OXIDATION AND ACETYLATION OF URSOLIC AND OLEANOLIC ACIDS ISOLATED FROM *Fragraea fragrans* FRUITS: ANTIPROLIFERATION OF P388 LEUKEMIA CELLS

Dasril Basir[1,*], Julinar[1], Eva Agustriana[1], and Budi Untari[2]

[1]*Department of Chemistry, Faculty of Science, University of Sriwijaya, Palembang 30662, South Sumatra, Indonesia*

[2]*Pharmaceutical Program, Faculty of Sciences, University of Sriwijaya, Palembang 30662, South Sumatra, Indonesia*

Received March 27, 2014; Accepted August 5, 2014

ABSTRACT

An interesting natural product chemistry aspect of Fragraea fragrans is that their fruits are richness with ursolic acid and its isomer oleanolic acid (3.05% of dried powder). As our continuous work on these inseparable structural isomeric triterpenes, this paper reports that 51.0% of inseparable 3-oxo-ursolic[3-oxo-oleanolic] acids and 48.6% of inseparable 3-acetyl-ursolic [3-acetyl-oleanolic] acids have already been made from those triterpenes as starting materials of the oxidized and acetylated compounds and evaluated their activity against P388 leukemia cells. The activity of 3-oxo-ursolic [3-oxo-oleanolic] acids with IC_{50} = 18.6 µg/mL exhibited three-fold more potent against P388 leukemia cell proliferations compared to ursolic [oleanolic] acids with IC_{50} = 53.5 µg/mL; while the 3-acetyl-ursolic [3-acetyl-oleanolic] acids with IC_{50} = 37.9 µg/mL showed two-fold more potent then their parent triterpenes (IC_{50} = 53.5 µg/mL) in the inhibition of P388 leukemia cell growth.

Keywords: *Fragraea fragrans; fruits; 3-oxo-ursolic[-oleanolic] acids; 3-acetyl-ursolic[-oleanolic] acids; P388 leukemia cells*

ABSTRAK

Suatu aspek yang menarik dari kimia organik bahan alam tumbuhan tembesu, Fragraea fragrans adalah buahnya yang kaya dengan asam ursolat dan isomernya asam oleanolat (3.05% terhitung dari bubuk kering). Sebagai pekerjaan lanjutan terhadap triterpen yang berupa isomer struktural yang tidak terpisahkan ini, maka artikel ini melaporkan bahwa sebanyak 52,8% asam 3-okso-ursolat[3-oxo-oleanolat] dan 48,6% asam 3-asetil-ursolat[3-asetil-oleanolat] telah berhasil dibuat dari triterpenoid buah tembesu sebagai bahan baku oksidasi dan asetilasi serta mengevaluasi aktivitasnya terhadap pertumbuhan sel leukemia P388. Asam 3-okso-ursolat [3-oxo-oleanolat] dengan nilai IC_{50} = 18,6 µg/mL menunjukkan aktivitas antiproliferasi tiga kali lipat lebih kuat dari asam ursolat [oleanolat] dengan IC_{50} = 53,5 µg/mL. Akan tetapi, asam 3-asetil-ursolat[3-asetil-oleanolat] dengan nilai IC_{50} = 37,9 µg/mL hanya memberikan aktifitasnya dua kali lipat lebih kuat dibandingkan triterpenoid induknya (IC_{50} = 53.5 µg/mL).

Kata Kunci: *Fragraea fragrans; buah; asam 3-okso-ursolat[-oleanolat]; asam 3-asetil- ursolat[-oleanolat]; sel leukemia P388*

INTRODUCTION

As part of our efforts to develop and promote ursolic and oleanolic acids coming from *Fragraea fragrans* fruits called *buah tembesu* [1], we design oxidation and acetylation reactions at C3-OH position of the ursolic and its isomer oleanolic acids of those fruits to become inseparable 3-oxo-ursolic acid and 3-oxo-oleanolic acid, and inseparable 3-acetyl-ursolic acid and 3-acetyl-oleanolic acid leading to medicinal benefit for domestic communities and industries, especially to increase their activity against P388 tumor cell proliferation [7]. Since the structure of ursolic acid is closely related to that oleanolic acid and differs only in the position of methyl groups at C-19 and C-20, the structures of oxidation and acetylation products could be written as 3-oxo-ursolic [3-oxo-oleanolic] acids, and 3-acetyl-ursolic [3-acetyl-oleanolic] acids, and both of these structural isomer products were also expected to be similar in P388 anticancer activity.

Such as apple peels, the fruits containing numerous ursolic acid and its derivates; 3-oxo-urs-12-en-28-oic acid, 3-oxo-1a-hydroxy-urs-12-en-28-oic acid, 3-oxo-1,19a-dihydroxy-urs-12-en-28-oic acid have been reported to give a number of potential health benefit for humans, especially for lipase-inhibitory activity [2]. In synthesis works, 3-oxo-oleanolic acid was also a useful triterpene as a pratarget molecule to make CDDO (2-cyano-3, 12-dioxoolean-1,9-dien-28-oi c acid); the compound at micro- and nano-molar (10^{-6}–

* Corresponding author.
Email address : debasril_chem@yahoo.com

10^{-10}) level inhibited proliferation of many tumor lines [3-4]. Because ursolic and oleanolic acids have the similar chemical structures but differ only in the positions of methyl group in ring E; thus they are relatively close in pharmacological activities with lower toxicity effect [5-6]; As a conclusion ursolic and oleanolic acids of *Fragraea fragrans* fruits can be the potential starting material to produced a number of their derivative triterpenes such as 3-oxo-ursolic [3-oxo-oleanolic] acids and 3-acetyl-ursolic [3-acetyl-oleanolic] acids for anticancer leukemia P388. Moreover the ursolic acid or oleanolic acid itself may be considered as novel inhibitors of cyclooxygenasse-2 (COX-2) in producing prostaglandins from arachidonic acid and as promising lead-compounds for developing new anti-inflammatory drugs because the shape of this acid matches excellent with the receptor pocket thus it has best fit in the COX-2 enzyme [8-9].

EXPERIMENTAL SECTION

Materials

The chemical were used in this experiment consisted of technical methanol, activated carbon, Whatman paper, *d*-DMSO, *d*-pyridine, pyridine for analysis Emsure, Ac_2O, THF dried SeccoSolv, CrO_3 (Aldrich), H_2SO_4, HCl (Unilab), DMSO (Aldrich), Silica gel Plate G60 F254, Silica gel G60 (70-230 mesh), Na_2SO_4 anhydrous, ethyl acetate, aquadest, and acetone, 3-(4,5-dimrthylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide (MTT), fetal bovine serum (FBS), penicillin, streptomycin, dimethyl sulfocide (DMSO), phosphor buffer solution (PBS), artonin E, and sodium dodecyl sulphate. P388 murine leukemia cell culture supplied by Department of Chemistry (ITB).

Instrumentation

Melting point was determined using Fisher Johns apparatus. UV (in absolute ethanol) and IR (in KBr form) spectra were recorded on a Beck DU-7500 UV and Shimadzu 8400 FTIR spectrometers, respectively. ^{1}H (*d*-DMSO) and ^{13}C-NMR (*d*-DMSO) were determined on JEOL ECA500 spectrometer operating at 500 MHz (^{1}H), and 125 MHz (^{13}C), respectively. Mass spectra were obtained with a JOEL HPLC JMS-LX 1000 spectrometer using FAB mode.

Procedure

Collection of the fruits and recovery of the isomeric triterpenes

The fruits of *Fragraea fragrans* were collected in Inderalaya swamp forest, South Sumatra, Indonesia on October 2012, dried at room temperature for three months and then extracted with methanol according to the procedures of the isolation of ursolic and oleanolic acids from these fruits in our previous work [1].

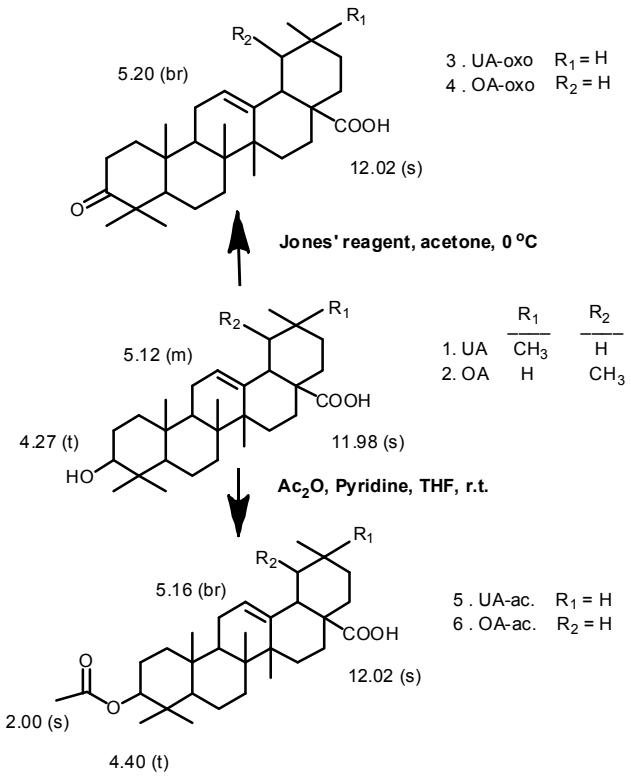

Fig 1. Oxidation and acetylation of C-3(OH) of the ursolic [oleanolic] acids isolated from *fragraea fragrans* fruits and their δ_H values: compound **1[2]** in *d*-pyridine; compound **3[4]** and **5[6]** in *d*-DMSO respectively

Oxidation of ursolic [its isomer oleanolic] acids to be 3 and 4

Oxidation was conducted according to the procedure Meng, et al. [4]. A solution of Ursolic [its isomer oleanolic] acids (1.697 g) in acetone (30 mL), Jones reagent (7 mL) was stirred slowly for one hour at 0 °C. Jones reagent was made by solving CrO_3 (2.67 g) with H_2SO_4 (2.3 mL) and distilled water (5.2 mL) [7]. The reaction was then allowed to be warming at room temperature and stirred for other three hours. The reaction was controlled by means of TLC and the oxidation and starting material spots gave respectively Rf value of 0.29 and 0.19 with 20% ethyl acetate in hexane. The reaction mixture was then cooled again in ice-salt water bath and 2-propanol (85 mL) was dropped to this last mixture and stirred for half hour.

The green precipitates was filtered from 2-propanol solution and washed with acetone (3 x 75 mL); Both of the 2-propanol and acetone filtrates were evaporated under reduced pressure to afford solid materials (2.3 g). This solid was then

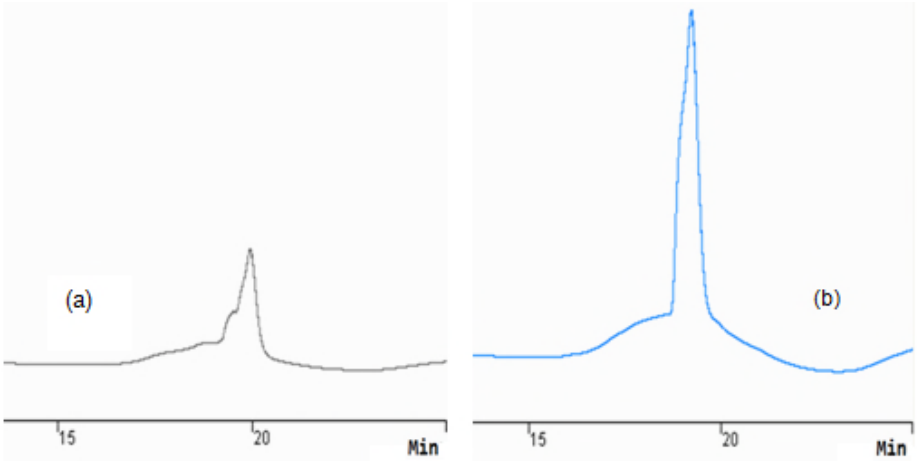

Fig 2. HPLC chromatogram of (a) **3[4]** and (b) **5[6]** by means of HPLC column with 10% DMSO in methanol

subjected to silica gel G60 column (75 g) with 10% ethyl acetate in hexane. Oxidation product (861.4 mg) were collected from vial number 85-146 (10 mL/vial) and then prepared to spectroscopy analysis including MS, ^{1}H-NMR, ^{13}C-NMR (DEPT 90 and DEPT 135), and tested for antitumor P388 leukemia cells.

Acetylation of ursolic [its isomer oleanolic] acids to be 5 and 6

Acetylation was conducted according to the procedure in reference [4]. A solution of Ursolic [its isomer oleanolic] acids (1.0 g) in THF (30 mL), pyridine (2 mL), and acetic anhydride (5 mL) was stirred for 4 h at room temperature. Reaction was controlled by TLC plates (Rf = 0.6 for acetylation products and Rf = 0.2 for starting materials) with 20% ethyl acetate in hexane. When reaction was complete, the solvent was concentrated in vacuo and the residues were dispersed in water, and then acidified to pH 3-4 with HCl. The acid solution was extracted with ethyl acetate (3 x 125 mL), dried with Na_2SO_4 anhydrous (7.5 g), filtered, and then concentrated in vacuo. The solids (740 g) were subjected to silica gel G60 column with increasing solvent polarity from 10 to 20% ethyl acetate in hexane. Acetylation product (486 mg) were collected, and then prepared to spectroscopy analysis including IR, MS, ^{1}H-NMR, ^{13}C-NMR (DEPT 90 and DEPT 135), and tested for antitumor P388 leukemia cells.

Antiproliferation evaluation

P388 murine leukemia cancer cell cultures (3 x 10^3 cell/mL) were suspended into RPMI 1640 media having contained FBS (Fetal Bovine Serum), penicillin, and streptomycin. Cells were inoculated in microplate 96 well plate and incubated in CO_2 incubator for one day. At the second day, the DMSO (dimethylsulfocide) solution of the crystals of **1[2]**, **3[4]**, and **5[6]** was diluted with PBS (phosphoric buffer solution, pH = 7.30-7.65) for variation concentrations of 100, 30, 10, 3, 1, 0.3, and 0.1 µg/mL media and then was dropped into those cells respectively. These last cells in microplate were incubated again in CO_2 incubator. The DMSO was used as negative control and artonin E (IC_{50} = 0.7 µg/mL) as positive control.

After 48 h the incubation process, MTT reagent was added into the cells, incubated during 4 h, and SDS was then added, shaked as well, and continuously incubated for other 24 h. The calor change of MTT in viable mitochondria cells from yellow to purple could be quantified with spectrophotometer at λ = 550 nm. The values of OD and concentration (µg/mL) of tested compounds were reported as the mean three of replicates. The IC_{50} value was noted from antilog graphics based on the correlation of tested compound concentrations (µg/mL) and color intensity of cell viable solution, respectively [11-13].

RESULT AND DISCUSSION

Ursolic [Its Isomer Oleanolic] Acids; 1 and 2

Isolation procedures, physical properties, and structure elucidation included molecular ion peak (M^+), δ_C, and δ_H values of triterpenoid-**1** and -**2** with m.p. = 284-286 °C, molecular ion peak (FAB-MS) = 456 have already been reported [1]. In this paper, NMR data in *d*-pyridine of **1** and **2** was shortly given as review, CMR = δ_C values; 78.08 (C-3), 122.51 (C-12), 144.78 (C-13), 180.12 (C-28) for ursolic acid (**1**); and 78.04 (C-3), 125.61 (C-12), 144.79 (C-13), 179.84 (C-28) for its isomer oleanolic acid (**2**); and PMR = δ_H values also differentiate signals between ursolic acid (**1**): 4.27 (t, 1H, H-3), 5.15 (t, 1H, H-12, J = 3.5 Hz), 11.98 (s, 1H, COOH); and its isomer oleanolic acid (**2**): 4.26 (t, 1H, H-3), 5.12 (t, 1H, H-12, J = 3.5 Hz), 11.98 (s, 1H, COOH).

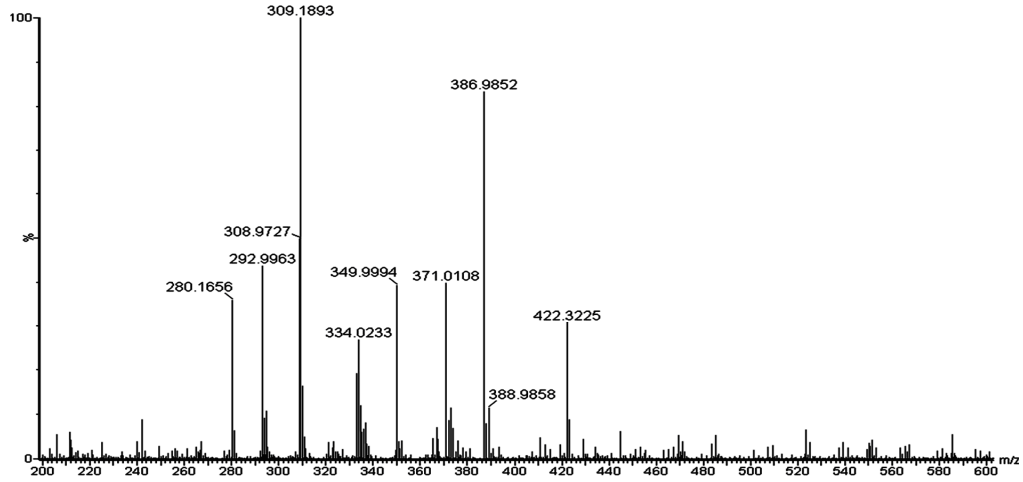

Fig 3. Mass spectral of 3-oxo-ursolic [oleanolic] acids

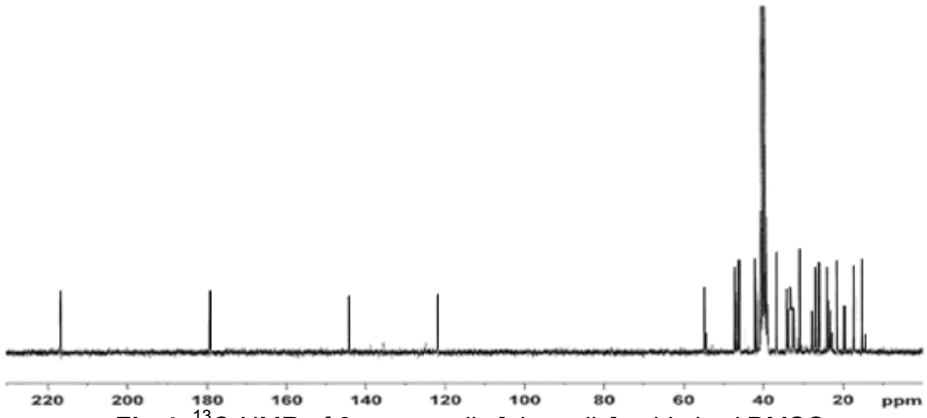

Fig 4. ^{13}C-NMR of 3-oxo-ursolic [oleanolic] acids in *d*-DMSO

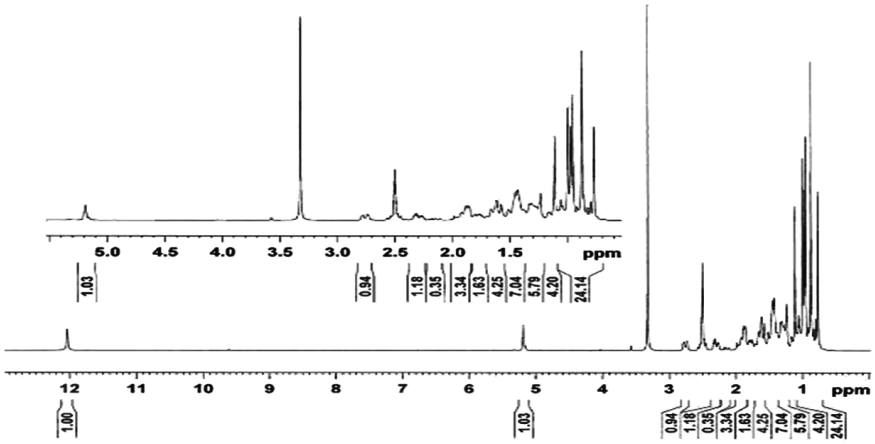

Fig 5. ^{1}H-NMR of 3-oxo-ursolic [oleanolic] acids in *d*-DMSO

3-Oxo-ursolic [3-oxo-oleanolic] Acids; 3 and 4

Compounds **3[4]**, 861.4 mg, 51% counted from compound **1** and **2**, gave m.p. = 187-189 °C. HPLC chromatogram was 20 min with 10% DMSO in methanol, Fig. 2a. The TOF MS ES$^+$ (1.20e5) gave fragment peaks (m/z): 422 [M – (CH_3 and$^{\cdot}$OH)]$^+$, 367, 371, 350, 334, 309 (base peak), 293, and 280, Fig. 3. Therefore, the peak (m/z) of 422 + CH_3OH (CH_3 and OH radicals) corresponds to molecular formula $C_{30}H_{46}O_3$ with molecular weight = 454, and DBE = 8 consisting of three functional groups of C=O, C=C, and COOH respectively,

Table 1. ^{13}C-NMR and ^{1}H-NMR data of 3-oxo-ursolic acid [its isomer 3-oxo-oleanolic acid] and 3-acetyl- ursolic acid [its isomer 3-acetyl-oleanolic acid] in *d*-DMSO

Carbon Number	3-oxo-UA [OA]		3-acetyl-UA [OA]	
	δ_C	δ_H	δ_C	δ_H
1	38.82	1.61	38.81	1.61
2	23.57		23.57	
3	218.90		80.60	4.40
4	37.32		37.32	
5	55.76		55.76	
6	18.74		18.74	
7	33.13		33.21	
8	39.71		39.71	
9	46.63		46.61	
10	37.31		37.31	
11	23.65	2.73	23.65	2.73
12	124.20	5.20	124.20	5.16
13	144.79		144.78	
14	41.95		41.95	
15	28.26		28.73	
16	23.71		23.77	
17	47.99		48.07	
18	53.49	2.82	53.49	2.82
19	39.32		39.32	
20	38.89		38.89	
21	30.44		30.91	
22	34.16		34.16	
23	28.04		28.04	
24	16.49		16.49	
25	15.51		15.51	
26	15.62		15.62	
27	26.12		26.12	
28	179.60	12.02	179.60	12.02
29	17.38		17.46	
30	21.37		21.37	
C=O			175.80	
CH_3			23.94	2.00

Note: UA = ursolic acid; OA = oleanolic acid; 3-oxo-UA [OA] = inseparable oxidation product; 3-acetyl-UA [OA] = inseparable acetylation product

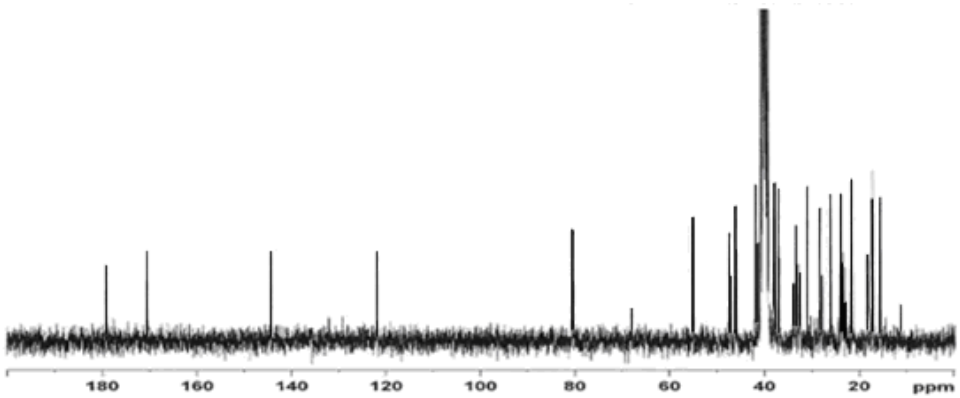

Fig 6. ^{13}C-NMR of 3-acetyl-ursolic [oleanolic] acids in *d*-DMSO

and five rings. The PMR of **3[4]** in *d*-DMSO gave the main functional groups at δ_H 5.20 (br, 1H, -CH=C- of C-12), and 12.02 (s, 1H, -COOH of C-28). While the methyl, methylene, and methine appeared at higher field in δ_H 3.0 to 0.5 range, Fig. 5. The CMR of oxidation products **3[4]** exhibited significant signals at δ_C 218.9 (C=O of C-3), 124.2 (-CH=C- of C-12), 144.78 (-CH=C- of C-13), and 179.60 (-COOH of C-28), (Table 1 and Fig. 4).

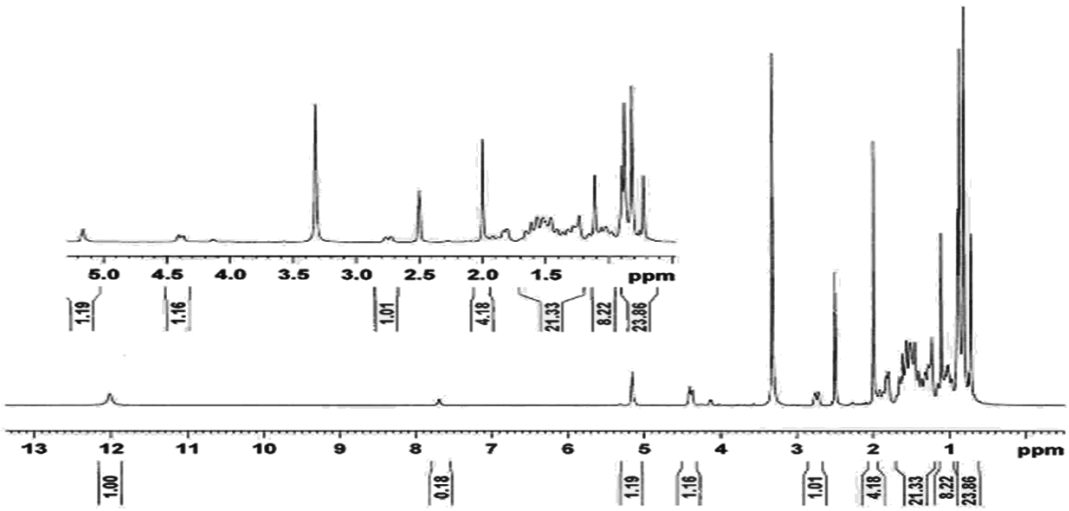

Fig 7. ^{1}H-NMR of 3-acetyl-ursolic [oleanolic] acids in *d*-DMSO

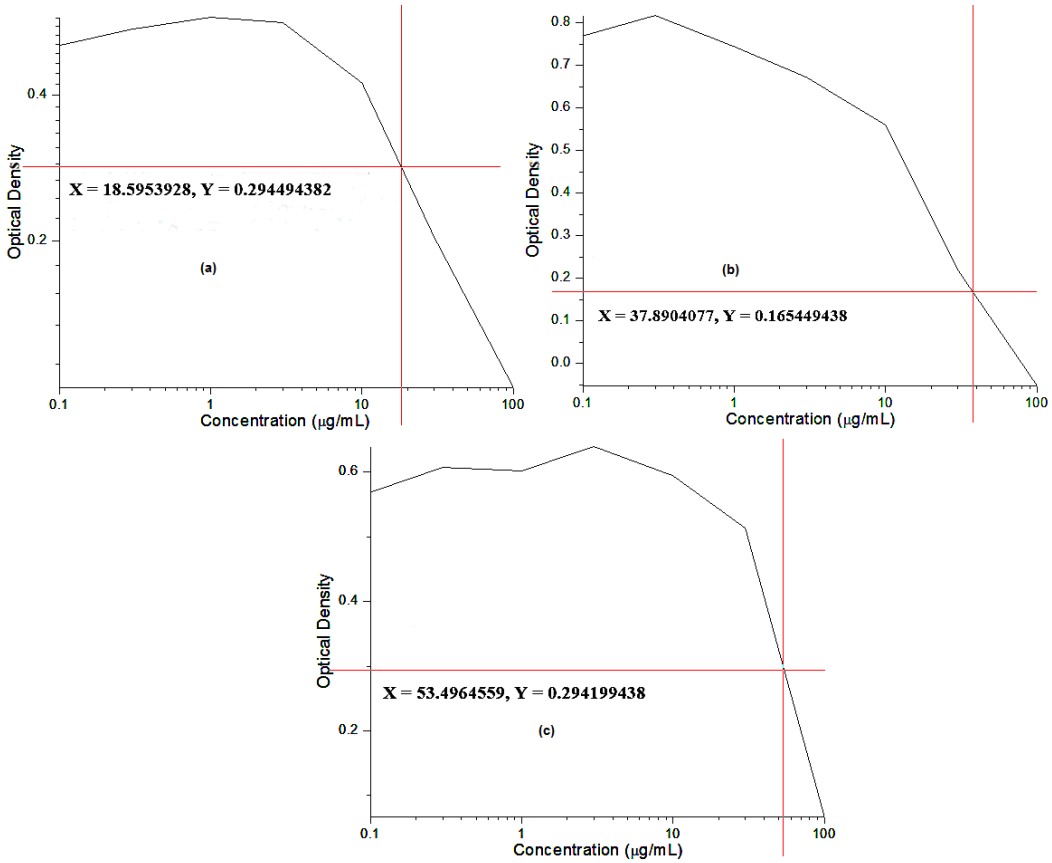

Fig 8. Graphics of the concentration of tested compound vs. the absorption of P388 leukemia cells. (a) = 3-oxo-usolic [3-oxo-oleanolic] acids, **3[4]**, with IC_{50} = 18.6 µg/mL and (b) = 3-acetyl-ursolic [3-acetyl-oleanolic] acids, **5[6]**, with IC_{50} = 37.9 µg/mL

3-Acetyl-ursolic [3-acetyl-oleanolic] Acids; 5[6]

Compounds **5[6]**, 486 mg, 48.6% counted from **1[2]**, gave m.p. = 190-193 °C. HPLC chromatogram was 18 min with 10% DMSO in methanol, Fig. 2b. The TOF MS ES^{+} (1.20 x 10^{5}) gave a molecular ion at (m/z): 567 $[M + 3Na]^{+}$ for $C_{32}H_{50}O_4Na_3$ and 521 $[M + Na]^{+}$ for $C_{32}H_{50}O_4Na$, 518, 517, 514, 441, 431 (base peak), 413

Table 2. IC_{50} and absorbance (optical density) of compound **1[2]**, **3[4]**, and **5[6]** at a wavelenght of 550 nm.

No	Concentration (µg/mL)	Compound **1[2]**		Compound **3[4]**		Compound **5[6]**	
		Optical density (OD)	IC_{50} (µg/mL)	Optical density (OD)	IC_{50} (µg/mL)	Optical density (OD)	IC_{50} (µg/mL)
1	100	0.067		0.099		0.051	
2	30	0.513		0.203		0.219	
3	10	0.594	53.5	0.424	18.6	0.559	37.9
4	3	0.639		0.566		0.671	
5	1	0.602		0.580		0.743	
6	0.3	0.607		0.548		0.817	
7	0.1	0.569		0.507		0.769	

OD of positive blank for **5[6]** = 0.1640 and for **1[2]** and **3[4]** = 0.2943

380, and 364. Therefore, the peak (m/z) at 521 – Na corresponds to molecular formula $C_{32}H_{50}O_4$ with molecular weight = 498, and DBE = 8 consisting of two C=O in acetyl and acid respectively, C=C, and five rings. The PMR of **5[6]** in *d*-DMSO gave the main functional groups at δ_H 2.00 (s, CH3 of acetyl), 4.40 (t, 1H, C-3 occupied by OH), 5.16 (br, 1H, -CH=C- of C-12), and 12.02 (s, 1H, -COOH of C-28). While the methyl, methylene, and methine appeared at higher field in δ_H 3.0 to 0.5 range, Fig. 7. The CMR of acetylation products **5[6]** exhibited significant signals at δ_C 175.80 (C=O of AcO- at C-3), 80.60 (C-3), 124.2 (-CH=C- of C-12), 144.78 (-CH=C- of C-13), and 179.60 (-COOH of C-28) [10], (Table 1 and Fig. 6). The IR (KBr) ν_{max} of acetylated compound **5[6]** showed absorption at 3563 sharp (methyl), 2921 (aliphatic C-H), 1767 (carbonyl ester), 1730 (carbonyl acid), 1630, 1453 (-C=CH-) and the rest peaks were at 1238, 1026, and 1006 cm^{-1}. While the hydroxyl on C-3 position of the unacetylated compound **1[2]** gave adsorption at 3385 cm^{-1} broad (hydroxyl). This improved that –OH was transformed to –OAc. As conclusion, the other IR peaks of unacetylated compound **1[2]** were 2937, 1656, 1564, 1460, 1356, 1269, 1182, 1050, 980, and 825 cm^{-1}. The spectroscopic data including MS (FAB), PMR, CMR, and IR indicated that hydroxyl group at C-3 position in compound **1** or **2** was changed to be its acetyl in **5[6]**; 3-acetyl-ursolic [3-acetyl-oleanolic] acids.

Antiproliferation Activities of 1[2], 3[4], and 5[6]

Compound **1[2]** as starting materials, and compound **3[4]**, and **5[6]** as C-3 modification products respectively gave IC_{50} values of the P388 leukemia cell antiproliferation: 53.5, 18.6, and 37.9 µg/mL. The results of this *in vitro* test were given in Table 2. Artonin E and DMSO were used as positive and negative controls, while the absorbance (OD) was read at a wavelength of 550 nm. The IC_{50} values of compound **1[2]**, **3[4]**, and **5[6]** were respectively determined from the graphics of the tested compound concentration versus the P388 leukemia cell absorption, see semilog graphics of 53.3818 (x-axis) as concentration (µg/mL) and 0.2937 (y-axis) as absorbance or optical density (OD), (Fig. 8c and Table 2); 18.2319 (x-axis) and 0.2847 (y-axis), (Fig. 8a and Table 2); and 37.8524 (x-axis) and 0.1685 (y-axis), (Fig. 8b and Table 2). As a result, the effect of C=O group at C-3 significantly increased the activities of P388 leukemia cell antiproliferation compared to acetyl and hydroxyl groups.

CONCLUSION

Ursolic [its isomer oleanolic] acids (IC_{50} = 53.5 µg/mL) as inseparable white solid crystals coming from *Fragraea fragrans* fruits has been successfully modified to be inseparable 3-oxo-ursolic [3-oxo-oleanolic] acids (IC_{50} = 18.6 µg/mL). These derivations are potential compounds for the antiproliferation of P388 leukemia cells rather then inseparable 3-acetyl-ursolic [3-acetyl-oleanolic] acids or their mother natural triterpenes.

ACKNOWLEDGEMENT

This work was fully supported by funds from Indonesian Higher Education Department, *Hibah Bersaing*, contract number; 1106.a/UN9.4.2/LK-ULP/2013-2014. MS spectral data were kindly furnished by Prof. Dr. Teruna Jaya Siahaan, Department of Pharmaceutical Chemistry, The University of Kansas, USA, and we also thank Prof. Dr. Naresh Kumar, School of Chemistry, The University of New South Wales, Kensington, Sydney, Australia for providing us with the PMR and CMR spectrals, *Rancak.*

REFERENCES

1. Basir, D., and Julinar, 2012, *Indo. J. Chem.*, 12 (1), 84–88.
2. McGhie, T.K., Hudault, S., Lunken, R.C.M., and Christeler, J.T., 2012, *J. Agric. Food Chem.*, 60 (1), 482–491.
3. Sun, H., Fang, W-S., Wang, W-Z., and Hu, C., 2006, *Bot. Stud.*, 47, 339–368.

4. Meng, Y., Song, Y., Yan, Z., and Xia, Y., 2010, *Molecules*, 15 (6), 4033–4040.
5. Feng, J-H., Chen, W., Zhao, Y., and Ju, X-L., 2009, *Open Nat. Prod. J*., 2 (1), 48–52.
6. Liu, J., 1995, *J. Ethnopharmacol.,* 49, 57–68.
7. Finlay, H.J., Honda, T., and Gribble, G.W., 2002, *ARKIVOC*, xii, 38–46.
8. Manikrao, A.M., Khatale, N.P., Jawarkar, R.D., Vias, J.V., Mahajan, D.T., Masand, V.H., and Habda, B.T., 2011, *J. Comput. Method. Mol. Design*, 1 (2), 9–13.
9. Vasconcelos, M.A.L., Royo, V.A., Ferreira, D.S., Crotti, A.E., Andrade e Silva, M.L., Carvalho, J.C., Bastos, J.K., and Cunha, W.R., 2006, *Z. Naturforsch. C: Biosci*, 61 (7-8), 477–482.
10. Naved, T., Ansari, S.H., Mukhtar, H.M., and Ali, M., 2005, *Indian J. Chem., Sect B*, 44 (5), 1088–1091.
11. Kwon, T.H., Lee, B., Chung, S.H., Kim, D-H., and Lee, Y.S., 2009, *Bull. Korean Chem. Soc.*, 30 (1), 119–123.
12. Basha, D.P., Ravishankar, K., Kiranmayi, G.V.N., and Subbarao, M., 2013, *World J. Pharm. Pharm. Sci.*, 2 (6), 4987–4996.
13. Husniati, and Hanafi, M., 2010, *Jurnal Teknologi Indonesia*, 33 (1), 27–31.