INTRODUCTION

In 2010, the rate of diabetes toll reached 6.6%, and it is predicted that by 2030 it will reach 7.4% of worldwide population.1,2 Regarding obesity, WHO has reported that more than 1 billion adult population worldwide suffer from overweight, and around 300 million of it is clinically obese.3,4 Protein tyrosine phosphatase 1B (PTP1B) is an enzyme present in some important insulin-targeted tissues such as liver, muscle, and fat. It plays a key role in the insulin signal transduction, in this case, as a negative regulator. Many studies have revealed that PTP1B inhibition is a validated target for curing type 2 diabetes (T2D) or reducing fat content on obese patients. Thus, inhibiting PTP1B will have a dual effect both as antidiabetes and as antiobesity. Nowadays, developing a small molecule as PTP1B inhibitor is getting of interest in a drug discovery for the T2D and obesity through a rational synthesis and natural resources.

MATERIALS AND METHODS

General experimental procedures

Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-LA400 spectrometer with tetramethylsilane as an internal standard. Meanwhile, medium pressured liquid chromatography...
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Plant material
M. leucadendron (Myrtaceae) was obtained from Genteng Market in Surabaya Indonesia in August 2006 and authenticated by one of the authors (Dr. Subehan). Its voucher specimen (TPMW 22276) is preserved in our previous institution the Museum of Materia Medica, Analytical Research Center for Ethnomedicines, Institute of Natural Medicine, University of Toyama, Toyama, Japan.

Extraction and isolation
One hundred and eight grams of powdered fruit of M. leucadendron was extracted with MeOH (3 × 400 mL, reflux, 2 h), and the MeOH solution was filtered and evaporated under reduced pressure to provide a MeOH extract (8.6 g). Following this, the MeOH extract was successively dissolved with ethyl acetate (EtOAc) and CHCl3 (3 × 250 mL each) to yield an EtOAc-soluble fraction (3.1 g), a CHCl3-soluble fraction (2.1 g), and a residue (2.2 g), respectively. The EtOAc-soluble fraction was rechromatographed by MPLC on a YMC silica gel column (4 cm × 30 cm, flow rate 20 mL/min) using a step gradient of CHCl3 – n-hexane (5:5, 5:4, 5:3, 5:2, 5:1, each 1.5 L, subfraction collection was 100 mL to afford 32 subfractions). Subfractions 12–15 were collected and concentrated under reduced pressure purposely to result in 640 mg of the fraction. The fraction was subsequently purified on Sephadex LH-20 (30 cm × 3 cm, collecting the volume of 50 mL) to result in 310 mg ursoic acid (2). While fraction 16–30 was rich in carbohydrates.

CHCl3-soluble fraction was separated on the same MPLC and eluted with CHCl3-MeOH (5:1, 5:2, 5:3, 5:4, 5:5, each 1.5 L) solvent systems to afford 25 subfractions. Subfractions 9–16 were collected to obtain 980 mg dry fraction. The fraction was purified on the Sephadex LH-20 column with MeOH as a solvent to afford betulinic acid (568 mg) and a mixture of ursoic acid and an ursoic acid derivative (258 mg). Subfractions 1–8 had so low quantity to separate, while subfractions 17–25 was rich in fatty acids.

Betulinic acid (1) - 1H NMR (CDCl3, 400 MHz) δ4.7 (1H, dd, J = 10.2; 5.6), 4.82 (s, H-17), 0.91 (s, H-23), 0.81 (s, H-25), 0.95 (s, H-26), and 0.93 (s, H-27); 13C NMR (CDCl3, 100 MHz) δ37.8 (C-1), 27.9 (C-2), 79.0 (C-3), 38.7 (C-4), 55.5 (C-5), 18.3 (C-6), 34.3 (C-7), 40.93 (C-8), 50.51 (C-9), 37.2 (C-10), 20.8 (C-11), 25.22 (C-12), 38.4 (C-13), 42.4 (C-14), 30.6 (C-15), 31.1 (C-16), 56.3 (C-17), 46.8 (C-18), 49.2 (C-19), 140.4 (C-20), 29.6 (C-21), 34.09 (C-22), 27.99 (C-23), 15.3 (C-24), 16.0 (C-25), 16.1 (C-26), 14.77 (C-27), 180.3 (C-28), 109.6 (C-29), and 19.4 (C-30).

Ursolic acid (1) - 1H NMR (CDCl3, 400 MHz) δ 3.43 (1H, br, H-3), 5.42 (1H, br, H-10), 2.52 (d, J = 10.2, H-18), 1.24 (s, H-19), 0.92 (s, H-20), 0.93 (s, H-25), 1.05 (s, H-26), 1.22 (s, H-27), 0.97 (s, H-29), and 0.99 (d, J = 6.1, H-30); 13C NMR (CDCl3, 100 MHz) δ 38.4 (C-1), 28.1 (C-2), 78.1 (C-3), 38.4 (C-4), 55.8 (C-5), 18.8 (C-6), 33.6 (C-7), 40.0 (C-8), 48.3 (C-9), 37.4 (C-10), 23.6 (C-11), 125.6 (C-12), 139.7 (C-13), 42.5 (C-14), 28.7 (C-15), 24.9 (C-16), 48.0 (C-17), 53.7 (C-18), 39.5 (C-19), 39.1 (C-20), 31.1 (C-21), 37.3 (C-22), 28.8 (C-23), 15.7 (C-24), 16.6 (C-25), 17.4 (C-26), 23.9 (C-27), 180.0 (C-28), 17.5 (C-29), and 21.4 (C-30).

Protein tyrosine phosphatase 1B inhibitory activity
All compounds were dissolved in dimethyl sulfoxide (DMSO). The final concentration of tested compound was 10 μg/mL, while DMSO in each well was 1% in which this concentration showed no influence on the activity of PTP1B. The assay was performed in 96-well clear polystyrene microplate (Corning, USA) according to a published procedure with a number of slight modifications.[11] Each well contained 0.05 μg PTP1B (Enzo Life Sciences, Inc., NY, USA), 2 mM p-nitrophenyl phosphate (pNPP; Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 50 mM citrate buffer containing 0.1 mM NaCl, 1 mM dithiothreitol (DTT), and 1 mM N, N', N'-ethylenediaminetetraacetate. The final volume of the mixture was 200 μL. The reaction was initiated by the addition of pNPP, incubated at 37°C for 30 min, and terminated with the addition of a stop solution (10 M NaOH). The amount of p-nitrophenol produced was estimated by measuring the absorbance at 405 nm using a Perkin-Elmer HTS 7000 bioassay reader. To identify the level of nonenzymatic substrate hydrolysis, the absorbance of wells only containing buffer and pNPP were measured for correction. The difference between the full enzymatic activity and the correction was arbitrarily set as 100% activity. The percent residual activity was calculated using the following formula: Residual Activity (%) = [(Absfull enzymatic) − Absinf sample − Abscorrection × (Absfull enzymatic) − Abscorrection)] × 100, where Absfull enzymatic was the absorbance of p-nitrophenol liberated by the enzyme in the system without a test sample in contrast to Absinf sample. The assays were performed in triplicate for all samples. A known phosphatase inhibitor, RK-682 (purity ≥98%; Enzo Life Sciences, Inc.) was used as the positive control then.

T-cell protein tyrosine phosphatase inhibitory activity
The assay was performed using a DTT concentration of 10 mM with an equal procedure as the PTP1B inhibition assay.

RESULTS AND DISCUSSION
During our preliminary study, the fruit extract of M. leucadendron exhibited the most active against PTP1B-more potent than positive control RK-682. It has also been mentioned in several Jamu medicine prescription records as a component for blood sugar control. Thus, it is interesting to reveal its chemical constituents, which could be related to its inhibitory activity. During the fractionation steps, most of the subfractions contained very polar materials, which were easily soluble in MeOH. In contrast, the chloroform fractions were rich of fatty acids. Under UV rays analyses of their TLC, it did not show the sufficient indication of the presence of certain compounds. Meanwhile, upon TLC spraying by cerium (IV) sulphate, the TLC plates exhibited some purple bands. However, due to oily matters present and deduced fatty acids based on NMR spectra, the material was collected based on TLC profiles. Compound 1 was isolated as white amorphous powder. Its 1H NMR displayed the signals of six tertiary protons (δ 0.81, 0.82, 0.91, 0.93, 0.95, 1.68) and two oxygenated secondary protons (δ 3.0, 4.66, and 4.7, respectively). The signals at δ 4.6 were exhibited a singlet signal, 3.0 was exhibited in a triplet (10.2 and 5.6, Hz), and 4.7 doublet of doublet (10.2 and 5.5), respectively. Its 13C NMR spectrum showed thirty carbons signals, mostly in the range of δ 14–56 ppm. Two vinylic carbons were present concurrently at δ 109.6 and 140.4 ppm. While the carbon at 79.01 was deduced as an oxygenated carbon. Finally, the carbon at 180 was arbitrarily as a carbonyl carbon. Upon the alert observation of their 1H and 13C NMR spectra, it was found that they had some good agreements with betulinic acid.[12] It was in a high concentration (7.6%). That could be a major compound of this material. Based on inhibitory examination, it exhibited 98.9% inhibition [Figure 1]. It had IC50 value of 1.5 μg/mL. That could be betulinic acid as the strong contributor toward the activity of methanol extract.

Compound 2 was present in both ethyl acetate and chloroform fractions. It has been found as a white amorphous powder. In certain fractions, it was present in a mixture with betulinic acid. Its 1H and 13C NMR spectra

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were similar to that of one except the position of two olefinic carbons $\delta$
125.6 and 139. 7 ppm. This spectrum showed a good agreement with that of ursolic acid.[11]

Both compounds exhibited some strong activities at 97% and 99% inhibition in a concentration of 10 $\mu$g/mL. [Figure 2]. Furthermore, IC$_{50}$ examination demonstrated a similar activity with positive control RK-682 with IC$_{50}$ values of 1.5 and 2.3 $\mu$g/mL, respectively [Table 1].

In this study, betulinic acid was isolated in 7.6% while ursolic acid was in 2.8%. Hence, betulinic acid and ursolic acid could be the corresponding constituents related to the strong activity of its extract inhibition. Interestingly, the recent study on diabetes-related scaffold, betulinic and ursolic acids have been becoming a skeleton lead. Their synthetic derivatives have demonstrated that the compound family has exhibited as potent agonist toward energy homeostasis TGR5 and insulin receptors and more potent compared to bile acids.[14] Intriguingly, a triterpene skeleton has been developed for current PTP1B inhibitor which results in trodusquemine, possessing a steroidal main building block. Therefore, further development triterpenoid has the interesting potential for PTP1B inhibitor modification. Hence, providing sufficient betulinic acid and ursolic acid for the development will be necessary. It is worthy noted that M. leucadendron fruit contains betulinic acid concentration at 7.6% enabling it to be the most readily potential source. Since trodusquemine has been developed from a natural product, which results in trodusquemine, possessing a steroidal main building block. Therefore, further development triterpenoid has the interesting potential for PTP1B inhibitor modification. Hence, providing sufficient betulinic acid and ursolic acid for the development will be necessary. It is worthy noted that M. leucadendron fruit contains betulinic acid concentration at 7.6% enabling it to be the most readily potential source.

Previously, Usia et al.[15] reported several Indonesian medicinal plants such as Zingiber aromaticum, Catharanthus roseus, and Melastoma malabratia containing the appreciable concentration of ursolic acid. That compound also present in South Asian plants such as Ziziphus jujuba,[16] Ziziphus spinosiloboides,[17] and Leucas aspera.[18] Thus, the notable ursolic concentration in M. leucadendron can be as a potential ursolic as well as betulinic acid source for pharmacutics or industrial purposes. On the basis of this work, the potent activity of compounds 1 and 2 as well as the MeOH extract should clearly demonstrate that M. leucadendron can be a potent medicinal plant for treating T2D and/or obesity.

**CONCLUSION**

That results demonstrated that the Indonesian medicinal plant M. leucadendron may be functionalized for the treatment and/or prevention of T2D and/or obesity. High quantities of betulinic and ursolic acid in this medicinal plant could be employed as chemical markers for its industrial processes.

### Table 1: Half maximal inhibitory concentration values of 1 and 2 against protein tyrosine phosphatase 1B and T-cell protein tyrosine phosphatase

| Compounds      | PTP1B (IC$_{50}$, μg/mL) | TCPTP (IC$_{50}$, μg/mL) |
|---------------|--------------------------|--------------------------|
| 1             | 1.5                      | 2.4                      |
| 2             | 2.3                      | 3.1                      |
| RK-682        | 2.05                     | -                        |

PTP1B: Protein tyrosine phosphatase 1B; TCPTP: T-cell protein tyrosine phosphatase; IC$_{50}$: Half maximal inhibitory concentration

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### Conflicts of interest

There are no conflicts of interest.

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