Free Solvent Amidation of Ursolic and Oleanolic Acids of Fagraea Fragrans Fruits: Their P-388 Antitumor Activity

To cite this article: D Basir et al 2018 J. Phys.: Conf. Ser. 1095 012006

View the article online for updates and enhancements.
Free Solvent Amidation of Ursolic and Oleanolic Acids of Fagraea Fragrans Fruits: Their P-388 Antitumor Activity

D Basir\textsuperscript{1}, M. Hanafi\textsuperscript{2}, Julinar\textsuperscript{1}, A Saputra\textsuperscript{1}, and T Wati\textsuperscript{1}

\textsuperscript{1}Department of Chemistry, Faculty of Sciences, The University of Sriwijaya, Palembang, Indonesia, email: debasril.chem@yahoo.com, Phone Number 0711-580927
\textsuperscript{2}Research Center for Chemistry, Indonesian Institute of Science, Kawasan PUSPIPTEK Serpong, Tangerang, Indonesia

E-mail email: debasril.chem@yahoo.com.

Abstract. This is an our laboratory effort in developing major compounds, ursolic acid (UA) and its isomer oleanolic acid (OA) of Fagraea Fragrans Roxb fruits, namely buah tembesu, Loganiaceae by free solvent amidation of the acids with \textit{n}-buthylamine and phenylamine. The reactions have been conducted by using thionyl chloride as a chlorination reagent to make their acid chlorides respectively. In this reaction, the secondary hydroxyl group at C-3 position was preferably converted to be olefinic while the carboxyl group at C-28 position was normally amidated to give \textit{N}-buthyl-urs-2,12-dien-28-amide [\textit{N}-buthyl-olean-2,12-dien-28-amide] with yield 45.5\% and \textit{N}-phenyl-urs-2,12-dien-28-amide [\textit{N}-phenyl-olean-2,12-dien-28-amide] with yield 39\%. Cytotoxic activity of amidation products toward P-388 murine leukemia cells was decreasing about half fold compared to starting material ursolic acid [oleanolic acid]. The IC\textsubscript{50} values of \textit{N}-butyl-urs-2,12-dien-28-amide [\textit{N}-butyl-olean-2,12-dien-28-amide] and \textit{N}-phenyl-urs-2,12-dien-28-amide [\textit{N}-phenyl-olean-2,12-dien-28-amide] were 81.4 \textmu g/mL and 83.6 \textmu g/mL respectively, whereas the IC\textsubscript{50} value of ursolic acid [oleanolic acid] was 53.4 \textmu g/mL.

Keywords ; Fagraea fragrans, fruits, oleanamide, ursolamide, P-388 marine leukemia cells

1. Introduction
This paper describes the thionyl chloride reactions on secondary alcohol and carboxyl groups in 3-hydroxyl-urs-12-en-28-oic acid and its isomer 3-hydroxyl-olean-12-en-28-oic acid isolating from the fruits of Fagraea fragrans, Loganiaceae \textsuperscript{[3,8]}. In addition to develop those natural triterpenes to be their derivates of \textit{N}-buthyl-urs-2,12-dien-28-amide [\textit{N}-buthyl-olean-2,12-dien-28-amide] and \textit{N}-phenyl-urs-2,12-dien-28-amide [\textit{N}-phenyl-olean-2,12-dien-28-amide] and their cytotoxic activity toward P-388 murine leukemia cells. The amidation reactions were conducted betwen ursolic acid [oleanolic acid], thionyl chloride, aliphatic amine, and aromatic amine separately without using organic solvent.

It was realizing that in nature the triterpenes such as ursolic acid and oleanolic acid were found in many plant species but they were rarely found in form of amides. Therefore these works were capable to develop the natural acid triterpenes to be their amide derivatives, see structure 3\textsuperscript{[4]} and 5\textsuperscript{[6]} in Figure 1. These derivatives still need other biological activity test because in this experimental they have lower cytotoxic activity toward P-388 murine leukemia cells compared to their mother triterpenes or the synthetic design must be changed. Some of ursolamide derivatives were reported to have antimalarial agents \textsuperscript{[1]}. 
As it has been shown in Figure 1 and referred to references of Gnoatto et al (2008) \cite{1} and Basir, D. et al (2014) \cite{2} dealing with biological activities, especially cytotoxic activity toward P-388 murine leukemia cells. These amidation reactions have involved eliminative reactions of hydroxyl group at C-3 position in both conditions of n-buthylamine and phenylamine eventhough those amines were different in base strength. As a conclusion, the hydroxyl group of carboxyl in C-28 was seem to have similar reactivity with secondary hydroxyl group at C-3 position toward thionyl chloride reagent and the heat of reaction (60 °C) might have been in powering the elimination reaction to give a double bond in C-2 and C-3 positions. In addition to those, N-buthyl-urs-2,12-dien-28-amide [N-buthyl-olean-2,12-dien-28-amide] and N-phenyl-urs-2,12-dien-28-amide [N-phenyl-olean-2,12-dien-28-amide] as amide derivatives of ursolic acid [its isomer oleanic acid] were the first report in this paper \cite{4,5,6,15}, and this works could also give additional data to improve that there is no doubt; Fagraea. Fragarans fruits contain ursolic acid and its isomer oleanolic acid as major metabolites.

To maintain the secondary hydroxyl group in the starting materials and to amidate the carboxyl group selectivity, the protecting group such as acetyl or oxo has to be inserted at C-3 position before the free solvent amidations\cite{12}. Therefore, some of N-buthyl-3-acetyl-urs-12-en-28-amide [N-buthyl-3-acetyl-olean-12-en-28-amide] and N-phenyl-3-acetyl-urs-12-en-28-amide [N-phenyl-3-acetyl-olean-12-en-28-amide]; and N-buthyl-3-oxo-urs-12-en-28-amide [N-buthyl-3-oxo-olean-12-en-28-amide] and N-phenyl-3-oxo-urs-12-en-28-amide [N-phenyl-3-oxo-olean-12-en-28-amide] can be produced from the triterpenes of Fagraea fragrans fruits \cite{2,9}. As a result, they are biological activities, especially toward P-388 murine leukemia cells, will be more potent then their mother triterpenes with IC$_{50}$= 53.4 µg/mL and 3-oxo-ursolic acid [its isomer 3-oxo-oleanolic acid] with IC$_{50}$= 18.3 µg/mL\cite{2}.

2. Materials and Methods

2.1 Materials

*Fagraea fragrans* fruits, technical methanol, activated carbon, *d*-pyridine, *d*-chloroform, *n*-buthylamin, anilin, thionyl chloride, silica gel G60 (70-230 mesh), Na$_2$SO$_4$ anhydrous, aquadest, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide (MTT), fetol bovine serum (FBS), penicillin, streptomycin, P388 murine leukemia cell culture supplied by Department of Chemistry (ITB), dimethyl sulfoxide (DMSO), phosphor buffer solution (PBS), artonin E, sodium dodecyl sulphate, whatmann paper, ethyl acetate, n-hexane, acetic acid, formic acid, and methanol p.a. for LC MS-ESI positive ion.

2.2 Instrumentation

Melting point was determined using Fisher Johns apparatus. $^1$C-NMR (*d*-CHCl$_3$) were determined on JEOL ECA500 spectrometer operating at 500 MHz ($^1$H), and 125 MHz ($^1$C), respectively. Mass spectra were obtained with a HITACHI L6200 LC-MS ESI positive ion spectrometer which was running on cullumn C-18 (15mm x 1 mm), flow 0.1 mL/min, injection volume 2 µl, and 0.3% formic acid in MeOH as solvents.

2.3 LC-MS Analysis

LC-MS analysis was performed using an Mariner Biospectrometry equipped with a binary pump. The HPLC was interfaced with a Q-tof mass spectrometer fitted with an ESI source. Full-scan mode from *m/z* 100 to 1200 was performed with a source temperature of 140 °C. HPLC column (Phenomenex 5µ C18, 150 × 1 mm i.d., ) was used for the analysis. Solvent was methanol with 0.3% formic acid. Solvents were delivered at a total flow rate of 0.1 mL/min. The solvent running by isocratic elution.
2.4 Compounds 1 [2]

The fresh *Fagraea fragrans* fruits were collected in Inderalaya swamp forest, Ogan Komring, South Sumatra on October 2014, see Figure 2b. The plant specimen was identified and deposited in the plant taxonomy laboratory of Department of Biology, Faculty of Science, Sriwijaya University. The fruits were then dried over two months at room temperature, and then milled to be powder. The powder were extracted with methanol according to the procedures of the isolation of the ursolic acid and its isomeric oleanolic acid from these fruits in our previous work, Basir, D. et al., (2012 and 2014) [2, 3], and then they (m.p.=284-286 °C) were used as starting materials to make the compounds 3 [4]. The spectroscopic elucidation data for these triterpenic acids as starting materials in this work has been given in those reference 2 as well.

2.5 Compounds 3 [4]

Amidation was conducted without using organic solvent according to the procedure of Kumagai et al., (2010) [7]. Thionyl chloride 0.5 mL (6.9 mmol) was added to white crystals of ursolic [its isomer oleanolic] acids (154 mg, 0.34 mmol) and then the mixture was stirred for three hours in round flask at 60 °C. This later solution was allowed to be cooled at room temperature, thus the n-buthylamine 0.2 mL (2 mmol) was gradually dropped to the solution while it was stirred again for 2 hours. The reaction was stopped whenever it gave different retention time (r.f. = 0.8) from the retention time of starting material (r.f. = 0.4) respectively with 20% ethyl acetate in n-hexane. The amidation product was then dissolved in chloroform (30 mL), washed with water (30 mL). The chloroform phase was dried with anhydrous Na₂SO₄, and filtered with whatmann paper, and later on the chloroform was evaporated under reduced pressure to give dry residue (130mg). This residue was then subjected to silica gel G60 column with increasing solvent polarity from 1 to 5 % ethyl acetate in n-hexane to give 70 mg (45.5%) of compound 3 [4], and prepared to spectroscopy analysis including MS, ¹H NMR, ¹³C NMR (DEPT 90 and 135), and tested for P-388 murine leukemia cells.

![Figure 1](image_url)

**Figure 1.** Free solvent amidation of ursolic acid (UA) and its isomeric oleanolic acid (OA) isolated from *Fagraea fragrans* fruits.

2.6 Compounds 5 [6]

Amidation was also conducted without using organic solvent according to the precedure of Kumagai et al., (2010) [7]. Thionyl chloride (0.3 mL, 3.5 mmol) was added to white crystals of ursolic [its isomer oleanolic] acids (100 mg, 2.2 mmol) and then the mixture was stirred for three hours in round flask at 60 °C. This later solution was allowed to be cooled at room temperature, thus the N-phenylamine 0.2 mL (3,5 mmol) was gradually dropped to the solution while it was stirred again for 2 hours. The reaction was stopped whenever it gave different retention time (r.f. = 0.7) from the retention time of starting material (r.f. = 0.4) respectively with 20% ethyl acetate in n-hexane. The amidation product was then dissolved in chloroform (50 mL), washed with water (50 mL). The chloroform phase was dried with anhydrous Na₂SO₄, and filtered with whatmann paper, and later on the chloroform was evaporated under reduced pressure to give dry residue (81.2). This residue was then subjected to silica gel G60 column with increasing solvent polarity from 1 to 5 % ethyl acetate in n-hexane to give 30.8 mg (39%) of compound 5 [6], and prepared to spectroscopy analysis including MS, ¹H NMR, ¹³C NMR (DEPT 90 and 135), and tested for cytotoxic activity against P-388 murine leukemia cells.
Compound 5[6] were also made in solvent (benzene) condition. The result was still similar to free solvent amidation condition that took place via C-2 and C-3 elimination on ring-A and C-28 amidation.

2.7 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 incubated in microplate 96 well plate and incubated in CO2 incubator for one day. At the second day, the DMSO (dimethylsulfoxide) solution of the crystals of 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 CO2 incubator. The DMSO was used as negative control and artonin E (IC50 = 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 (Sodium Dodecyl Sulphate) was then added, shacked as well, and continuously incubated for other 24 h. The color 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 IC50 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].

3. Results and Discussion

Compounds 3[4] consisted of of N-buthyl-urs-2,12-dien-28-amide [N-buthyl-olean-2,12-dien-28-amide, They had LC chromatogram of 3[4] with r.t. = 13.6 minutes by LC column with 10% DMSO in methanol, while the compounds 5[6] consisted of N-phenyl-urs-2,12-dien-28-amide [N-phenyl-olean-2,12-dien-28-amide with LC chromatogram of 5[6] with r.t. = 34.6 minutes by LC column with 0.5% acetic acid in methanol.

Compounds 3[4] had some peaks of LCMS-ESI positive ion and methanol as eluent at m/z = 998.68 or 990.67 as protonated dimeric ions [2M + H+], 516.84 as pseudomolecular ion [M + Na – H], and 494.85 as molecular ion peak [M+H]+. while the compounds 5[6] gave some peaks at m/z = 1028.64 or 1050.66 as protonated dimeric ions [2M + H+], 536.78 as pseudomolecular ion [M + Na – H], and 514.80 as molecular ion peak [M+H]+.

Compounds 3[4] had some peaks of 1H-NMR at δ (ppm) = 5.99, 5.95, 5.93, 5.41, 5.40, 5.39, 5.38, 4.74, 4.70, 4.68, 4.44, 3.90, 3.88, 3.37, 3.35, 3.34, 3.04, 3.03, 3.02, 3.01, 2.51, 2.49, 2.27, 2.22, 1.66, 1.62, 1.45, 1.44, 1.43, 1.42, 1.34, 1.33, 1.32, 1.31, 1.25, 1.17, 0.98, 0.95, 0.94, 0.92, 0.90, 0.89, and 0.85. while the compounds 5[6] gave some main peaks at δ (ppm) = 7.75, 7.73, 7.47, 7.46, 7.30, 7.29, 7.26, 7.22, 6.74, 6.72, 6.58, 6.53, 5.62, 5.61, 5.60, 4.25, 4.23, 2.88, 2.68, 2.66, 2.37, 2.35, 2.05, 1.71, 1.70, 1.41, 1.40, 1.37, 1.25, 1.22, 1.03, 0.95, 0.94, 0.93, and 0.74. The 13C NMR of the compounds 3[4] was at δ (ppm) = 178.5 (C-28 of CONH for ursolamide, 178.3 [C-28 of CONH for its isomeric oleanamide ] ), 145.8 (C-13), 138.1 (C-2), 122.9 (C-12), 121.4 (C-3), 56.0, 52.1, 47.0, 46.5, 46.2, 42.5, 39.3, 34.4, 33.2, 32.6, 31.6, 31.0, 26.0, 24.1, 23.7, 20.4, 19.7, 14.0, while the compounds 5[6] was at δ (ppm) = 176.8 (C-28 of CONH- for ursolamide, 176.7 [C-28 of CONH- for its isomeric oleanamide ] ), 145.7 (C-13 for for ursolamide, 145.3 [C-28 for its isomeric oleanamide ] ), 138.3 (C-2 for for ursolamide, 138.1 [C-2 for for ursolamide ] ), 136.1 (C-3), 124.1 (C-12), 140.1, 129.1, 124.2, and 119.9 ( carbons of phenylcy), 46.2, 34.5, 34.1, 33.1, 32.6, 32.5, 30.9, 27.8, 25.6, 24.4, 23.7, 23.6, 19.7. Some of the chemical shifts of carbon peaks of compounds 5[6] could be clearly differentiated ursolamide peaks from its isomeric oleanamide peaks but they were not detected fully in the compounds 3[4], exception the carbonyl peaks of those amides at 178.5 (C-28 of CONH for ursolamide, 178.3 [C-28 of CONH for its isomeric oleanamide ] ) respectively.

3.1 Ursolic [Its Isomer Oleanolic] Acids, 1 [2]

Isolation procedures, physical properties, and structure elucidation included δC, and δH values of triterpenes 1 [2] with m.p. = 284-286 °C, and molecular ion peak (FAB-MS) = 457 (M + H)+ have

IOP Conf. Series: Journal of Physics: Conf. Series 1095 (2018) 012006 doi:10.1088/1742-6596/1095/1/012006
already been reported in reference 3, Basir D. et al. (2012). In this paper, $^{13}$C NMR data of 1 [2] in d-pyridine was given in Table 1.

3.2 N-buthyl-urs-2,12-dien-28-amide [N-buthyl-olean-2,12-dien-28-amide], 3 [4]

Compound 3 [4], 70 mg, 42 % counted from compound 1 [2], gave m.p. 141-143°C. TLC (r.f.) was 0.37 in 20% ethyl acetate in n-hexane and LC chromatogram was 13.6 minutes with 0.5 % acetic acid in methanol. The LC-MS ESI gave molecular ion peak (M + H)$^+$ at $m/z = 494.8$, (M + Na) at $m/z = 516.8$, (2M + H)$^+$ at $m/z = 988.7$, and (2M + Na) at $m/z = 1010.7$. Molecular weight (MW) calculated was 493 which relatively correspond to the experimental (494.8 – H = 493.8) data with empirical formula, C$_{34}$H$_{55}$O$_1$N$_1$, double bond equivalent (DBE) = eight (8), consisted of five rings and three double bonds i.e. two of C=C and one of C=O. The chemical shifts of 3 [4] in ppm were given in Table 1. Table 1 indicated that $^1$H NMR of 3 [4] at $\delta$H = 5.9 (br, 1H, $J$ = 8.4 Hz) for its amide functional as reported in N-alkyl/aryl acetamide in reference 4 was coming from the loss of hydroxyl group of carboxyl at C-28 with $\delta$H =11.98 (s, 1H) in compound 1 [2]. Those were also supported by changing $\delta$C value of –COOH at 179.6 [180.1] to $\delta$C value of –CONH- at 178.3 [178.5]. The 3 [4] also has $\delta$H = 5.1 (1H at C-3) and 5.1 (1H at C-2) resulted in the elimination of H and OH coming from hydroxyl at C-2 and hydrogen at C-3 of 1 [2] with $\delta$H = 4.27 (t, 1H at C-3) and 2.98 (m, 1H at C-2) as well as $\delta$C of 3 [4] at 121.4 (C-3) and 138.1 (C-2) coming from $\delta$C of 1 [2] at 79.6 (C-3) and 23.6 (C-2).

3.3 N-phenyl-urs-2,12-dien-28-amide [N-phenyl-olean-2,12-dien-28-amide], 5 [6]

Compound 5 [6], 30.8 mg, 38 % counted from compound 1 [2], gave m.p. 122-123°C. TLC (r.f.) was 0.4 in 20% ethyl acetate in n-hexane and LC chromatogram was 34.6 minutes with 10% DMSO in methanol[12], Figure 2b. The LC-MS ESI gave molecular ion peak (M + H)$^+$ at $m/z = 514.8$, (M + Na) at $m/z = 536.8$, (2M + H)$^+$ at $m/z = 1028.6$, and (2M + Na) at $m/z = 1050.6$. Molecular weight (MW) calculated was 513 which relatively correspond to the experimental (514.8 – H = 513.8) data with empirical formula, C$_{36}$H$_{51}$O$_1$N$_1$, double bond equivalent (DBE) = twelfth (12), consisted of six rings and three double bonds i.e. five rings in triterpenenoid skeleton, one ring of aromatic, two of C=C and one of C=O. In addition to the above free solvent condition, the LC-MS ESI of solvent (benzene) amidation condition gave protonated molecular ion peak (M + H)$^+$ at $m/z = 514.83$, and protonated dimeric ions [2M + H+]$^+$ = 1029.07, see Figure 2, with a single peak on retention time = 0.6 minutes (injection volume = 5 µL , flow 0.1 mL/min, and MeOH eluent). The chemical shifts of 5 [6] in ppm were given in Table 1. Table 1 indicated that $^1$H NMR of 5 [6] at $\delta$H = 7.8 (s, br, 1H) for its amide functional was coming from the loss of hydroxyl group of carboxyl at C-28 with $\delta$H =11.98 (s, 1H) in compound 1 [2]. Those were also supported by changing $\delta$C value of –COOH at 179.6 [180.1] to $\delta$C value of –CONH- at 176.8 [176.7]. The 5 [6] also has $\delta$H = 5.6 (1H at C-3) and 5.6 (1H at C-2) resulted in the elimination of H and OH coming from hydroxyl at C-2 and hydrogen at C-3 of 1 [2] with $\delta$H = 4.27 (t, 1H at C-3) and 2.98 (m, 1H at C-2) as well as $\delta$C of 5 [6] at 136.1 (C-3) and 138.3 (C-2) coming from $\delta$C of 1 [2] at 79.6 (C-3) and 23.6 (C-2).

As it has been described in the introduction that the action of amine base in rate of elimination of H and OH from C-2 and C-3 positions of the starting materiales 1[2] to be a double bond via the chlorinated intermediate, see Figure 3a, might be affected significantly. Moreover aliphatic amine forced forming of the double bond faster then aromatic amines, thus they gave 3[4], 45.5% then 5[6], 39%. These are the in situ reaction for both of elimination and amidation reactions so that the presumable reaction mechanism of this works are written as below, see Figure 3a. As shown in Figure
3a, both of hydroxyl at C-3 and carbonyl at C-28 of 1[2] had been converted to their chloronated compounds as well as those chlorinated intermediates were converted to be 3[4] and 5[6] as elimination and amidation products respectively [10]. Amidation of 1[2] in both solvent (benzene) and free solvent conditions has given the same target compounds, 5[6]. This is indicated that secondary amine can not be made by means of solvent and free solvent conditions from secondary alkyl halide of ring-A. As a result, this elimination and amination products belong to new derivatives of ursolamide and oleanamide.

Table 1. $^{13}$C-NMR and $^1$H-NMR data of ursolic acid [its isomer oleanolic acid], 1 [2] in pyridine, N-buthyl-urs-2,12-dien-28-amide [its isomer N-buthyl-olean-2,12-dien-28-amide], 3 [4], and N-phenyl-urs-2,12-dien-28-amide [its isomer N-phenyl-olean-2,12-dien-28-amide], 5 [6] in CDCl$_3$

| C. Number | $^1$C, δC | $^1$H, δH | $^1$C, δC | $^1$H, δH | $^1$C, δC | $^1$H, δH |
|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| 1 | 38.8 | 39.2 | 40.9 |
| 2 | 23.6 | 2.98 (m) | 138.1 | 5.38 (m) | 138.3 | 5.64 (d, 1H) |
| 3 | 79.6 | 4.27 (t, 1H) | 121.4 | 5.39 (m) | 136.1 | 5.68 (d, 1H) |
| 4 | 37.3 | 36.2 | 38.3 |
| 5 | 55.7 | 55.8 | 52.1 |
| 6 | 18.7 | 16.6 | 19.72 |
| 7 | 33.1 | 33.1 | 32.6 |
| 8 | 39.7 | 39.2 | 39.7 |
| 9 | 46.6 | 46.4 | 46.2 |
| 10 | 37.3 | 39.2 | 36.3 |
| 11 | 23.7 | 23.7 | 24.4 |
| 12 | 124.2 | 5.15 (t, 1H) | 122.9 | 5.4 (t) | 124.1 | 5.62 (t, 1H) |
| 13 | 144.8 | 145.8 | 145.3 |
| 14 | 41.9 | 42.3 | 40.3 |
| 15 | 28.3 | 30.8 | 28.1 |
| 16 | 23.7 | 24.0 | 24.4 |
| 17 | 48.0 | 46.4 | 49.5 |
| 18 | 53.5 | 52.1 | 47.6 |
| 19 | 39.3 | 39.2 | 42.9 |
| 20 | 38.9 | 34.3 | 40.2 |
| 21 | 30.4 | 1.27 (m) | 31.5 | 1.3 (d) | 31.1 | 1.25 (m) |
| 22 | 34.2 | 34.3 | 34.4 |
| 23 | 28.0 | 25.9 | 33.1 |
| 24 | 16.5 | 25.8 | 16.8 |
| 25 | 15.5 | 20.6 | 15.8 |
| 26 | 15.6 | 16.7 | 15.9 |
| 27 | 26.1 | 19.5 | 26.5 |
| 28 | 179.6 | 180.1 (OA) | 111.98 (s, 1H) | 178.3 (UA), 178.5 (OA) | 176.8 (UA), 176.7 (OA) |
| 29 | 17.4 | 15.6 | 17.1 |
| 30 | 21.4 | 16.6 | 21.5 |

| NH- | 5.9 (br, J=8.4 Hz) | 7.8 (s, br, 1H) |

*Not; UA = ursolic acid and OA = oleanolic acid*
**Figure 2.** LC-MS ESI mass spectra of compound 5[6] made in the solvent (benzene) amidation condition of ursolic acid (UA) and its isomer oleanolic acid (OA) isolated from *Fagraea fragrans* fruits.

**Figure 3.** (a). Free solvent elimination and amidation reaction mechanisms of the H and OH in ring-A and COOH at C-28 of 1[2] to give 3 [4] or 5 [6] via chlorinated intermediates. (b). The *Fagraea fragrans* Roxb fruits as a potential resource of 3-hydroxyurs-12-en-28-oic acid [1] and its structural isomer 3-hydroxyolean-12-en-28-oic acid [2].

### 3.4 Antiproliferation Activities of 3[4] and 5[6]

Compound 1[2] as starting materials have IC$_{50}$ values = 53.5 µg/mL, see reference 2, Basir D.et al. (2014) while the compound 3[4], and 5[6] as the products of C-28 amidation including C-2 and C-3 elimination respectively gave IC$_{50}$ values of the cytotoxic againsts P-388 murine leukemia cells: 81.4, and 83.6 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 3[4] and 5[6] were respectively determined and the results were given in Table 2. Compound 3[4], N-buthyl-urs-2,12-dien-28-amide [N-buthyl-olean-2,12-dien-28-amide], and compound 5[6], N-phenyl-urs-2,12-dien-28-amide [N-phenyl-olean-2,12-dien-28-amide], gave IC$_{50}$ = 81.4 µg/mL and IC$_{50}$ = 83.6 µg/mL respectively as well as their semilog graphics of the tested compound concentration versus the P-388 murine leukemia cells absorption; 81.4335 (x-axis)
as concentration (µg/mL) and 0.1735 (y-axis) as absorbance or optical density (OD) for 3[4] and and 83.6331 (x-axis) and 0.1750 (y-axis) as absorbance or optical density (OD) for 5[6], see Table 2. As a result, the effect of elimination of H and OH in ring-A of these amide products, 3[4] and 5[6] decreased half fold of the activities of P-388 murine leukemia cell antiproliferation compared to the their mother natural triterpenes 1[2] with IC₅₀ =53.4 µg/mL. Eventhough the N-buthyl-urs-2,12-dien-28-amide (3), N-buthyl-olean-2,12-dien-28-amide (4) as isomeric molecule of 3; and N-phenyl-urs-2,12-dien-28-amide (5), N-phenyl-olean-2,12-dien-28-amide (6) as isomeric of 5. They have similar P-388 murine leukemia cell antiproliferation activity.

As a conclusion, ursolic [its isomer oleanolic] acids (IC₅₀ = 53.5 µg/mL ) as inseparable white solid crystals coming from *Fragraea fragrans* fruits has been succesfuly amidation to be inseparable N-buthyl-urs-2,12-dien-28-amide [N-buthyl-olean-2,12-dien-28-amide] (IC₅₀ = 81.4 µg/mL ), and N-phenyl-urs-2,12-dien-28-amide [N-phenyl-olean-2,12-dien-28-amide] (IC₅₀ = 83.6 µg/mL). In addition to our previous works on *Fragraea fragrans* (called tembesu) fruits that are richly containing acid pentacyclic triterpenes [2,3], There is no doubt that those fruits are still the potential as resources of ursoic acid and its isomeric oleanolic acid to be developed to be other amide derivatives with more active anticancer compound by means of other amidation reaction routes eventhough in this works they gave lower antiproliferation activity against P388 leukemic cells. In short, the amide derivatives containing hydroxyl or amine group at C-3 are more prefered rather then alkene groups at C-2 and C-3 positions to be strong P-388 murine leukemia cell antiproliferation candidates.

Table 2. IC₅₀ and absorbance (optical density) of compound 3[4], and 5[6] at a wave length of 550 nm.

| Concentration (µg/ml) | Compound 3[4] | Compound 5[6] |
|----------------------|---------------|---------------|
|                       | Optical Density [OD] | IC₅₀ (µg/ml) | Optical Density [OD] | IC₅₀ (µg/ml) |
| 1                     | -0.0163        | -0.0106       |
| 2                     | 1.1822         | 1.2920        |
| 3                     | 1.9617 81.43   | 1.5203 83.63 |
| 4                     | 1.3972         | 1.6270        |
| 5                     | 1.7417         | 1.6926        |
| 6                     | 1.7202         | 1.5060        |
| 7                     | 2.0812         | 1.2970        |

OD of positive blank for 1[2] = 0.2943 and for 3[4] and 5[6] = 0,1778

**Acknowledgment**
This work was fully supported by funds from Indonesian Higher Education Department, and my Institution, the Sriwijaya University Research Center; rancak.

**References**
[1] Gnoatto SCB, Susplugas S, Vechia L D, Ferreira TB, Klimpt A D, Zimmer K R, Demailly C, Nascimento S D, Grollion J, Grellier P, Verli H, Gosmann G and Sonnet P 2008 *Bioorganic & Medicinal Chemistry*, 16 771.

[2] Basir D, Julinar, Agustriana E and Untari B 2014 *Indonesian J. of Chemistry*, 14(3) 269.

[3] Basir D and Julinar 2012 *Indonesian J. Chem.*, 12(1) 84

[4] Katke S A, Amrutkar S V, Bhor R J and Khainar M V 2011 *International J. of Pharma Sciences and Research (IJPSR)*, 2(7) 148.

[5] Chen H, Gao Y, Wang A, Zhou X, Zheng Y and Zhou J 2015 *European J. of Medicinal Chemistry*, 92, 648.

[6] Meng Y, Song Y, Yan Z and Xia Y 2010 *Molecules*, 15 4033.

[7] Kumagai, Takashi, Ebi T, Konishi A, Matsumoto K, Kurata H, Kubo T, Katsumoto K, Kitamura C and Kawase T, 2010 *Tetrahedron*, 66 8968.

[8] He X, and Liu R H 2007 *J. Agr.Food Chem.*, 55 4366.

[9] Mazumder K, Tanaka K and Fukase K 2013 *Molecules*, 18 8929.

[10] Montalbetti A G, Christian N, and Falque V 2005 *Tetrahedron*, 61 10827.

[11] Hanaf M, Salahuddin, and Haryanti 2013 *Indo. J. Chem*, 13(2) 166.

[12] Zou S., and Wu Chen 2008 *Journal Braz.Chem.Soc.*, 19(7) 1429.

[13] Batool A., Shahid K., and Muddasir M 2015 *Int. J. Pharm. Sci. Rev. Res.*, 30(2) 25.

[14] Bi Y., Xu J X, Sun F, Wu X M, Ye W C, Sun Y J, Meng Q G, and Huang W W 2013 *Rec. Nat. Prod.*, 7(4) 254.

[15] Wang C, Lu Lu, Heya Na, Lifeng Cai, and Liu K 2014 *J.Med.Chem*, 57 7342.

[16] Spom, M. B., Lybi, K. T., Yore, M. M., Fu, L., Lopchuk, J. M., and Gribble, G. W J. 2015 *Nat. Prod.* 74(3) 537.