 0.1%. The results of Minimum Inhibitory Concentration (MIC) by Dilution Agar from fraction B of *Colocacia esculenta*leaves against enteric gram negative bacteria can be seen in the Table 2.

Table 2: Minimum Inhibitory Concentration (MIC) activity of Bis (2-Ethylhexyl) phthalate leaves fraction *Colocacia esculenta* L against enteric gram negative bacteria, (-) Bactariostatic (+) Bactericidal

Bacteria	Concentration (%)		
	0,1%	**0,5%**	**1%**
Vibrio cholerae	+	+	+
Eschericia coli	+	+	+
Pseudomonas aeruginosa	+	+	+
Salmonella typhi	+	+	+
Streptococcus mutans	+	+	+

The determination of the minimum bactericidal concentration apply Nutrient Agar medium with an incubation time of 1 x 24 hours using a temperature of 37^0C. The MBC value of the leaves fraction of *Colocacia esculenta* L against *Escherichia coli, Pseudomonas aeruginosa, Salmonella thypi, Streptococcus mutans*, and *Vibrio cholera* is at a concentration of 1%. Table 3 represents the results of the Minimum Bactericidal Concentration (MBC) Test.

Table 3: Minimum Bactericidal Concentration (MBC) activity of *Colocacia esculenta* L leaves fraction against enteric gram negative bacteria, (-) Bactariostatic (+) Bactericidal

Bacteria	Concentration (%)		
	0,1%	**0,5%**	**1%**
Vibrio cholerae	-	-	+
Eschericia coli	-	-	+
Pseudomonas aeruginosa	-	-	+
Salmonella typhi	-	-	+
Streptococcus mutans	-	-	+

Figure 1 indicates the isolation of the active compound from fraction B (active fraction) of the leaves of *Colocacia esculenta* L by TLC-Preparative with n-hexane: ethyl acetate (10: 1) eluting fluid obtains three isolates/compounds. Compound 3 as the active compound is analyzed by purification of a single compound using TLC-Two Dimensions in the 1 (one) direction using n-hexane: ethyl acetate (10: 1) eluting liquid and 2 (two) using n-hexane: ethyl acetate (1: 1) and a multi-eluent system elution analysis using a variety of elusive fluid, namely n-hexane: ethyl acetate (10: 1), the value of Rf = 0.7, n-hexane: ethyl acetate (15: 1) obtained Rf = 0.5, n-hexane: chloroform (10: 1) resulting in a value of Rf as 0.95. It indicates that compound 3 is a single compound.

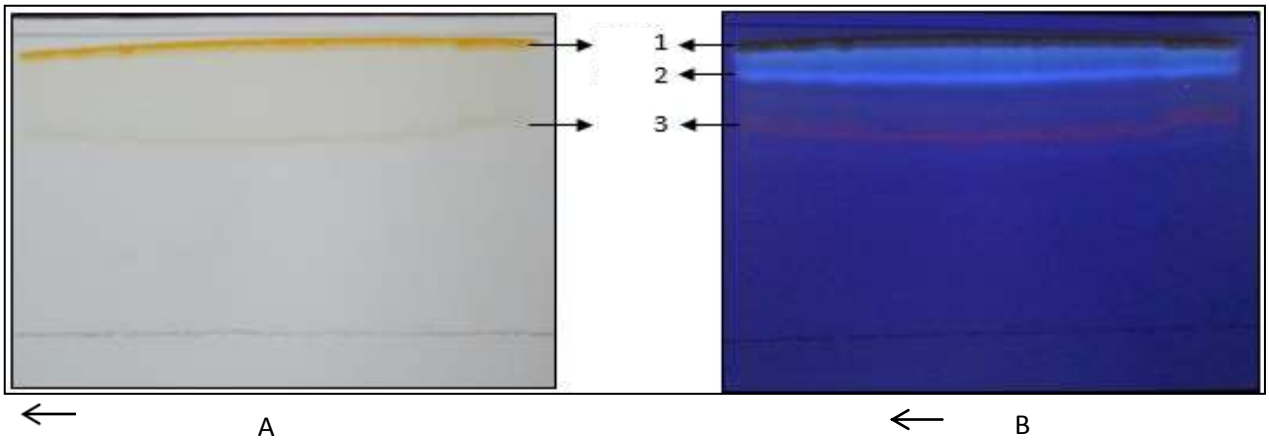
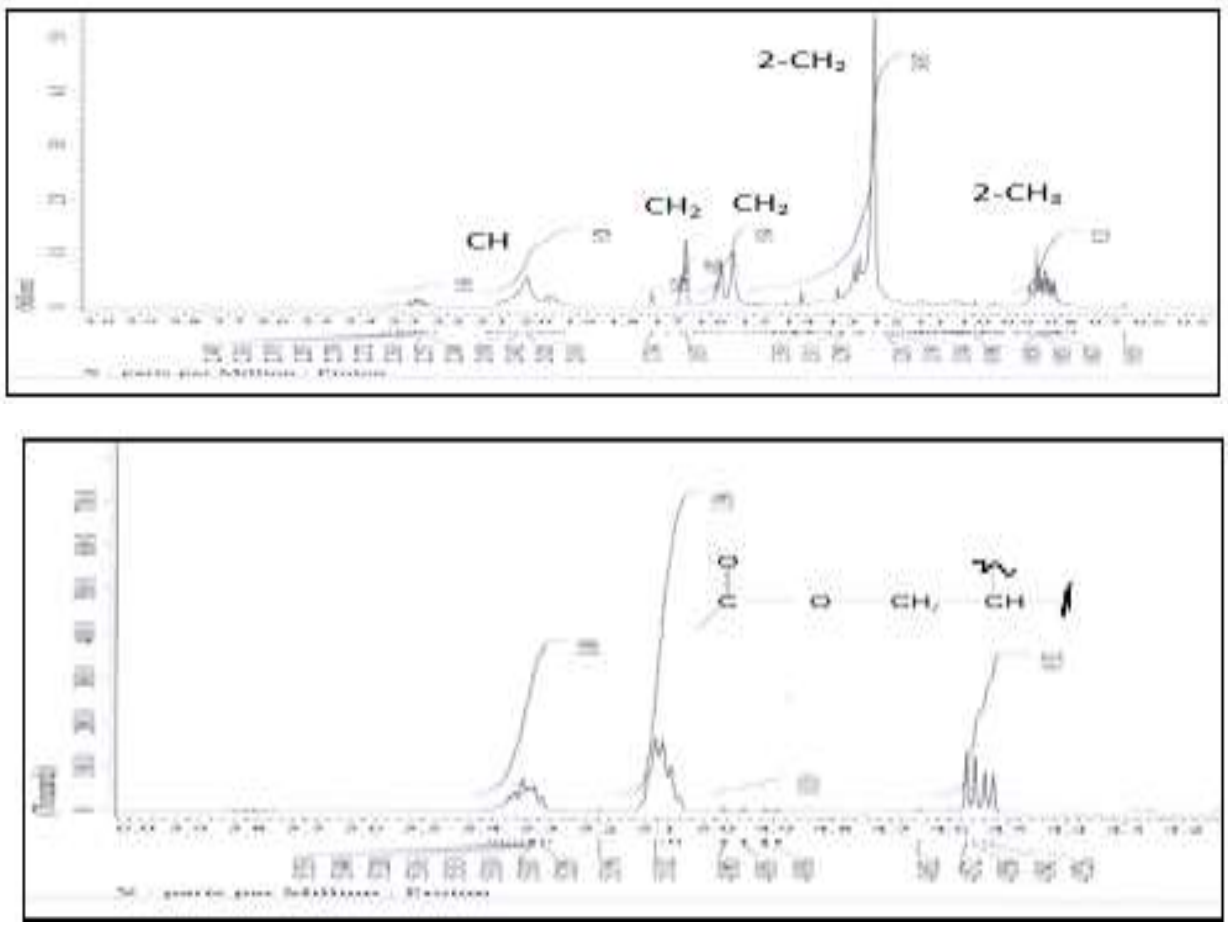


Fig. 1: Isolation of the active compound from fraction B (active fraction) of *Colocacia esculenta*leaves by TLC-Preparative, A = UV 254 nm, B = UV 366 nm.

The ^{1}H-NMR data interpretation (400 MHz) can determine the number of types of atomic environments in the molecule, the number of hydrogen atoms in each type of hydrogen environment, and the number of hydrogen atoms presenting in neighboring carbon atoms. The appearance of various resonance signals is caused by the presence of protons in a molecule spread in different chemical environments. The resonant signals are separated by a chemical shift, vary from 0.655 ppm (d, 1H), 0.858 ppm (k, 3H), 0.998 ppm (d, 1H), 1,262 ppm (d, 1H), 1,550 ppm (d, 1H,), 1,657 (d, 1H), 1,734 ppm (d, 1H). They indicate that the presence of a methyl group existed in various chemical shift starting from 2,034 ppm (d, 1H), 2,260 ppm (k, 3H), 2,315 ppm (d, 1H), 3,645 ppm (d, 1H), 3,856 ppm (d, 1H), and 4,031 ppm (k, 3H). Moreover, the chemical shift of 4,454 ppm (t, 2H) indicates the presence of a methoxy group (OCH3). Meanwhile, the chemical shift of 5,102 ppm (d, 1H), 5,328 ppm (t, 2H), 6,570 ppm (d, 1H), and 6,587 ppm (d, 1H) indicates the presence of an alkene group. Besides, a chemical shift of 7,038 ppm and 7,115 ppm indicates an aromatic group.



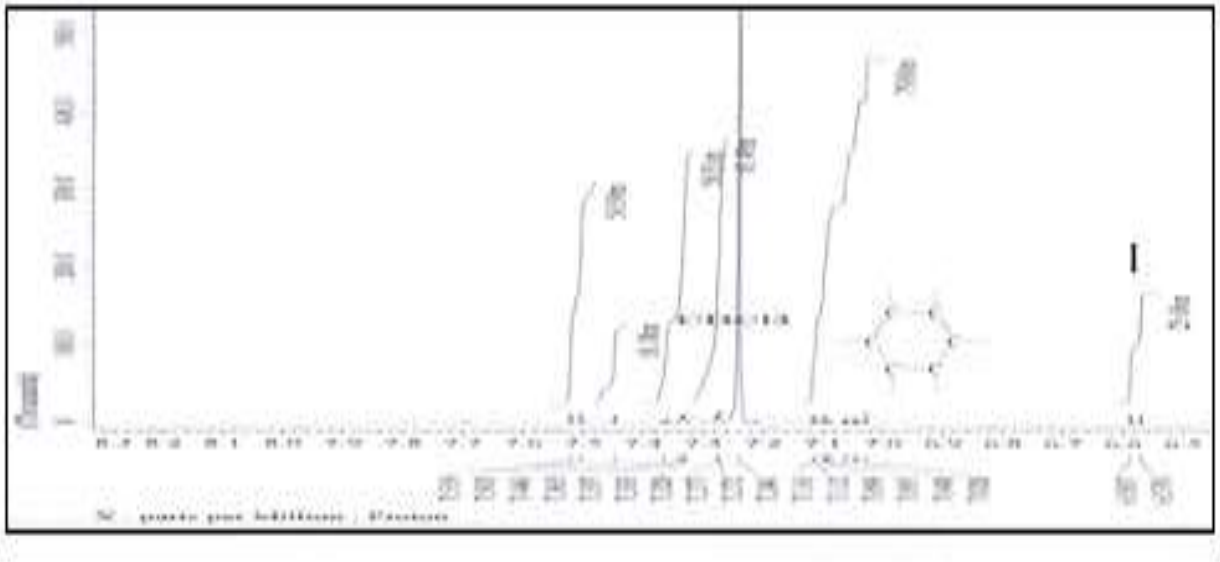


Fig. 2: ^{1}H-NMR spectrum fragment of fraction B of *Colocacia esculenta* leaves

The ^{13}CNMR (100 MHz) spectra interpretation describes the chemical shift of the carbon atoms in the form of sp3, sp2, and sp. Thus, it can determine the type of carbon for the compound and the total number of carbon atoms. Besides, the ^{13}CNMR spectral characteristics of the *Colocacia esculenta* L leaves isolate are chemical shifts of 135.60 ppm, 125,233 ppm, and 119,541 ppm indicating the presence of aromatic carbon (C-Aromatic). Moreover, a chemical shift of 61,431 ppm indicating the presence of methoxy, and a shift of 32,155 ppm, 29,310 ppm, 26,616 ppm, 23,660 ppm, 22,921 ppm, 19,974 ppm, 17,910 ppm, 16,595 ppm, and 14,359 ppm indicates the presence of methyl. The results of the analysis of the spectra data above reveal that the isolated leaves of *Colocacia esculenta* L with a molecular weight of 390 obtains the molecular formula $C_{24}H_{38}O_4$ and obtains a compound called Bis (2-Ethylhexyl) phthalate.

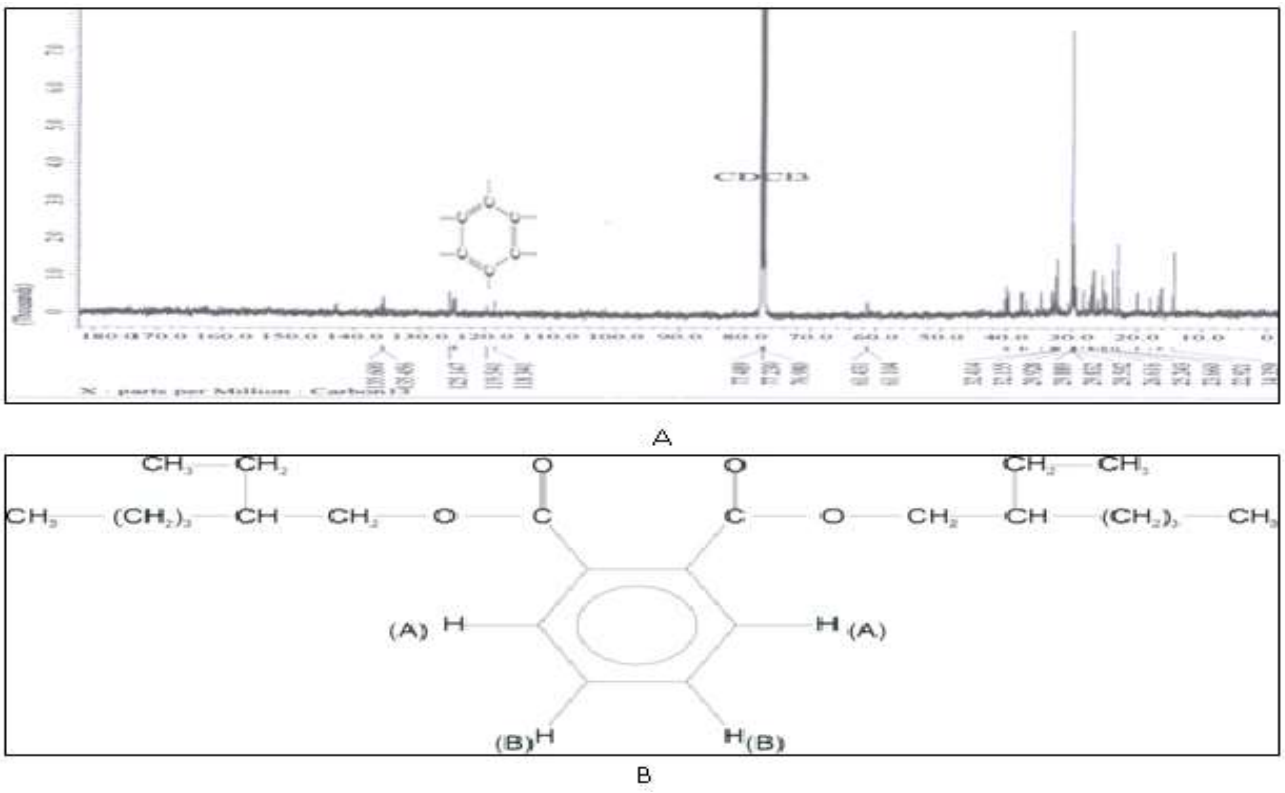


Fig.3: A) ^{13}C-NMR spectrum of *Colocacia esculenta* L leaves fraction, B) Chemical structure of Bis (2-Ethylhexyl) phthalate leaves fraction *Colocacia esculenta* L

The results of antibacterial activity for the compound Bis (2-Ethylhexyl) phthalate leaves fraction of *Colocacia esculenta* L at a concentration of 1%, 30 mg of tetracycline antibiotics, 5 mg of Ciprofloxacin, 15 mg of Eritromicyn, and 30 mg of chloramphenicol against *Escherichia coli, Pseudomonas aeruginosa, Salmonella thypi, Streptococcus mutans, and Vibrio cholera* obtain the largest inhibition zone diameter. It can be inhibited by the Bis (2-Ethylhexyl) phthalate compound from the leaves fraction of *Colocacia esculenta* L at a concentration of 1% as 15 mm against bacteria against *Salmonella thypi.* The results obtained from the antibacterial activity can be seen in the Table 4.

Table 4: Antibacterial activity of the Bis (2-Ethylhexyl) phthalate compound from the leaves fraction of *Colocacia esculenta* against Enteric Gram Negative Bacteria by the agar diffusion method

Sample	Dose (mg)	Inhibition Zona (mm)				
		VC	EC	PA	ST	SM
Bis (2-Ethylhexyl) phthalate leaves fraction of *Colocacia Esculenta*	10	11	13	11	15	11
Tetraciklin	30	12	15	10	13	10
Ciprofloxacin	5	26	30	38.3	41	41.6
Eritromicyn	15	25	30	30	36.6	39
Kloramfenikol	30	27	28	38	36.6	39

VC = *Vibrio cholerae*, EC = *Escherichia coli*, PA = *Pseudomonas aeruginosa*, ST = *Salmonella thypi*, SM = *Streptococcus mutans*

The results of testing the compound Bis (2-Ethylhexyl) phthalate extract of *Colocacia esculenta* L using TLC-Bioautography method with a value of Rate of Flow (Rf) 0.7 using n-hexane: ethyl acetate (10: 1) elution fluid indicate that it is effective against *Escherichia coli, Pseudomonas aeruginosa, Salmonella thypi, Streptococcus mutans*, and *Vibrio cholerae*. Table 5 indicates the antibacterial activity of the compound Bis (2-Ethylhexyl) phthalate from the leaves fraction of *Colocacia esculenta* L against Enteric Gram Negative Bacteria by TLC-Bioautography.

Table 5: Antibacterial activity of Bis (2-Ethylhexyl) phthalate extract of *Colocacia esculenta* L against Enteric Gram Negative Bacteria using TLC-Bioautography method

Compound	Color		Rf	Bacteria
	UV 254	UV 366		
Bis(2-ethylhexyl)phthalate	Yellow	Purple	0,7	*EC, PA, ST, SM, VC*

VC = *Vibrio cholerae*, EC = *Escherichia coli*, PA = *Pseudomonas aeruginosa*, ST = *Salmonella thypi*, SM = *Streptococcus mutans*

Discussion

The ethanol extract of *Colocacia esculenta* L contains various chemical compounds, including alkaloids, glycosides, terpenoids, flavonoids, saponins, and phenols [24-25]. The results obtain from the dilution test for fraction b of *Colocacia esculenta* L leaves against enteric gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Salmonella thypi, Streptococcus mutans, and Vibrio cholerae*) are that the MIC values obtained at a concentration of 0.1% and the MBC value at a concentration of 1%.

It indicates that the fraction b of *Colocacia esculenta* L leaves can be bacteriostatic and bactericidal. Besides, extracts of *Colocacia esculenta* L shows good antimicrobial activity against several bacteria and fungi. They are tested at low concentrations, namely against *Escherichia coli, Aeromonas Hydrophila, Flavobacterium sp., Edwardsiella Tarda, Klebsiella sp., Salmonella sp., Vibrio Alginolyticus, V. Parahaemolyticus, V. Cholerae, and Pseudomonas Aeruginosa* [26].

The isolation of the active compound from fraction B (active fraction) of the leaves of *Colocacia esculenta* L in TLC-Preparatively obtains three (3) bands/isolates. Then, the third isolate is purified by the two-dimensional TLC method with the multi-eluent system resulted in isolate three as a

single compound. Analysis of bioactive compounds fraction B of *Colocacia esculenta*

L leaves using infrared spectroscopy, magnetic core resonance spectroscopy (proton NMR and carbon NMR), and chromatography-mass spectroscopy gas obtain molecular formula $C_{24}H_{38}O_4$ showing the presence of Bis (2-Ethylhexyl) phthalate. Added to this, antibacterial activity test of Bis (2-Ethylhexyl) phthalate fraction B of *Colocacia esculenta* L leaves uses the agar diffusion and TLC-Bioautography testing methods. After that, compound Bis (2-Ethylhexyl) phthalate fraction B of *Colocacia esculenta* L leaves is dissolved with DMSO at concentrations less than 2% DMSO.

It has low activity and toxicity. Besides, DMSO also has solvency for various chemicals [27]. Based on diffusion testing, the Bis (2-Ethylhexyl) phthalate compound fraction B of *Colocacia esculenta* L leaves has the same activity as tetracycline antibiotics against *Salmonella thypi* bacteria. Meanwhile, the test results using the TLC-Bioautography method obtain the Rate of Flow (RF) value of 0.7 against *Escherichia coli, Pseudomonas aeruginosa, Salmonella thypi, Streptococcus mutans,* and *Vibrio cholerae.* Compound Bis (2-Ethylhexyl) phthalate extract *Colocacia esculenta* L can inhibit the growth of enteric gram negative bacteria. Apart from plant bioactivity of phthalate derivatives, it also is found in algae and marine microorganisms [28].

Several reports have shown the antibacterial potential of phthalates of plant origin [29]. Moreover, several studies have reported the antibacterial activity of Bis (2-Ethylhexyl) phthalate and among them is Bis (2-Ethylhexyl) phthalate from *Streptomyces bangladheshiensis* extract containing antibacterial activity against *Pseudomonas aeruginosa Bacteria* [30]. The (2-Ethylhexyl) phthalate from *Alchornea sp* is able to reduce anti-inflammatory activity [31]. Besides, Bis (2-Ethylhexyl) phthalate has proven to have potent antifungal activity against major fungal pathogens such as *Candida, Cryptococcus, and Aspergillus sp* [32].

Conclusion

Based on the research results, it is concluded that the results of UV-Vis, H-NMR, C-NMR, and mass spectroscopic analysis shows the presence of alkane (CH), carbonyl (C = O), CH aromatic, and ester (CO) functional groups with a molecular weight of 390.5 is to obtain the chemical structure of Bis (2-Ethylhexyl) phthalate with a molecular formula of $C_{24}H_{38}O_4$ and a molecular weight of 390.5. Antibacterial activity results of the Bis (2-Ethylhexyl) phthalate compound fraction B of *Colocacia esculenta* L leaves by diffusion to obtain the larges inhibition zone diameter against *Salmonella thypi* bacteria, by TLC-Bioatography the Rate of Flow value is obtained (Rf) as 0.7 actives against bacteria *Escherichia coli, Pseudomonas aeruginosa, Salmonella thypi, Streptococcus mutans*, and *Vibrio cholerae.*

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