ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at www.jgpt.co.in

RESEARCH ARTICLE

Cytotoxic Activity of 2- iminoethyl 2-(2-(1-hydroxypentan-2-yl) phenyl) Acetate from Sterculia Quadrifida R.Br Ethyl Acetate Fraction

Rollando Rollando*, Rehmadanta Sitepu, Eva Monica

Program of Pharmacy, Faculty of Science and Technology, Ma Chung University, MalangVilla Puncak Tidar N-01, Malang 65151.

*** Corresponding Author: Rollando Rollando**

Abstract

Faloak (*Sterculia quadrifida* R.Br) was used empirically as a traditional medicinal plant. The information of the active compounds contained in the faloak bark was not specifically published. This study aims to determine the active compounds contained in the fraction of ethyl acetate of faloak bark which has cytotoxic effect on breast cancer cell type T47D. Extraction method use maceration, isolation compound using gradient isolation method, structural elucidation using information from IR, H-NMR, C-NMR and LC-MS. Cytotoxic activity test on breast cancer cell line T47D using MTT method. The result show the isolate compound was 2-iminoethyl 2- (1-hydroxypentan-2-yl) phenyl) acetate with IC_{50} value on T47D breast cancer cell 7, 12 µg/mL and with selectivity index value was 47, 53.

Keywords: *Sterculia quadrifida R.Br, 2-iminoethyl 2-(2-(1-hydroxypentan-2-yl) phenyl) acetate, T47D.*

Introduction

One of the plants used by a traditional community as multipurpose efficacious medicinal plants was faloak (*Stercuila quadrifida* R.Br). Faloak bark herb has long been used by the people of East Nusa Tenggara as a medicinal plant [1]. Empirically, the water decoction of the bark of plants used faloak East Nusa Tenggara people as a cure hepatitis, typhoid, ulcers, and recovery of stamina [2].

In the previous study it was found that the extract of ethanol bark of *Stercuila quadrifida* R.Br had antibacterial activity against *Salmonella thypi*, *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella thypi* and *Candidia albicans* which confirmed the potency of antibiotics [2]. Some antibiotics may be further developed into an anticancer drug such as doxorubicin [3], daunorubicin, carminomycin, bleomycin, enediynes, mitomycin [4], and Kigamicin D [5]. In addition, the fraction of separation results by preparative thin layer chromatography method was studied to have very high antioxidant effect with IC_{50} equivalent to vitamin C [2].

In this research, isolation and identification of active compounds were conducted to find the active isolate that have cytotoxic activity on T47D and Vero cells. Comparison of IC_{50} values between Vero and T47D cells will yield a selectivity index value. The value is used to assess the ability that the isolate is not toxic in normal but toxic cells in cancer cells.

Material and Instrument

Ethyl acetate fraction of faloak bark, DMSO 0,1%, cisplatin (Wako), High glucose DMEM (Dulbecco's Modified Eagle Media) (Gibco), (FBS) 10% (v/v) (Qualified FBS, Gibco, Invitrogen USA), penicillin-streptomycin 1,5% (v/v) (Gibco, Invitrogen USA and Fungizone 0,5% v/v Gibco).

Tripsin-EDTA 0, 25% (Gibco, Invitrogen Canada), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, USA).MTT was prepared with a concentration of 5 mg/mL dissolved in phosphate buffer saline (PBS) 1 x pH 7, 4. 0, 01 N HCl (Merck, Darmstadt, Germany).PBS containing 1 mg/ml (minimum 95% (HPLC),

Sigma-Aldrich Co., St. Louise, MO, 63178, USA) 10 mg/ml RNAse (obtained from Laboratory of Animal Sciences, NAIST, Japan) and Triton X-100 for GC, E. Merck, 64271, Darmstadt, Germany). Autoclave (Hirayama HV 25 020585175, Hirayama Manufacturing Co., Japan), liquid nitrogen, Labconco purifier class II biosafety cabinets (Delta Series, Labconco Corporation, Missouri, USA), CO2 incubator (Heraeus), inverted microscope (Nikon, Eclipse, TE 2000-U), hemocytometer (Nebauer improved 0.100 mm Tiefe Depth Profondeur 0.0025 mm2, Germany). cell counter, micropipet (Pipetman® la Gilson, France), digital camera (Sony), centrifuge (Sigma 203, B.Braun Biotech International), digital balance sheets (Mettler Toledo, AG204 Delta Rang®), Stirrer (Nuova, Thermolyne), Mixer (Maxi Mix II, Thermolyne type 37600 mixer, Iowa, USA), oven (Memmert), ELISA reader (Bio-Rad microplate reader Bencmark serial No. 11565, Japan), FACTS calibur flow cytometer.FTIR (FTIR-100 Perkin Elmer) MS (Mariner Biospectrometry System HRESIMS) and NMR spectrometer (Delta 2 400MHz for 1H-NMR and 100 MHz for 13C-NMR).

Procedure

Fractionation and Purity Test

The ethyl acetate fraction was fractionated using preparative thin layer chromatography with the stationary phase (silica gel 60 PF 254) and mobile phase (chloroform: methanol (9:1 v/v)).

Three stages of testing the purity, the first to be identified by TLC using three kinds of mobile phases of different polarity, ie n-hexane: ethyl acetate (2:1, v/v), chloroform: ethyl acetate (9:1, v/v), and chloroform: ethyl acetate (1:1, v/v). Second, the identification with the two dimensional TLC with the first mobile phase, chloroform: ethyl acetate (9:1, v/v) and second mobile phase, ethyl acetate: hexane (3:7 v/v).Third, using LC-MS with mobile phase of methanol: water (95: 5). Pure fraction and proved the most active (called active isolates) were identified with spray reagent, FTIR spectrophotometer, LC-MS, ^{1}H-NMR spectrometer, ^{13}CNMR and DEPT.

Cytotoxic Test

Cells were harvested with 8 × 10^3 cell concentration and diluted with culture medium, then implanted into a microplate 96 well of100 µL/well and incubated for 24 h in a 5% CO_2 incubator. Before being used for treatment, the media in the plate is removed and then washed using PBS of 100 µL/well.Then the PBS was removed and given test solution of 100 µL/ well.

The cells were then incubated for 24 hours. After incubation, washed with PBS and 100 mL/ml reagent was added and incubated for 3-4 hours at 37 °C. Thereafter, a 100 µL/ ater solution was added to the stopper solution (SDS 10% in HCl 0.01N) and incubated overnight at room temperature, then read with ELISA reader at λ 595 nm and obtained an absorption which states the absorption of T47D cells alive. Single treatment absorbance data was converted to percent viability and used to calculate IC50.

Result and Discussion

Purity of Fraction

The main compounds of fraction 2 purity test conducted using the method of stationary phase TLC with silica gel and several mobile phases with a different polarity. Fraction 2 was tested with the mobile phase of n-hexane: ethyl acetate (2:1, v/v), chloroform: ethyl acetate (9:1, v/v), and chloroform: ethyl acetate (1:1, v/v). Produce a single spot with hRf respectively 20, 35, and 60.

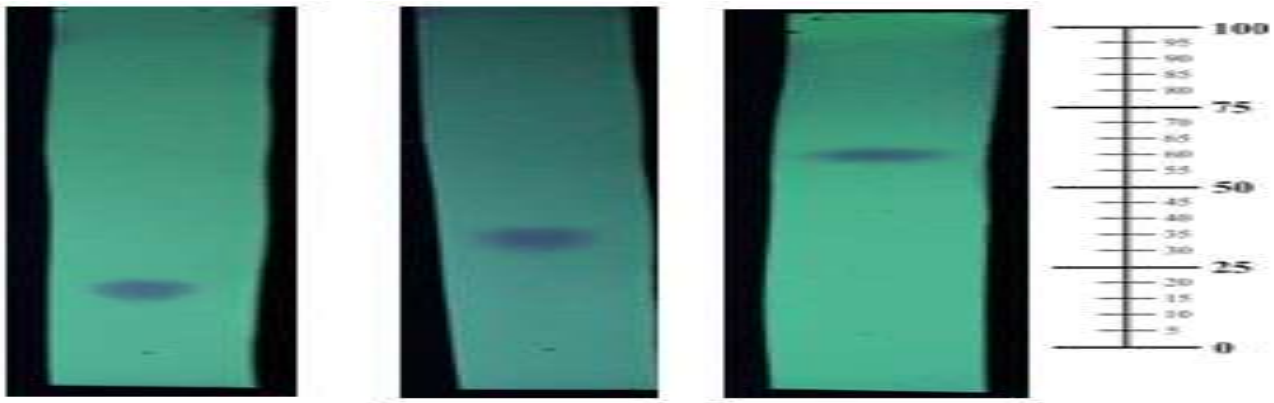

Figure 1: TLC Chromatogram fraction 2 on UV 254

The melting point check was performed to determine the purity of the compound. The fraction powder that has been tested shows that the melting distance of fraction 2 was 175, 21 – 175, 90 °C. The results showed that the powder of fraction 2 had a melting distance of 0, 69 °C. The relatively short distance of melting point fraction 2 with a distance of 1-2 °C indicates that fraction 2 is relatively pure [6].

Table II: Melting Point Checking

Fraction	Melting Point Distance		Standard deviation	
	Start of melting (°C)	End of melting (°C)	Start of melting	End of melting
2	175,21	175,90	0,40	0,41

The purity test was continued by using the LC-MS instrument. Chromatogram fraction 2 has retention time of 2, 2 minutes (Figure 2) with 100% purity. The TLC test result, melting point test, and liquid chromatography chromatogram from fraction 2 can be concluded that fraction 2 has been relatively pure KLT, melting point, and liquid chromatography and can called isolate 2.

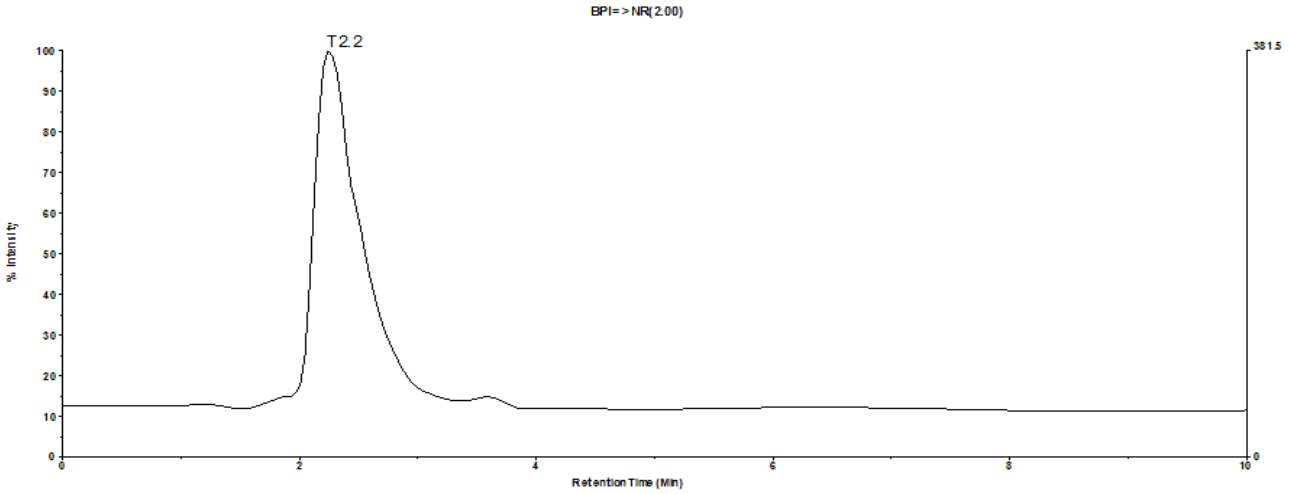

Figure 2: Faction 2 Chromatogram

Infrared Spectrum Analysis

The infrared spectrum of isolate 2 shows the widening band in the 3303 cm^{-1} region indicating the presence of an OH-hydroxy group, low-intensity stretch vibration at 2150 cm^{-1} indicates an imine group (HN=CH-) [7] , strong absorption with sharp band form at 1650 cm^{-1} indicates the presence of C=O [8], weak absorption at 1375 cm^{-1} indicates the presence of methyl groups. A weak peak at the fingerprint region of 1025 and 1050 cm^{-1} wave numbers indicates the presence of a C-O bond and indicates the presence of an ester compound.

^{1}H-NMR Spectrum Analysis

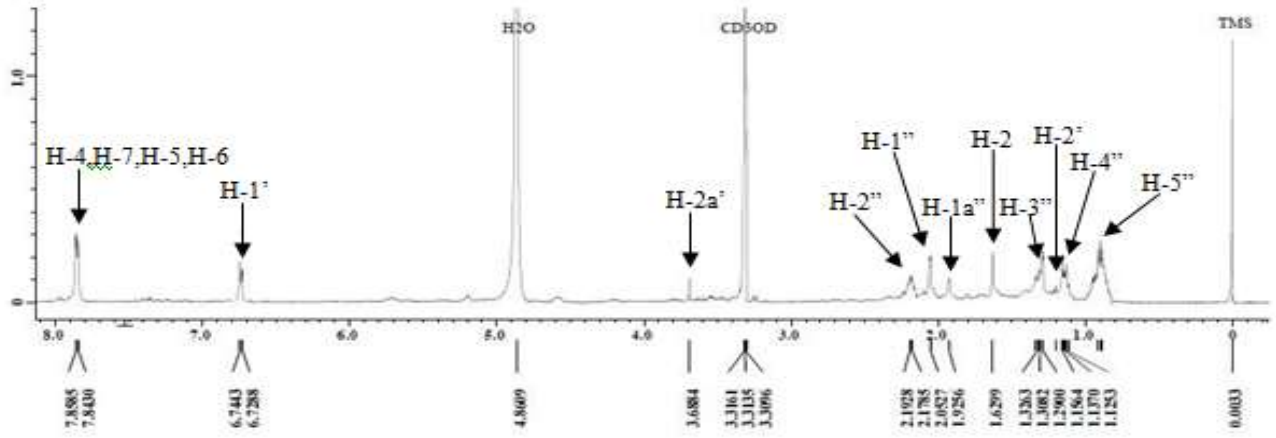

Figure 3: Spectrum of ^{1}H-NMR Isolate 2

Table III: Spectrum Data ^{1}H-NMR Isolate 2

δ_H (ppm)	Code	Multiplicity	Functional groups
7,85 dan 7,84	H-4, H-7, H-5, H-6	*doubled of doubled*	-CH=CH- (aromatis)
6,73	H-1'	*doublet*	-CH_2-
3,68	H-2a'	*singlet*	=NH-
2,16; 2,17; 2,19; 2,21; dan 2,24	H-2"	*quintet*	-CH-
2,0	H-1"	*doublet*	-CH_2-
1,92	H-1a"	*singlet*	-OH
1,62	H-2	*singlet*	-CH_2-
1,29; 1,30; 1,32; dan 1,39	H-3"	*quartet*	-CH_2-
1,20; 1,21; dan 1,21	H-2'	*triplet*	-CH=
1,11; 1,12; 1,13; 1,142; 1,149; dan 1,15	H-4"	*sextet*	-CH_2-
0,88; 0,90; dan 0,91	H-5"	*triplet*	-CH_3

The ^{1}H-NMR spectrum of isolate 2 shows protons with δ 7,85 ppm (H-4 and H-7, d, J = 7) and 7,84 ppm (H-5 and H-6, d, J = 7) benzene protons were substituted with ortho or benzene ortho substitution positions with four total integration, which comprises two linear sets of hydrogen (H-4 liniear with H-7, H-5 linear with H-6). Protons that appear at δ 6,73 ppm (H-1 ') with splitting doublet pattern, two integrations and coupling constant (J) 7,75 Hz indicate the presence of methylene (-CH_2-) group, methylene protons with more downfiled chemical shifts indicate that the methylene group binds a more electronegative atom (oxygen atom).

The electronegative substituents in the carbon will reduce the diamagnetic protection near the bounded protons, since they will reduce the electron density around the protons. The proton attached to the carbon that binds the electronegative element, the chemical shift of the proton will rise with the increase in electronegativity of the element bonded by the carbon atom [9]. Protons with δ 3,68 ppm (H-2a') exhibit a splitting singlet pattern with one integration indicating a proton attached to primary N atom [5], and holds clutch with H-2'.

Protons bound to N atoms do not really know their neighbor's protons so come out with a splitting singlet. Protons that appear at δ 2, 16; 2, 17; 2, 19; 2, 21; and 2, 24 ppm (H-2") with splitting quintet pattern and one integration indicate that this proton was a methyl proton (-CH-) having four neighboring protons. Proton neighbors of proton H-2" were either methylene protons (-CH_2) or methyl protons (-CH_3) and metin (-CH-).Proton with δ 2,0 ppm (H-1") with two integrations and splitting doublet pattern indicate the presence of methylene group (-CH_2-) adjacent to one proton or methyl group (-CH-). Protons that appear on δ 1,92 ppm (H-1a) with singlet splitting pattern with one integration were characteristic of protons attached to oxygen atoms having electronegative properties or protons of the hydroxy group (-OH) on the side chain. More common condition occurs in the uptake of hydroxy protons with higher concentrations, uptake appears in the region of δ 0,5 – 3,0 ppm because of the hydrogen bond between the hydroxy group or -NH with the solvent used in the analysis ie methanol and D_2O [7].

Protons with δ 1,62 ppm (H-2) with singlet splitting pattern and total two integration indicate the presence of a methylene group (-CH_2-), where protons with upfield chemical shifts indicate that the methylene group was attached to the carbonyl group (C=O) , while the more downfield protons indicate the presence of a methylene group binding at tertiary N atom. Proton that appear at δ 1, 29; 1, 30; 1, 32; and 1, 39 ppm (H-3") with a splitting quartet pattern with two integrations indicate the presence of a methylene group (-CH_2-) adjacent to three protons ie methyl and methylene protons or methyl protons.

Proton with δ 1, 20; 1, 21; and 1, 21 ppm (H-2') with one integration and triplet splitting pattern indicate the presence of methyl group (-CH=) adjacent to two protons ie methylene group. Molecular group having electrons π (phi) produces a secondary anisotropy magnetic field. The metin group having π electrons, the magnetic field generated by the induction of electrons rotation π has geometry in such a way that protons of the metin group are protected. The methyl proton has a resonance in the higher field than expected so that it appears in the upfield area [9]. Proton in upfield area with splitting pattern sextet at δ 1,11; 1,12; 1,13; 1,142; 1,149; and 1,15 ppm (H-4") with two integration representing the methylene group (-CH_2-). Five protons adjacent to the secondary protons, three on the other hand

and two on the other, are not equivalent, but the combined constants of J ab (5 hertz) and J BC (6 hertz) are almost identical. Proton at very upfiled area with δ 0, 88; 0, 90; and 0, 91 ppm (H-5") with three integration and triplet splitting patterns referred to the methyl (-CH_3) group adjacent to the two secondary protons. Protons in the upfield region were protons that are highly protected or away from electronegative atoms.

^{13}C-NMR and DEPT Spectrum Analysis

^{13}C-NMR and DEPT spectra were used to show the number, type, and position of the carbon atoms found in the isolates.^{13}C-NMR spectrum (Figure 4) isolate 2 detected 15 carbon resonance signal. Signals that appear in highly downfield areas with δ 177, 68 ppm (C-1) were characteristic for carbon-carbonyl resonance with conjugated double bonds. Signals that appear in highly upfield areas with δ22, 09 ppm (C-5 ") were characteristic of methyl carbon. The chemical shifts in ^{13}C-NMR spectroscopy were similar to ^{1}H-NMR spectroscopy, methyl carbon and TMS absorb in upfield while carbon carboxyl and aldehyde absorb in the downfield [7].

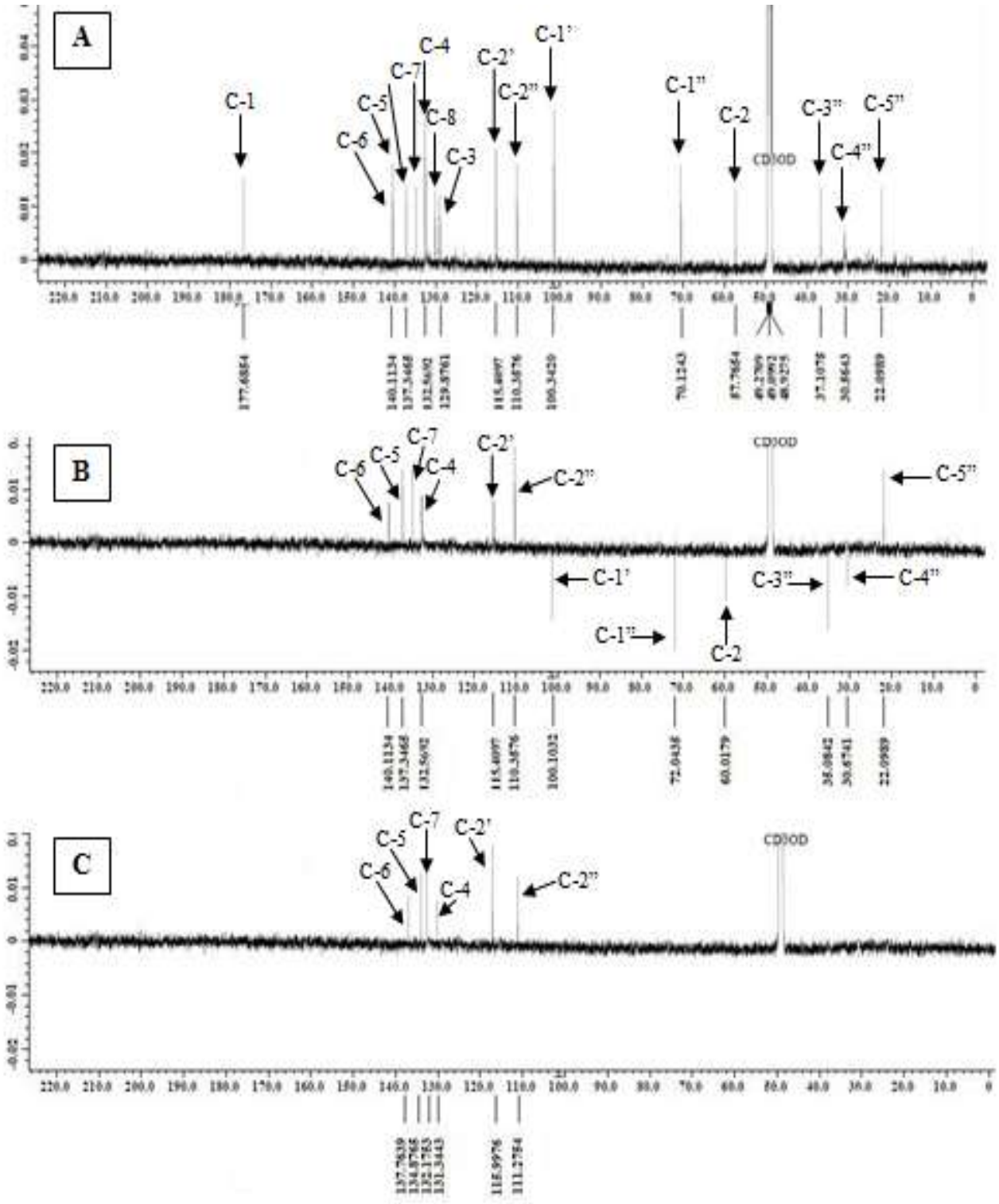

Figure 4:^{13}C-NMR spectra (A), DEPT-135 (B), and DEPT-90 (C) Isolate 2

Table IV: ^{13C}-NMR Spectrum Data Isolate 2

δ_c (ppm)	Code	Functional Group
177,68	C-1	C=O
22.09	C-5"	$-CH_3$
129,87; 130, 54; 132,56; 134,98; 137,34; dan 140,11	C-3, C-8, C-4, C-7, C-5, dan C-6	-C=C- (aromatis)
30,67; 35,08; 60,01; 72,04; dan 100,10	C-4", C-3", C-2, C-1", dan C-1'	$-CH_2-$ (metilen)
111,27 dan 115,99	C-2" dan C-2'	-CH- (metin)

Signal of the carbon spectrum with δ 129, 87; 130, 54; 132, 56; 134, 98; 137, 34; and 140, 11 ppm (C-3, C-8, C-4, C-7, C-5, and C-6) showed the carbon characteristics of the benzene group. The carbon signal from the benzene group will exit adjacent to a sufficiently downfield area. Hybridation of ^{13}C atoms was essential for determining the chemical shift, the carbon atoms of benzene which are the carbon atoms of sp and sp^2 will absorb simultaneously on the weak magnetic field so that it exits in a sufficiently downfield region [7].

The DEPT-135 analysis shows that there were five methylene car ($-CH_2$) with negative peak (Figure 4) at δ 30,67; 35,08; 60,01; 72,04; and 100,10 ppm (C-4", C-3", C-2, C-1", and C-1'). The three quartener carbons characterized by the missing peaks (Fig. 4A and B) ie those that exit at the highly downfield region at 177,68 ppm chemical shift were carbon from the carbonyl group (C-1) and at δ 129,87 and 130,54 ppm which was the two carbons of the benzene group at the substituted position (C-3 and C-8). One signal of carbon in the highly upfield region at δ 20, 09 was a carbon characteristic of the methyl group (C-5"). The DEPT-135 spectrum (θ3 = 135°) gives a negative signal for the methylene group ($-CH_2$), the positive signal for the methyl and methyl groups, but does not signal to the quartener carbon (signal loss) [7].

The DEPT-90 analysis showed six methyl carbon (Figure 4C) at δ 111,27; 115,99; 131,24; 132,17; 134,87; and 137,76 ppm (C-2", C-2', C-4, C-7, C-5, and C-6). One carbon signal at δ 20,09 ppm at DEPT-135 was thought to be the carbon of the methyl group, the DEPT-90 was not appear and it reinforces that the signal at δ 20,09 ppm was the signal of the methyl group. The DEPT-90 spectrum (θ2 = 90°) gives a positive signal for the methyl group, but carbon signals from quartener, methyl and methylene carbon do not appear [7].

LC-MS Mass Spectrum Analysis

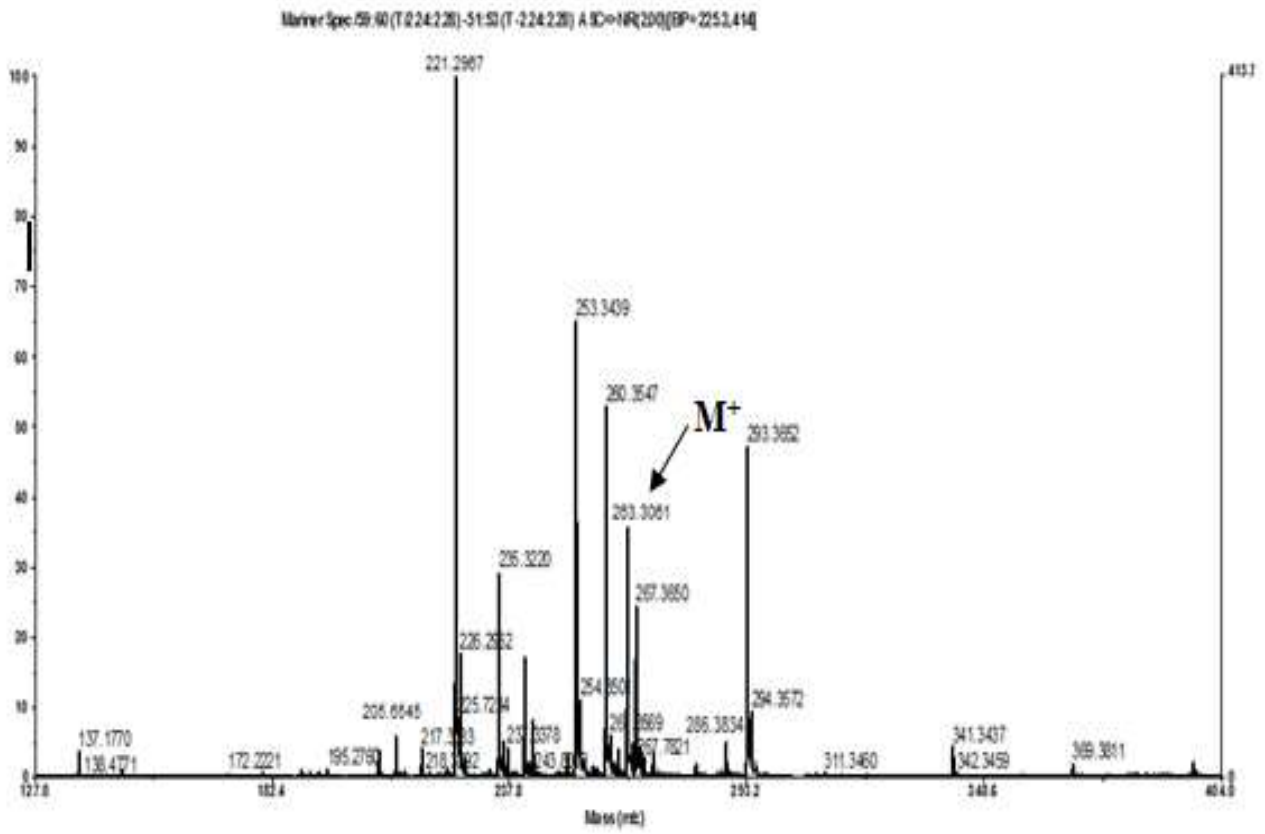

Figure 5: Mass spectrum Isolate 2 with methanol: water (90:10, v/v)

Identification of isolate 2 by LC-MS method showed a dominant peak at retention time of 2,2 minutes (Figure 2). From the peak, then mass spectral recording was performed using Electrospray Ionzation-Mass Spectrometry method (Figure 5). The mass spectrum of isolate 2 shows the presence of a fragment at m/z 221 with 100% abundance, it represents a stable ion fragment detected by a mass spectrometer. Fragmentation isolates showed fragments of m/z 235 (37%), 253 (66%), 260 (61%), 263 (35%), 293 (47%) and 137 m/z smallest fragment. Peak m/z 263 was corresponding to $[M]^+$ of isolate 2 have the molecular formula $C_{15}H_{21}NO_3$ with molecular weight of 263,33 g/mol. Based on the peak produced on the spectra, it appears that $C_{15}H_{21}NO_3.^+$ ions undergo several fragmentation patterns. In the first fragmentation pattern, $C_{13}H_{17}O_3^+$ ions were generated at peak with m/z 221 with 100% abundance (figure 25) and $C_2H_4N^+$ ions with m/z 42. Subsequent fragmentation produced $C_{13}H_{17}O_2^+$ ions in peak with m/z 205 with an abundance of 8% (Figure 5) and $C_2H_4ON^+$ ions with m/z 58. In the last fragmentation path a special peak produced with m/z 76 was characteristic of the benzene group.

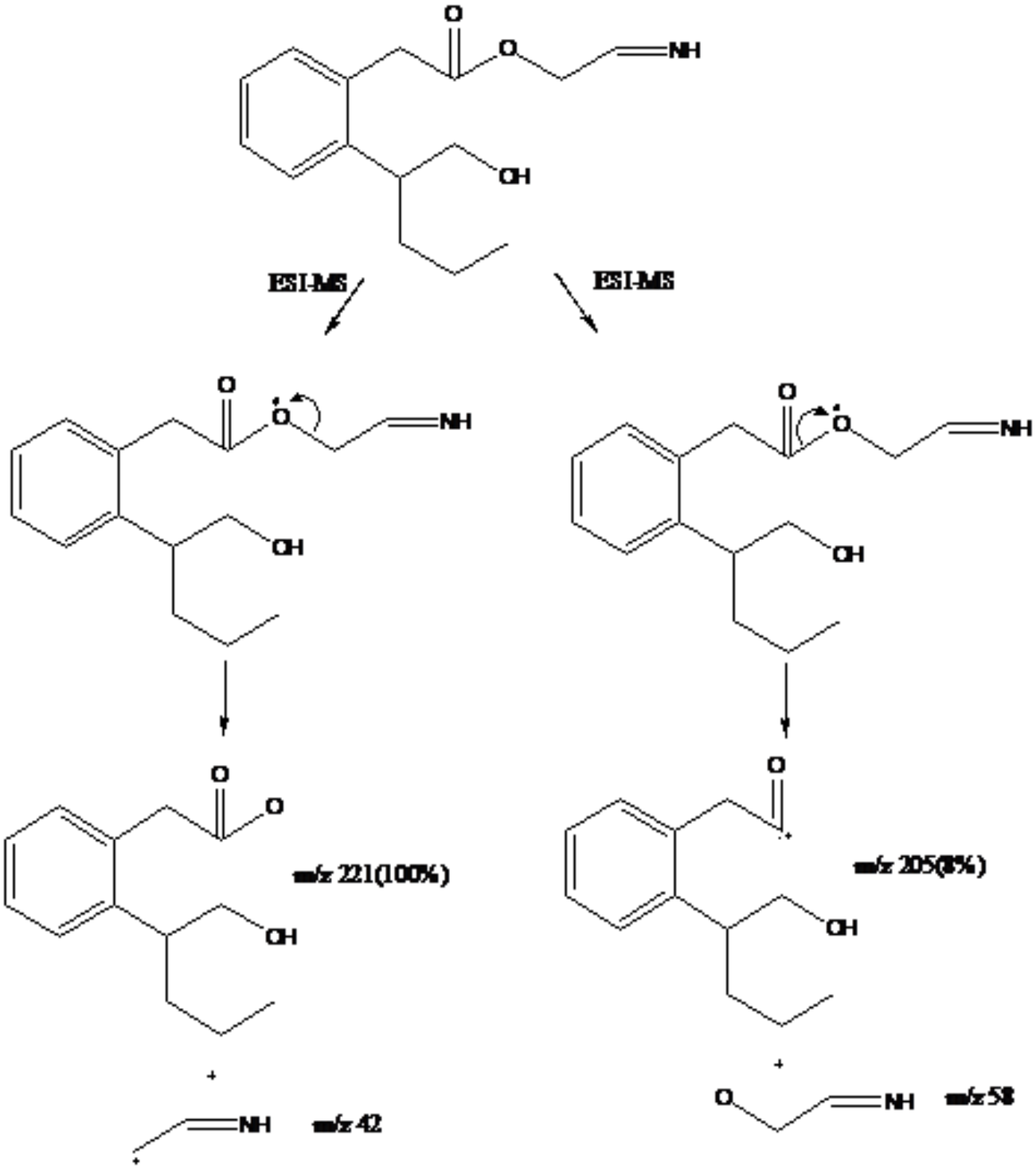

Figure 6: Estimation fragmentation pattern of isolate 2

Based on the four analysis of isolate 2 structure described above, it can be assumed that isolate 2 was an alkaloid derivative compound of L-serine and L-phenylalanine with $C_{15}H_{21}NO_3$ molecular formula and wake formula as shown in Figure 7. The name of the isolate compound 3 was obtained from ChemDraw Ultra 8.0, ie 2-iminoethyl 2-(2-(1-hydroxypentan-2-yl) phenyl) acetate. Further analysis was needed to confirm and strengthen the abstraction of the 2D-NMR isolate formulas such as COSY (Correlated Spectroscopy), HMQC (Heteronuclear Multiple Bond Connectivity) and HMBC (Heteronuclear Multiple Bond Connectivity).

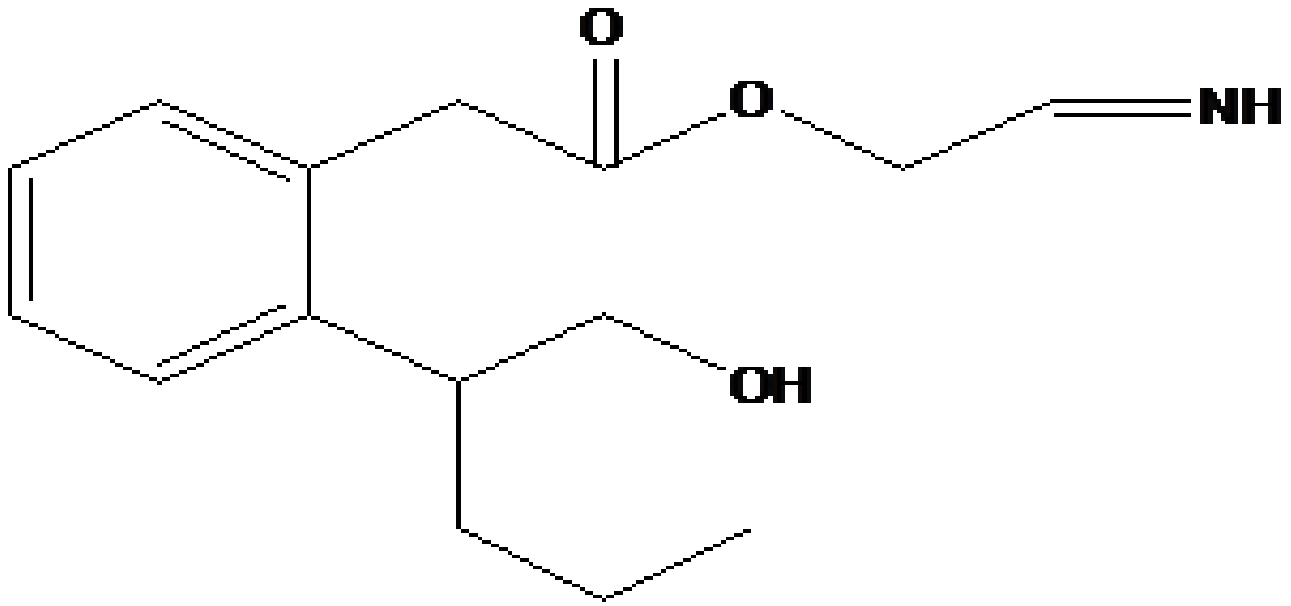

Figure 7: Structure Isolate 2 2-iminoethyl 2-(2-(1-hydroxypentan-2-yl) phenyl) acetate

Cytotoxic Activity

The data in Table V shows the IC_{50} value parameter, obtained from the test result that isolate 2 has IC_{50} value on T47D cancer cell of 7, 12 µg/ml with active category. The smaller the IC_{50} value of a compound the greater the cytotoxic effect. IC_{50} values obtained in the treatment of isolate 2 showed that it could be developed as a chemopreventive agent because IC_{50} values were less than 100 µg/mL [10].The criteria for selecting an anticancer compound against breast cancer cells were based on the potential, selectivity, ease of isolation and the adequacy of the compound to be tested and further developed.

The value of selectivity of a compound aims to determine the level of safety of an anticancer compound against normal cells. The required selectivity index value was > 3, indicating that extract, fraction, or isolate have cytotoxic activity against cancer cells but with minimal influence on normal cells, and can be further developed as a chemopreventive agent (Prayong *et al.*, 2008).

Isolate 2 selectively kills T47D breast cancer cells. It was seen from the value of selectivity index isolate 1 with value > 3. Strong anticancer activity categorized as follows: IC_{50} = 5 µg/mL (very active); IC_{50} = 5-10 µg/mL (active); IC_{50} = 11-30 (medium); IC_{50} = 30 µg/mL (inactive)[10].

Table V: Result of Cytotoxic Test of Isolates

Isolate	IC_{50} T47D (µg/ml)	IC_{50} Vero (µg/ml)	*Selectivity index* (SI)
1	7,12	338,44	47,53

Aside from the MTT assay results, the cytotoxicity caused by the treatment of isolates 2 can be observed through cell morphological changes. Treatment of isolates 2 caused T47D cells to undergo morphological changes ie shrunken cell nuclei, visible cell death, and cell number decreased, while cells without treatment showed normal morphology (Figure 8b).

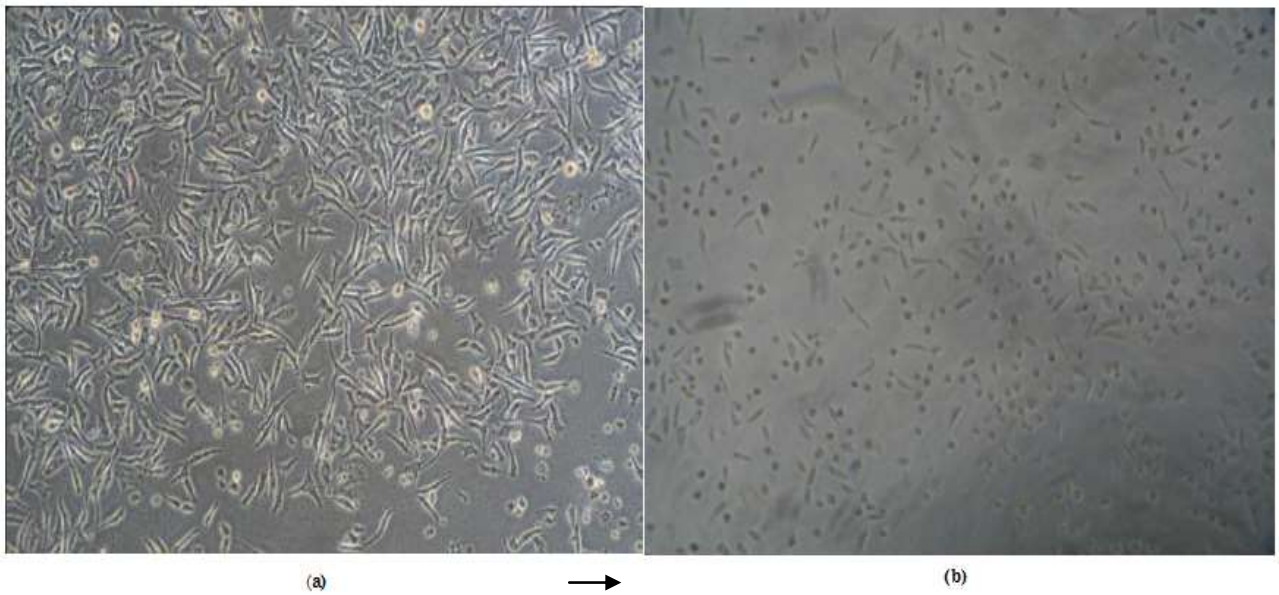

Figure 8: Effects of treatment of isolates 2 on Vero cells. The observations were performed under an inverted microscope with 100x magnification. (a) Cells without acting; (b) Isolate 2 with a concentration of 10 µg/mL. Vero cell morphological changes are shown with arrows ( )

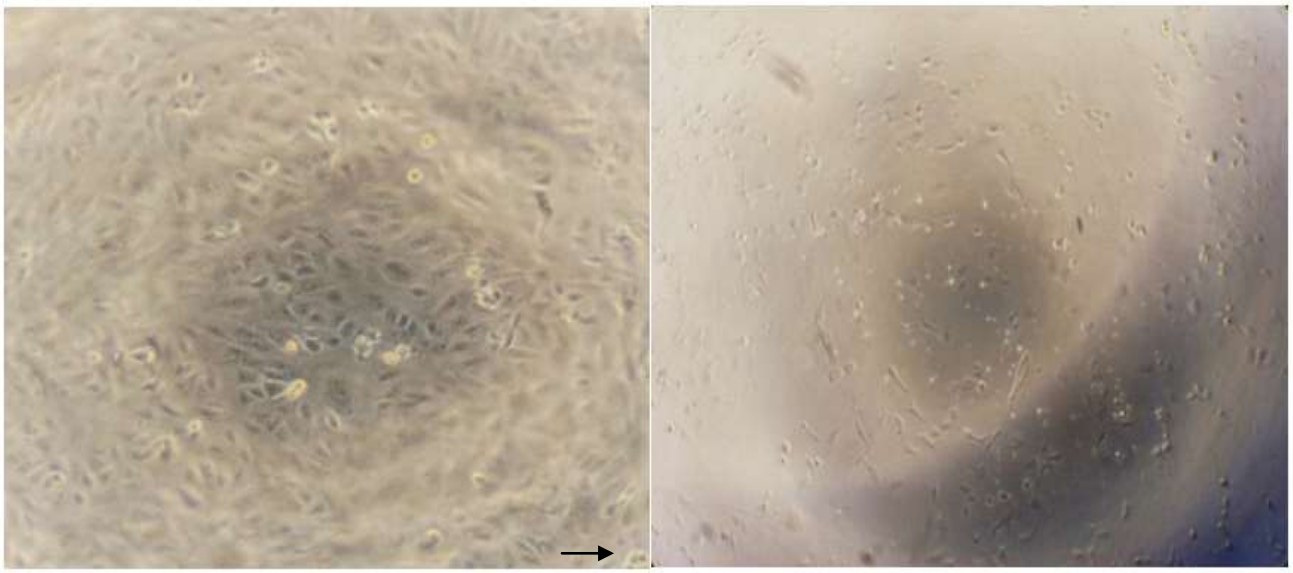

Figure 9. Treatment effect of isolate 2 on Vero cell. The observations were performed under an inverted microscope with 100x magnification. (a) cells without acting; (b) Isolate 2 with a concentration of 300 µg/mL. Vero cell morphological changes are shown with arrows ( )

The cytotoxic effects of isolates 2 on Vero cells based on IC_{50} values and cell morphology profiles showed a lower effect when compared to their effect on T47D cells. Treatment of isolates 2 also caused Vero cells to undergo morphological changes, cells were smaller and rounded but higher concentrations were required to produce the same effect on T47D cells, whereas untreated cells show normal morphology (Fig. 9b).

Cells indicate a possible morphological change because the cytoskeleton was cut off and the proteins that play a role in cell attachment do not undergo polymerization so that the cell bonds are released and the lipid membrane will be rounded (Fig. 8 and Fig. 9) [11]. The decrease in cell viability and cell density was seen in the higher doses used, as well as with shrinking morphological changes are the cell markers leading to death.

Conclusion

The results showed that isolate 2 was *2-iminoethyl 2-(2-(1-hydroxypentan-2-yl) phenyl) acetate* having activity cytotoxic in breast cancer cell type T47D with active category with IC_{50} 7,12 µg/mL.

Acknowledgements

Gratefully acknowledge for the financial support from Institute for Research and Community Service Ma Chung University from Ma Chung Research Grant 2018 (No: 06/MACHUNG/LPPM-MRG-MAD/III/2018).

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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