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## Antidiabetic, cytotoxic and antioxidant activities of *Rhodomyrtus tomentosa* leaf extracts

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As part of our project on exploring Indonesian medicinal plants for antidiabetic and anticancer agents, this study was conducted to investigate the total phenolic and flavonoid contents, and antioxidant, cytotoxic and antidiabetic properties of *R. tomentosa* leaf extracts. The antioxidant activity was tested using DPPH, ABTS, and FRAP methods. *In vitro* cytotoxic assay was performed against MCF-7, HeLa, A549, and B16 cancer cell lines. The *in vitro* antidiabetic testing was determined using  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibitory evaluation, while STZ-induced diabetic rats were used for *in vivo* study. The highest values of total phenolic ( $191.97 \pm 0.19$  mg GAE  $g^{-1}$ ) and flavonoid ( $29.11 \pm 0.05$  mg QE  $g^{-1}$ ) contents were recorded in methanolic extract. This extract also showed the highest DPPH and ABTS activities with  $IC_{50}$  values of  $7.79 \pm 0.03$  and  $4.03 \pm 0.02$   $\mu g$   $mL^{-1}$ , respectively, as well as the highest FRAP activity with a value of  $64.05 \pm 0.54$   $\mu M$   $Fe^{2+}$   $g^{-1}$ . The methanol extract had cytotoxicity against MCF-7, HeLa, A549, and B16 cancer cell lines with  $IC_{50}$  values of  $123.49 \pm 0.79$ ,  $28.28 \pm 0.17$ ,  $168.88 \pm 1.14$ , and  $42.44 \pm 0.18$   $\mu g$   $mL^{-1}$ , respectively. *In vitro* antidiabetic evaluation indicated that the MeOH extract inhibited  $\alpha$ -glucosidase and  $\alpha$ -amylase with  $IC_{50}$  values of  $45.73 \pm 1.06$  and  $41.31 \pm 1.12$   $\mu g$   $mL^{-1}$ , respectively. A dose of  $400$   $mg$   $kg^{-1}$  body weight of the MeOH extract reduced rats' blood glucose rate and serum blood glucose by 48.51% and 17.73%, respectively after 15 days of treatment. Taken together, these findings suggested that the methanolic extract of *R. tomentosa* leaves can be used as a potential source of antioxidant, cytotoxic, and antidiabetic agents.

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### Introduction

Diabetes type 2 is called non-insulin-dependent or adult-onset diabetes, and is caused by the body's ineffective use of insulin. Ineffective use of insulin by the body causes blood sugar levels to exceed normal limits. Diabetes is a dangerous disease because it is the main cause of heart disease, kidney failure and stroke.<sup>1</sup> Traditional medicines using medicinal plants as a therapeutic agent are still frequently used today to treat diabetes through several mechanisms. The common way is to inhibit the enzymes such as  $\alpha$ -glucosidase<sup>2</sup> and  $\alpha$ -amylase<sup>3</sup> that are responsible in hydrolytic breakdown of carbohydrates, and therefore the absorption of glucose into the blood is slowed down. Meanwhile, cancer is one of the main causes of death in the world with about 9.6 million deaths were recorded in 2018.<sup>4</sup> Cancer is caused by abnormal growth of cells due to various

gene mutations and unstoppable cell division. An effort in searching of effective cancer drugs becomes main research focus, as the number of new cancer cases increases per year.<sup>5</sup>

*R. tomentosa* has been known as a potential medicinal plant. *R. tomentosa* is widely distributed in Asia such as Vietnam, Japan, China, Philippines, Malaysia and Thailand. The leaves, roots, buds and fruit of this plant have been used as traditional medicine. Vietnamese, Chinese and Malaysians use this plant to cure stomachaches, dysentery, abscesses, and sepsis. In Thailand, this plant is used as an antipyretic, anti-diarrheal, and antidysentery. In Indonesia, *R. tomentosa* is used for wounds healing,<sup>6</sup> while Kalimantan people use the leaves as a blood sugar-lowering drug.

*R. tomentosa* contains various phytochemical compositions in many parts of the plant including terpenoids, phenols and lipids. Triterpenoids have been identified from the leaves of *R. tomentosa*, including lupeol,  $\beta$ -amyryn,  $\beta$ -amyrenonol, and botulin. The phenolic compounds (major component) were identified from *R. tomentosa*, such as rhodomyrtosone A, rhodomyrtosone B, rhodomyrtosone C, and rhodomyrtosone D.<sup>7</sup> In addition, *R. tomentosa* is used as an antioxidant, antibacterial, anticancer, and anti-inflammation. The  $IC_{50}$  value of DPPH radical scavenging activity for anthocyanins-rich extract of *R. tomentosa* fruits and ascorbic acid were  $6.27 \pm 0.25$   $\mu g$   $mL^{-1}$  and

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$17.4 \pm 0.31 \mu\text{g mL}^{-1}$ , respectively. The  $\text{IC}_{50}$  value of ABTS radical scavenging activity for the same extract and ascorbic acid were  $90.3 \pm 1.52 \mu\text{g mL}^{-1}$  and  $206 \pm 2.37 \mu\text{g mL}^{-1}$ , respectively.<sup>8</sup> However, only a few comprehensive studies were reported on the antioxidant and antidiabetic evaluation of the leaves extract of *R. tomentosa*. Therefore, this study was conducted to evaluate Total Phenolic Content (TPC) and Total Flavonoid Content (TFC), antioxidant activities (DPPH, ABTS, and FRAP), cytotoxicity against MCF-7, HeLa, A549, and B16 cancer cell lines and antidiabetic activities *in vitro* and *in vivo* of leaves extracts of *R. tomentosa*.

## Materials and methods

### Animals

Male albino Wistar rats (160–200 g) used were given a standard pellet diet and placed in cages at a relative humidity of  $55 \pm 10\%$  and temperature of  $25 \pm 1^\circ\text{C}$ . All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of “Stem Cell Laboratory, Airlangga University, Surabaya, Indonesia” and approved by the Animal Ethics Committee of “Health Research Ethics Committee of School of Medicine, Airlangga University (161/EC/KEPK/FKUA/2021)”. Humans have not been used as subjects in this study.

### Plant materials

The leaves of *R. tomentosa* were collected in June 2019 at Banjarbaru, South Kalimantan, Indonesia, and identified by Mr Edi Suroto, a botanist at Purwodadi Botanical Garden, Pasuruan, Indonesia. The voucher specimen was deposited at Laboratory of Natural Products and Synthetic Chemistry, Institut Teknologi Sepuluh Nopember, Surabaya, Indonesia (012/VI/HT-KIBAS/2019).

### Extraction

The extraction process was carried out separately for each sample using four different solvents, including *n*-hexane, dichloromethane ( $\text{CH}_2\text{Cl}_2$ ), ethyl acetate (EtOAc), and methanol (MeOH). The dried leaves of *R. tomentosa* (each 20.0 g) were extracted with 250 mL of *n*-hexane,  $\text{CH}_2\text{Cl}_2$ , and EtOAc for 24 h in room temperature, while the dried leaves of *R. tomentosa* (5.0 kg) were extracted with 30 L of MeOH using the same treatment. Each extract was filtered using filter paper and concentrated with a rotary evaporator to give crude *n*-hexane (0.37 g),  $\text{CH}_2\text{Cl}_2$  (0.90 g), EtOAc (0.70 g), and MeOH (723.0 g) extracts.

### Total phenolic content (TPC)

The total phenolic content of the MeOH, EtOAc,  $\text{CH}_2\text{Cl}_2$ , and *n*-hexane extracts of *R. tomentosa* was determined using Follin–Ciocalteu's method.<sup>9</sup> Each extract with various concentrations in methanol was taken 0.5 mL and then mixed with 2.0 mL of 7.5% sodium carbonate solution and 10% Follin–Ciocalteu's reagent from Sigma-Aldrich (St. Louis, MO, USA). Absorbance was measured using a UV-vis spectrophotometer at 765 nm after incubation at  $40^\circ\text{C}$  for 1 h. Next, gallic acid (Sigma-Aldrich) with concentrations of 0–200  $\text{mg L}^{-1}$  was used as the standard curve.

The TPC of the extracts were calculated as mg GAE (gallic acid equivalent)  $\text{g}^{-1}$  of dry extract.

### Total Flavonoid Content (TFC)

The TFC of the MeOH, EtOAc,  $\text{CH}_2\text{Cl}_2$ , and *n*-hexane extracts of *R. tomentosa* leaves was measured by spectrophotometer, as previously reported method<sup>10</sup> with minor modification. Each extract with concentrations of 100–1000 ppm was taken 0.5 mL and then mixed with 0.5 mL of a solution of 2%  $\text{AlCl}_3$  (Sigma-Aldrich) in methanol. The mixture was incubated for 1 hour and the absorbance was measured at 415 nm using a UV-vis spectrophotometer. Quercetin (Sigma-Aldrich) with concentrations of 0–50  $\text{mg L}^{-1}$  was used as the standard curve. The TFCs of the extracts were calculated as mg QE (quercetin equivalent) per g of dry extract.

### DPPH radical scavenging assay

DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals scavenging activity was determined as previously described.<sup>4</sup> 33.3  $\mu\text{L}$  of each extract at different concentrations ( $159.73\text{--}4.99 \mu\text{g mL}^{-1}$ ) was added to 1 mL of DPPH  $6.0 \times 10^{-5} \text{ M}$  and the mixture were incubated at  $37^\circ\text{C}$  for 20 minutes in dark room. The absorbance was measured at 517 nm using UV-visible Spectrophotometer (Thermo Fisher Scientific, USA) with three replications. Blank sample was a mixture of 33.3  $\mu\text{L}$  of DPPH and methanol solution, while gallic acid was used as the positive control. The percentage of DPPH capacity was estimated by applying the following equation.

$$\text{Inhibition (\%)} = (\text{blank abs} - \text{sample abs}) / (\text{blank abs}) \times 100\% \quad (1)$$

### ABTS radical scavenging assay

ABTS, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay was performed using previous method.<sup>11</sup> Briefly, 88  $\mu\text{L}$  of 140 mM potassium peroxydisulfate ( $\text{K}_2\text{S}_2\text{O}_8$ ) and 5.0 mL of 7 mM ABTS stock solution were mixed and stored for 12–16 h before use in the dark at room temperature. The mixture was added with an ethanol solution (99.5%) until a working solution was obtained with an absorbance of 0.7 ( $\pm 0.02$ ) at 734 nm. Each extract with various concentrations ( $49.51\text{--}1.55 \mu\text{g mL}^{-1}$ ) was added 1 mL onto the working solution and then stirred manually for 10 s and incubated for 4 min at  $30^\circ\text{C}$ . Each solution was measured the absorbance at 734 nm with a UV-vis spectrophotometer. Gallic acid and ethanol were used as a positive control and blank sample, respectively. The ABTS Radical Scavenging Assay was calculated with the eqn (1).

### Ferric-reducing antioxidant power (FRAP) assay

The FRAP assay was determined using the reported method<sup>12</sup> with slight modification. The reduction of colorless of  $\text{Fe}^{3+}$ -tripridyltriazine to strongly blue-colored of  $\text{Fe}^{2+}$ -tripridyltriazine is often determined by measuring the absorbance change at 593 nm. A total of 300 mM acetate buffer pH 3.6, 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) (Sigma-Aldrich) solution in



40 mM HCl, and 20 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  were mixed in the ratio of 10 : 1 : 1 to get working FRAP reagent.  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  was used for FRAP standard curve. Sample solution (100  $\mu\text{L}$ ), FRAP reagent (2 mL), and distilled water (900  $\mu\text{L}$ ) were mixed and incubated at 37 °C in a dark room for 30 min. The mixture absorbance was measured at 593 nm. A mixture of  $\text{H}_2\text{O}$  (1 mL) and FRAP reagent (2 mL) was used as the blank sample. Finally, the FRAP values were stated as  $\text{mM Fe}^{2+} \text{ g}^{-1}$  of sample (FRAP value of sample ( $\mu\text{M}$ ) =  $\text{abs (sample)} \times \text{FRAP value of std (}\mu\text{M)/abs (std)}$ ).

### *In vitro* $\alpha$ -glucosidase inhibitory activity

The anti- $\alpha$ -glucosidase assay was carried out using rat intestinal enzyme.<sup>13</sup> The rat enzyme solution was prepared from rat intestinal acetone powder (Sigma, St. Louis). Rat intestinal acetone powder (1 g) was homogenized in 30 mL of normal saline. After centrifugation (12 000g  $\times$  30 min), the aliquot was subjected to assay. Each extract of *R. tomentosa* leaves (5 mg  $\text{mL}^{-1}$  in DMSO, 10  $\mu\text{L}$ ) was added with 50  $\mu\text{L}$  of the 0.1 M phosphate buffer (pH 6.9), 20  $\mu\text{L}$  of the maltose substrate solution (10 mM) in 0.1 M phosphate buffer, 80  $\mu\text{L}$  of glucose assay kit (SU-GLLQ2, Human), and 20  $\mu\text{L}$  of the rat enzyme solution. The reaction mixture was then incubated at 37 °C for 10 min. A microplate reader (BioTek ELx800TM, BioTek Instruments, Inc, Winooski, VT, USA) was used to measure the absorbance of enzymatic activity at 520 nm. Inhibition (%) was calculated based on eqn (2).

$$\text{Inhibition (\%)} = (\text{blank abs} - \text{sample abs}) / (\text{blank abs}) \times 100\% \quad (2)$$

where;  $\text{Abs}_{\text{blank}} = \text{Abs}_{\text{enzyme reaction}} - \text{Abs}_{\text{blank of enzyme reaction}}$

where;  $\text{Abs}_{\text{sample}} = \text{Abs}_{\text{sample reaction}} - \text{Abs}_{\text{blank of sample reaction}}$

### *In vitro* $\alpha$ -amylase inhibitory activity

The anti- $\alpha$ -amylase assay was adapted from ref. 14 with a slight modification. Each sample (10 mg) was dissolved in 1 mL of DMSO and the  $\alpha$ -amylase enzyme was prepared by dissolving 5 mg of porcine pancreas  $\alpha$ -amylase in 1 mL of 0.1 M phosphate buffer (pH 6.9). 100 mg of starch potato (substrate) was warmed in 5 mL of 0.1 M phosphate buffer (pH 6.9) for 5 minutes and cooled at room temperature. 10  $\mu\text{L}$  of sample and 250  $\mu\text{L}$  of substrate were mixed into 150  $\mu\text{L}$  of 0.1 M phosphate buffer (pH 6.9). After preincubation for 5 minutes, 100  $\mu\text{L}$  of  $\alpha$ -amylase enzyme was added into the mixture and further incubated at 37 °C for 15 minutes. The reaction was stopped by adding 250  $\mu\text{L}$  of 1M HCl and 250  $\mu\text{L}$  of 10% iodine solution. Acarbose was used as a positive control and the absorbance was measured at 650 nm by microplate reader (BioTek ELx800TM, BioTek Instruments, Inc, Winooski, VT, USA), and inhibition percentage was calculated using eqn (2).

### *In vivo* antidiabetic activity assay

The *in vivo* antidiabetic assay was adapted from (ref. 15) with slight modifications. The rats used were male albino Wistar rats. The rats were fasted for 8–12 hours before streptozocin (STZ)

injection. The rats were induced by STZ (60 mg  $\text{kg}^{-1}$ ) was induced *via* single intraperitoneal injection. The blood glucose rate was observed on the third day after STZ injection. Rats with blood sugar rates more than 200 mg  $\text{dL}^{-1}$  were used for further testing. Diabetic rats were classified to 5 groups as follows: G1 (0.5% of NaCMC as negative control), G2 (10 mg  $\text{kg}^{-1}$  of glibenclamide as positive control), G3 (200 mg  $\text{kg}^{-1}$  of extract), G4 (400 mg  $\text{kg}^{-1}$  of extract), and G5 (600 mg  $\text{kg}^{-1}$  of extract). Additionally, one group is used for normal control (without treatment), named as G6. One group consists of four rats. The extract, glibenclamide, and NaCMC were administered orally for 15 days. Blood glucose was measured at 0, 3, 6, 9, 12, and 15 days by ACCU Check Instant and the serum glucose was measured at 0 and 15 days by spectrophotometer UV-vis. Serum glucose analysis was performed using the glucose test method. First, 1 mL of rat blood was transferred to centrifuge tube and centrifuged at 4000 rpm for 15 minutes to separate the blood plasma and serum. The serum (10  $\mu\text{L}$ ) was taken and mixed with 1000  $\mu\text{L}$  of kit reagent, then put into a test tube and vortexed to make it homogeneous. The mixture was incubated at 37 °C for 10 minutes or at 30 °C for 20 minutes, while the absorbance calculation was conducted at 500 nm. The absorbance measurement of the blank and standard (glucose) was conducted in the same way as the sample, and the serum glucose (mg  $\text{dL}^{-1}$ ) was calculated using eqn (3).

$$\text{Glucose (mg dL}^{-1}\text{)} = (\text{Abs}_{\text{sample}}) / (\text{Abs}_{\text{standard}}) \times \text{glucose standard (mg dL}^{-1}\text{)} \quad (3)$$

### *In vitro* cytotoxic activity assay

The *in vitro* cytotoxic assay against the human cervix adenocarcinoma (HeLa), human breast adenocarcinoma (MCF-7), human lung carcinoma (A549), and murine melanoma (B16) cancer cell lines was performed using the PrestoBlue (PB) method.<sup>16</sup> The cells were preserved with a complete RPMI medium containing 50  $\mu\text{L}/50 \text{ mL}$  antibiotics and 10% (v/v) FBS. Cell cultures with a density of 170 000 cells per mL of medium were then incubated in 96 well plates at 37 °C for 24 hours in 5%  $\text{CO}_2$ . Next, samples with concentrations from 2.34 to 300  $\mu\text{g mL}^{-1}$  were added to fresh medium and incubated for 48 hours. PrestoBlue reagent (Thermo Fisher Scientific, Uppsala, Sweden) was added to the medium. Then, the absorbance of the mixture's was measured using a multimode reader at 570 nm. Cisplatin is used as positive control.

### Phytochemical profiling using HPLC analysis

The analysis was performed on a Ultimate 3000 instrument (Thermo Fisher Scientific) with a UV-vis detector. The system was equipped with an Hypersil GOLD  $\text{C}_{18}$  column (250  $\times$  4.6 mm) with the flow rate of 1.0  $\text{mL min}^{-1}$  at 30 °C. Approximately 1.0 mg of each extract was dissolved with methanol (1.0 mL) and filtered using PTFE filter before sample injection. The mobile phase used was methanol (A) and water (B) with a gradient elution of 60–75% A at 0–20 min, 75–85% A at 20–35 min, 85–100% A at 35–50 min, and 100% at 50–60 min. The injection



volume was set to 20  $\mu\text{L}$  and the UV detector was set to 254, 280, 310, and 325 nm for the analysis of phenolic and non-phenolic contents.

### Statistical analysis

The data were summarized using the formula of mean  $\pm$  standard deviation. Linear regression equation was used to determine concentrations of antioxidant and cytotoxicity. Nonlinear regression equation used to determine concentrations of anti- $\alpha$ -glucosidase and anti- $\alpha$ -amylase activities. Statistical differences between groups in anti-oxidant were analyzed by Student's *t*-test in antioxidant evaluation and *in vitro*  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibition assay. A *p*-value of  $<0.01$  was considered to be statistically significant difference. Meanwhile, the results of *in vivo* antidiabetic assay were analyzed by ANOVA followed by Least Significance Different (LSD) where a *p*-value of  $<0.05$  was considered as statistically significant difference.

## Results and discussion

### Total phenolic and flavonoid contents

Table 1 shows the TPC and TFC values of *n*-Hexane,  $\text{CH}_2\text{Cl}_2$ , EtOAc, and MeOH extracts from the *R. tomentosa* leaves.

The TPC values of the *R. tomentosa* extracts were determined from a linear gallic acid standard curve. Table 1 shows that the MeOH extract had the highest TPC value, followed by the  $\text{CH}_2\text{Cl}_2$  and EtOAc extracts. This shows that the phenolic compounds in the leaves of *R. tomentosa* are mostly in polar and semi-polar. This fact was supported by T. S. Vo and D. H. Ngo in 2019 (ref. 7) who reported the presence of phenolic derivatives as major metabolites isolated from the alcoholic extract of *R. tomentosa* leaves. Meanwhile, the TFCs of the *R. tomentosa* extracts were determined from a linear quercetin standard curve. The result indicated that the most flavonoid compounds are exist in polar part of *R. tomentosa*. The MeOH extract had the highest TFC value followed by the  $\text{CH}_2\text{Cl}_2$  and EtOAc extracts. Flavonoid compounds such as kaempferol, quercetin-7,4'-diglucoside, dihydromyricetin, vitexin, myricetin, and quercetin were isolated from MeOH extract from aerial parts of *R. tomentosa*.<sup>6</sup>

### Antioxidant activity

Table 2 illustrates the DPPH radical scavenging assay conducted on *n*-hexane,  $\text{CH}_2\text{Cl}_2$ , EtOAc, and methanol extracts of *R. tomentosa* leaves, and gallic acid (positive control) with  $\text{IC}_{50}$  values of  $25.09 \pm 0.14$ ,  $20.81 \pm 0.21$ ,  $13.95 \pm 0.08$ ,  $7.79 \pm 0.03$ ,

Table 1 The TPC and TFC values of *R. tomentosa* leaves<sup>a</sup>

Extract	TPC (mg GAE $\text{g}^{-1}$ extract)	TFC (mg QE $\text{g}^{-1}$ extract)
<i>n</i> -Hexane	$25.62 \pm 0.11$	$10.55 \pm 0.04$
$\text{CH}_2\text{Cl}_2$	$119.11 \pm 0.19$	$25.73 \pm 0.05$
EtOAc	$113.95 \pm 0.30$	$22.32 \pm 0.04$
MeOH	$191.97 \pm 0.19$	$29.11 \pm 0.05$

<sup>a</sup> Data are expressed as mean  $\pm$  SD of triplicate experiments.

Table 2 The results of antioxidant testing of *R. tomentosa* leaves<sup>a</sup>

Extracts	DPPH	ABTS	FRAP ( $\mu\text{M Fe}^{2+} \text{g}^{-1}$ )
	$\text{IC}_{50}$ ( $\mu\text{g mL}^{-1}$ )	$\text{IC}_{50}$ ( $\mu\text{g mL}^{-1}$ )	
<i>n</i> -Hexane	$25.09 \pm 0.14^*$	$18.21 \pm 0.04^*$	$12.15 \pm 0.14^*$
$\text{CH}_2\text{Cl}_2$	$20.81 \pm 0.21^*$	$5.05 \pm 0.02^*$	$32.91 \pm 0.27^*$
EtOAc	$13.95 \pm 0.08^*$	$12.80 \pm 0.01^*$	$44.09 \pm 0.09^*$
Methanol	$7.79 \pm 0.03^*$	$4.03 \pm 0.02^*$	$64.05 \pm 0.54^*$
Gallic acid	$5.29 \pm 0.02$	$0.12 \pm 0.01$	Nt
Ascorbic acid	Nt	Nt	$30.62 \pm 0.27$

<sup>a</sup> Data are expressed as mean  $\pm$  SD of triplicate experiments, \**p*  $<0.01$  vs. positive control, Nt: not tested.

and  $5.29 \pm 0.02 \mu\text{g mL}^{-1}$ , respectively. The result indicated that the methanol extract showed the highest antioxidant activity compared to the less polar extracts and comparable to the positive control. The activity might be due to the presence of phenolic constituents which abundant in polar part. This is in accordance with the result of total phenolic content analysis of the methanol extract which was higher than the less polar extracts (Table 1).

A typical approach for testing antioxidant activity is DPPH method. The free radical in DPPH takes an electron or a hydroxy radical from another source and becomes stable diamagnetic molecule.<sup>17</sup> The color change from purple to yellow indicates that the radicals are taken up by antioxidants through a hydrogen contribution to stabilize molecules of DPPH.<sup>18</sup>

Table 2 shows that the highest ABTS assay result was methanol extract with an  $\text{IC}_{50}$  value of  $4.03 \pm 0.02 \mu\text{g mL}^{-1}$  followed by the  $\text{CH}_2\text{Cl}_2$  extract with an  $\text{IC}_{50}$  value of  $5.05 \pm 0.02 \mu\text{g mL}^{-1}$ . The  $\text{CH}_2\text{Cl}_2$  and MeOH extracts showed weaker activity in the DPPH test than the ABTS assay, possibly due to the reaction differences of phenolic compounds and free radicals in the organic phase of the DPPH assay and the aqueous phase of the ABTS assay.<sup>19</sup>

FRAP assay (Table 2) shows that the methanol extract has the value approximately 2-fold higher than ascorbic acid as the positive control with a value of  $64.05 \pm 0.54 (\mu\text{M Fe}^{2+} \text{g}^{-1})$  followed by the EtOAc and  $\text{CH}_2\text{Cl}_2$  extracts with values of  $44.09 \pm 0.09$  and  $32.91 \pm 0.27 (\mu\text{M Fe}^{2+} \text{g}^{-1})$ , respectively. The FRAP evaluation indicates a sample's capacity to participate in a one-electron redox reaction.<sup>20</sup> FRAP is directly proportional to the TPC and TFC values, possibly due to the presence of phenolic and flavonoids contents, which act as electron donors to neutralize free radicals.

The results of the DPPH, ABTS, and FRAP assay showed significant differences between the extracts (*n*-hexane,  $\text{CH}_2\text{Cl}_2$ , EtOAc, and MeOH) and the positive control based on the *t*-Test (*p*  $<0.01$ ).

Table 3 describes the correlation of the TPC, TFC, and antioxidant activities of *R. tomentosa* leaves extracts. Pearson correlation was used to calculate the relationship. A strong correlation was observed between TPC and TFC with  $r = 0.961$  (*p*  $<0.05$ ), which means that the higher the TPC, the higher the TFC. A positive correlation was observed between DPPH vs. TPC



**Table 3** Pearson correlation coefficient of total phenolic, total flavonoids, and antioxidants activities<sup>a</sup>

	TPC <sup>b</sup>	TFC <sup>c</sup>	DPPH <sup>d</sup>	ABTS <sup>d</sup>	FRAP <sup>d</sup>
TPC	1				
TFC	0.961*	1			
DPPH	0.091*	0.215*	1		
ABTS	0.114*	0.054*	0.612*	1	
FRAP	0.969*	0.884*	0.017*	0.255*	1

<sup>a</sup> \*Correlation is significant at 0.05 level. <sup>b</sup> Total phenolics content. <sup>c</sup> Total flavonoids content. <sup>d</sup> Coefficient of antioxidant.

and DPPH vs. TFC with  $r = 0.091$  ( $p < 0.05$ ) and  $r = 0.215$  ( $p < 0.05$ ), respectively. There was a positive correlation between ABTS and TPC with  $r = 0.114$  ( $p < 0.05$ ). ABTS vs. TFC showed a positive correlation with  $r = 0.054$  ( $p < 0.05$ ). ABTS vs. DPPH showed a fairly strong correlation with  $r = 0.612$  ( $p < 0.05$ ). A strong correlation was observed between FRAP and TPC with  $r = 0.969$  ( $p < 0.05$ ) as well as FRAP and TFC with  $r = 0.884$  ( $p < 0.05$ ). FRAP vs. DPPH showed a positive correlation with  $r = 0.017$  ( $p < 0.05$ ). A positive correlation was observed between FRAP and ABTS with  $r = 0.225$  ( $p < 0.05$ ). Generally, TPC and TFC play an important role in the antioxidant activity acting as an electron donor, chain breaker, and free radical scavenger.<sup>21</sup> This data concluded that there is a positive correlation between TPC and TFC with antioxidant activities.

### In vitro antidiabetic assay

The antidiabetic activity of the extract was tested using the  $\alpha$ -glucosidase and  $\alpha$ -amylase enzyme inhibition method. The results of *in vitro* antidiabetic assay are shown in Table 4.

Table 4 shows the results of screening for the anti- $\alpha$ -glucosidase activity of *n*-hexane, CH<sub>2</sub>Cl<sub>2</sub>, EtOAc and methanol extracts as well as acarbose as the positive control. The methanolic extract displayed the highest activity with inhibition of 81.91%, which was comparable to acarbose at the same concentration of 5 mg mL<sup>-1</sup>, while the other extracts were less active with inhibition lower than 50%. A concentration-dependent experiment was conducted on the methanol extract and gave the IC<sub>50</sub> values of 45.73 ± 1.06 µg mL<sup>-1</sup>, which was 6-fold weaker than that of acarbose as the positive control (IC<sub>50</sub> = 7.67 ± 1.14 µg mL<sup>-1</sup>). The *in vitro*  $\alpha$ -glucosidase inhibition data

has not been reported previously and it can provide insight for further studies on *R. tomentosa* leaves extract as an antidiabetic therapy. The screening result of anti- $\alpha$ -glucosidase assay showed that the methanol extract had the highest inhibitory potential and thus was used for *in vitro* anti- $\alpha$ -amylase evaluation and *in vivo* antidiabetic study.

Table 4 shows that  $\alpha$ -amylase inhibition of the methanol extract was comparable to acarbose with the inhibition percentage of 82.41 ± 2.21% and 94.01 ± 1.56%, respectively at 5 mg mL<sup>-1</sup>. Further analysis indicated that the MeOH extract inhibited  $\alpha$ -amylase enzyme with IC<sub>50</sub> value of 41.31 ± 1.12 µg mL<sup>-1</sup>, which was 5-fold higher than acarbose.

The results of the anti- $\alpha$ -glucosidase and anti- $\alpha$ -amylase activities showed significant differences between the extracts and the positive control based on the *t*-Test ( $p < 0.01$ ).

The inhibition of  $\alpha$ -glucosidase is one of therapies for treating type 2 diabetes mellitus. This enzyme is commonly found at the border of the small intestine.<sup>22</sup> Sugar production is inhibited by an  $\alpha$ -glucosidase inhibitor, which function to delay the absorption of complex carbohydrates, and thus gradually reduces glucose absorption and level in blood.<sup>23,24</sup>

Treatment of diabetes can be performed using alternative medicines from medicinal plants with less side effects. Pancreatic and salivary  $\alpha$ -amylases are responsible for breaking down large and insoluble starch molecules into smaller and more easily absorbed units.<sup>25</sup> Meanwhile, an effective way to manage diabetes mellitus is to reduce postprandial hyperglycemia by slowing glucose absorption. This is obtained when  $\alpha$ -glucosidase and  $\alpha$ -amylase activities in hydrolyzing carbohydrates in the digestive organs are inhibited.<sup>26</sup>

### In vivo antidiabetic assay

**Effect of *R. tomentosa* extract on rat blood glucose.** STZ is a toxic glucose analog that gathers in pancreatic beta cells. STZ is absorbed by beta cells through the glucose transporter GLUT2, and then poisoning occurs in beta cells through alkylation. Later, beta cells die through necrosis, resulting in insulin-dependent diabetes mellitus.<sup>27</sup> Therefore, the increase in blood glucose levels after STZ induction may be due to a state of resistance in diabetic rats or insulin deficiency.<sup>28</sup> STZ is a diabetogenic agent with a wider range of effectiveness than alloxan. STZ cause permanent damage to diabetes while large doses of alloxan killed the rat and did not result in permanent diabetes.<sup>29</sup>

**Table 4** The *in vitro* antidiabetic activity of *R. tomentosa* leaves extracts<sup>a</sup>

Extracts	$\alpha$ -Glucosidase		$\alpha$ -Amylase	
	% Inhibition ± SD (5 mg mL <sup>-1</sup> )	IC <sub>50</sub> (µg mL <sup>-1</sup> )	% Inhibition ± SD (5 mg mL <sup>-1</sup> )	IC <sub>50</sub> (µg mL <sup>-1</sup> )
<i>n</i> -Hexane	13.63 ± 1.80*	Nt	Nt	Nt
CH <sub>2</sub> Cl <sub>2</sub>	15.32 ± 1.21*	Nt	Nt	Nt
EtOAc	31.79 ± 0.78*	Nt	Nt	Nt
MeOH	81.91 ± 1.09*	45.73 ± 1.06*	82.41 ± 2.21*	41.31 ± 1.12*
Acarbose	87.14 ± 2.36	7.67 ± 1.14	94.01 ± 1.56	8.70 ± 0.79

<sup>a</sup> Data are expressed as mean ± SD of triplicate experiments, \* $p < 0.01$  vs. positive control, Nt: not tested.



Table 5 Effect of *R. tomentosa* extract on blood glucose rate in diabetic rats<sup>a</sup>

Treatment groups	Blood glucose level (mg dL <sup>-1</sup> ) on day						Blood glucose loss (%)
	0	3	6	9	12	15	
G1 (negative control)*	559.50 ± 28.20	428.50 ± 55.67	416.75 ± 54.41	458.25 ± 41.04	443.25 ± 54.39	556.50 ± 26.50	0.53 ± 0.41
G2 (positive control)*	475.75 ± 60.72	466.00 ± 116.20	402.25 ± 62.14	377.50 ± 63.40	409.25 ± 53.27	340.00 ± 35.59	28.53 ± 5.02
G3 (dose I (200 mg kg <sup>-1</sup> ))*	477.50 ± 141.51	425.50 ± 71.11	404.75 ± 56.79	367.00 ± 40.61	370.75 ± 99.21	327.25 ± 90.56	31.46 ± 5.05
G4 (dose II (400 mg kg <sup>-1</sup> ))*	502.00 ± 113.27	341.75 ± 25.28	360.00 ± 14.99	288.75 ± 21.82	302.25 ± 20.98	258.50 ± 22.81	48.51 ± 7.23
G5 (dose III (600 mg kg <sup>-1</sup> ))*	564.00 ± 45.28	395.80 ± 71.32	357.50 ± 31.98	350.00 ± 39.20	344.30 ± 37.60	474.50 ± 48.06	15.96 ± 2.72
G6 (normal control)	109.5 ± 5.32	97.00 ± 5.10	97.50 ± 14.06	100.50 ± 6.86	109.00 ± 6.88	109.25 ± 5.62	0.00 ± 0.00

<sup>a</sup> Values are means ± SD ( $n = 4$ ), \* $p < 0.05$  vs. normal control.

The effect of methanol extract of *R. tomentosa* leaves on rat blood glucose levels for 15 days treatments is shown in Table 5. The results showed that a dose of 200 mg kg<sup>-1</sup> of the extract reduced the blood glucose level by 31.64%, while doses of 400 and 600 mg kg<sup>-1</sup> reduced by 48.51% and 15.96%, respectively.

Table 5 describes the blood glucose decline in rats given methanol extract from *R. tomentosa* leaves. Blood glucose levels in normal rat was relatively stable during the 15 days experiment, while the negative control experienced a slight decrease in blood glucose levels (0.53%), as NaCMC does not function to lowering blood glucose levels. Meanwhile, positive control can reduce rat blood glucose by 28.53%. This data implied that glibenclamide at a 10 mg kg<sup>-1</sup> dose can lower rats blood glucose. The percentage of lowering blood glucose at a 400 mg kg<sup>-1</sup> dose was as much as 48.51% and showed the highest as compared to the other two doses. It indicated that the extract with an optimal dose at 400 mg kg<sup>-1</sup> was able to repair and protect  $\beta$ -cells. Meanwhile, the higher dose of 600 mg kg<sup>-1</sup> can lower blood glucose rates by only 15.96%, which indicated a downward trend in protecting and repairing  $\beta$ -cells, and the insulin production is thus inhibited. The blood glucose levels in diabetic rats decreased significantly ( $p < 0.05$ ) with the administration of *R. tomentosa* leaves methanol extract. These results were significantly different from the normal group based on the results of ANOVA and LSD analysis. Plant have anti-hyperglycemic activity due to their ability to restore pancreatic tissue function resulting an increase of insulin production,

inhibits intestinal glucose absorption, and facilitates metabolism in insulin-dependent processes.<sup>28</sup> Therefore, herbal medicines to treat diabetes can protect  $\beta$ -cells and reduce fluctuation in blood glucose rates. Biological knowledge about the mechanism of action in diabetes treatment is still scarce. However, many plants have been known to contain substances such as flavonoids, terpenoids, and glycosides, which are often associated with antidiabetic effects.<sup>30</sup>

**Effect of *R. tomentosa* extract on rat body weight.** The effect of methanol extract from *R. tomentosa* leaves on rat body weight is described in Table 6. The results showed that the body weight of rats given the extract at a dose of 400 and 600 mg kg<sup>-1</sup> were increased up to 10.32% and 10.23%, respectively, while a dose of 200 mg kg<sup>-1</sup> caused the body weight loss by 23.79%.

The weight measurement results showed that the normal control had only a slight change in body weight. Meanwhile, the percentage of weight loss from negative control, positive control, and dose of 200 mg kg<sup>-1</sup> were 19.05, 7.67, and 23.79%, respectively. The percentage of weight loss from negative control and a 200 mg kg<sup>-1</sup> dose was relatively high. The negative control was only given NaCMC, which did not affect on reducing blood glucose levels and it affected weight loss. Increased blood glucose levels in people with diabetes are generally accompanied by weight loss, polyuria, polydipsia, and polyphagia. Weight loss may be due to impaired fat and protein catabolism.<sup>28</sup> Meanwhile, the percentage of weight loss from the positive control was smaller than the negative control and the

Table 6 Effect of *R. tomentosa* extract on body weights in diabetic rats<sup>a</sup>

Treatment groups	The rat body weight (g) on day							Body weight loss (%)
	Beginning	0	3	6	9	12	15	
Normal control	158.00 ± 6.06	158.50 ± 6.35	159.25 ± 6.18	159.75 ± 5.44	158.50 ± 5.45	158.50 ± 3.70	156.75 ± 4.99	↓0.79 ± 0.59
Negative control*	215.25 ± 15.69	200.75 ± 24.60	201.25 ± 27.97	196.50 ± 28.01	195.0 ± 26.52	192.75 ± 29.84	174.25 ± 10.36	↓19.05 ± 1.35
Positive control	166.25 ± 6.55	161.75 ± 7.89	160.50 ± 6.61	158.25 ± 3.50	157.75 ± 4.72	155.50 ± 4.73	153.50 ± 5.00	↓7.67 ± 1.45
Dose I (200 mg kg <sup>-1</sup> )*	165.00 ± 1.63	151.00 ± 8.21	147.00 ± 12.57	146.25 ± 13.89	148.50 ± 8.02	142.50 ± 6.45	125.75 ± 2.06	↓23.79 ± 1.01
Dose II (400 mg kg <sup>-1</sup> )*	169.50 ± 4.20	160.75 ± 7.37	182.75 ± 26.59	175.00 ± 16.27	165.50 ± 29.96	184.50 ± 18.43	187.00 ± 4.97	↑10.32 ± 1.12
Dose III (600 mg kg <sup>-1</sup> )*	171.00 ± 6.68	161.25 ± 9.95	166.75 ± 17.95	174.50 ± 18.66	177.25 ± 19.29	186.25 ± 15.33	188.50 ± 7.33	↑10.23 ± 0.70

<sup>a</sup> Values are means ± SD ( $n = 4$ ), \* $p < 0.05$  vs. normal control, ↓ = decrease, ↑ = increase.



Table 7 Effect of *R. tomentosa* extract on serum blood glucose<sup>a</sup>

Treatment groups	Serum blood glucose (mg dL <sup>-1</sup> ) on day	
	0	15
Normal control	1.39 ± 0.19	1.40 ± 0.19
Negative control*	3.14 ± 0.60	4.76 ± 1.02
Positive control*	5.34 ± 1.33	4.43 ± 0.41
Dose I (200 mg kg <sup>-1</sup> )*	2.90 ± 0.20	3.70 ± 0.13
Dose II (400 mg kg <sup>-1</sup> )*	4.23 ± 0.19	3.48 ± 0.13
Dose III (600 mg kg <sup>-1</sup> )*	4.10 ± 0.29	4.68 ± 0.32

<sup>a</sup> Values are expressed by means ± SD ( $n = 4$ ), \* significantly different from normal control, ( $p < 0.05$ ).

200 mg kg<sup>-1</sup> dose treatment. This shows that the positive control has a better effect on lowering blood glucose than the two doses. In addition, the treatment using 400 and 600 mg kg<sup>-1</sup> doses increased body weight. This indicates that the two doses have a protecting impact on protein structure degradation.<sup>28</sup> The body weight of diabetic rats changed significantly ( $p < 0.05$ ) on the administration of *R. tomentosa* leaves methanol extract. These results were significantly different from the normal group based on the results of ANOVA and LSD analysis.

**Serum blood glucose.** Based on the analysis of serum glucose levels as shown in Table 7, a dose of 400 mg kg<sup>-1</sup> reduced serum glucose level in rats by 17.73%, which was from 4.23 mg dL<sup>-1</sup> to 3.48 mg dL<sup>-1</sup>.

The normal control did not experience changes in serum glucose levels. Meanwhile, negative control, 200 and 600 mg kg<sup>-1</sup> doses experienced an increased serum blood glucose level. It indicates that these treatments did not have the effect of reducing serum glucose levels in the rats. However, a dose of 400 mg kg<sup>-1</sup> could reduce serum glucose levels in rats which is in line with decreased blood glucose rates in plasma and serum.

The above analysis revealed that the blood glucose in serum when given *R. tomentosa* extract at a 400 mg kg<sup>-1</sup> dose was tended to decrease glucose rates better than other doses. The serum blood glucose of diabetic rats also decreased significantly ( $p < 0.05$ ) on the administration of *R. tomentosa* leaves methanol extract with the same dose. These results were significantly different from the normal group based on the results of ANOVA and LSD analysis.

### *In vitro* cytotoxic activity assay

The methanol extract was evaluated for its *in vitro* cytotoxicity against MCF-7, HeLa, A549, and B16 cancer cell lines using

PrestoBlue method. Table 8 shows that the methanol extract has cytotoxicity on four human cancer cells with IC<sub>50</sub> values ranging from 28.28 to 168.88 μg mL<sup>-1</sup>. Notably, the IC<sub>50</sub> values of the extract against A549 and B16 cells were only 1-3-fold weaker compared to cisplatin as the positive control, indicating its promising activity on the two types of cancer cells.

The morphology of MCF-7, HeLa, A549, and B16 cells treated with methanol extract are shown in Fig. 1–4, respectively. The results showed that the toxicity effect of methanol extract against MCF-7, HeLa, A549, and B16 cells increased with increasing extract concentration, which means that the number of living cells was reduced. The morphology of the cells looked like needles with tight spacing at low concentration of extracts. Meanwhile, the morphology of the dead cells can be seen at a high concentration of extracts with round and floating. Currently, research on non-resistant anticancer drugs becomes the main target. Bioactive compounds from plants with their chemically diverse structures have the potential to fight cancer with fewer side effects compared to standard therapies.<sup>5</sup> The anticancer effect of *R. tomentosa* extract obtained in this result may be attributed to the combination of polyphenols such as flavonoids, tannins, and glycosides, as well as terpenoids, and other compounds present in *R. tomentosa*.<sup>31</sup>

Based on the results of the cytotoxicity test, the methanol extract has potential use for the treatment of cancer cells.

HPLC analysis was performed to analyze the presence of phytochemicals in each extract. Fig. 5 showed HPLC chromatogram of *n*-hexane (a), CH<sub>2</sub>Cl<sub>2</sub> (b), EtOAc (c), and MeOH (d) extracts using methanol : H<sub>2</sub>O as a mobile phase and with wavelength at 325 nm. The hexane extract was observed to have lack of chemical content with smallest absorbance compared other extracts as in Fig. 5a, whereas several compounds were present with increasing the solvent polarity. Each extract exhibited distinct HPLC peak profiles, indicating the existence of different chemical constituents (Fig. 5a–d). The methanol extract contained two major components with highest absorbance compared other extract which might be phenolic derivatives and responsible to its potent antioxidant and antidiabetic properties. Chromatograms of *n*-hexane, CH<sub>2</sub>Cl<sub>2</sub>, EtOAc, and MeOH extracts looked similar because all extracts were dissolved in methanol, but the abundance of compounds increased according to the polarity of the extracts.

Flavonoid and phenolic compounds are often associated with several bioactivities such as antioxidant, antidiabetic, and anticancer. In previous report, phenolic compounds such as rhodomirtosone D,<sup>32</sup> rhodomirtone,<sup>32</sup> and kaempferol 3-*O*-β-

Table 8 *In vitro* cytotoxicity of methanol extract against four cancer cell lines<sup>a</sup>

Samples	IC <sub>50</sub> (μg mL <sup>-1</sup> )			
	MCF-7	HeLa	A549	B16
MeOH extract	123.49 ± 0.79	28.28 ± 0.17	168.88 ± 1.14	42.44 ± 0.18
Cisplatin	15.96 ± 0.13	5.72 ± 0.50	150.58 ± 0.27	12.97 ± 0.11

<sup>a</sup> Data are expressed as mean ± SD of twice experiments.



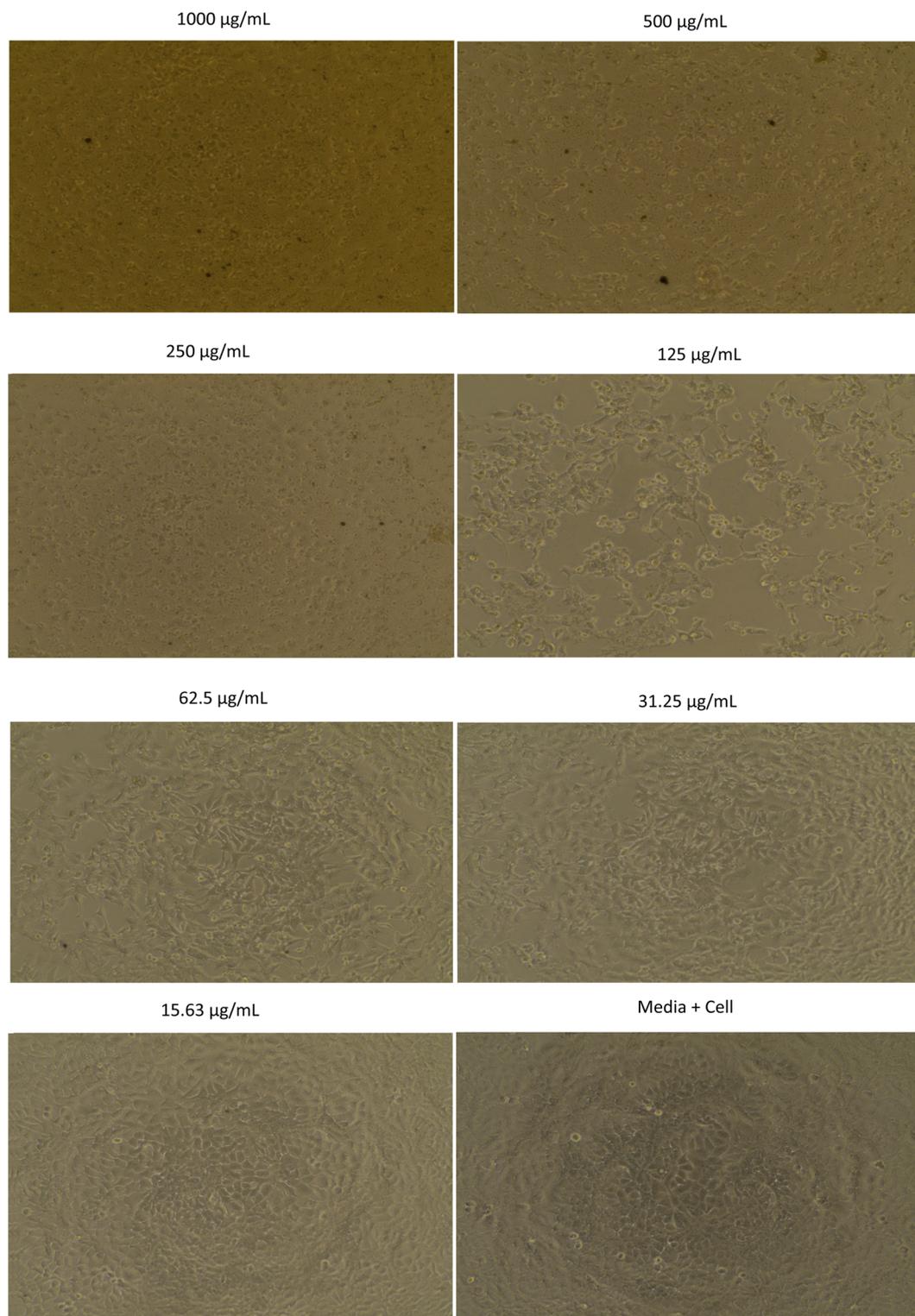


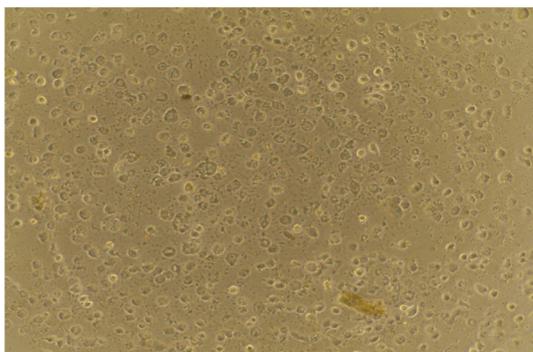
Fig. 1 The morphology of MCF-7 cells assayed with methanol extract of *R. tomentosa* leaves at various concentrations.

sambubioside<sup>33</sup> were exist in the methanolic fraction of *R. tomentosa*. On the other hand, non-phenolic constituents were also observed and some of which showed promising anticancer effect, such as  $\beta$ -amyrin (triterpenoid) isolated from the leaves

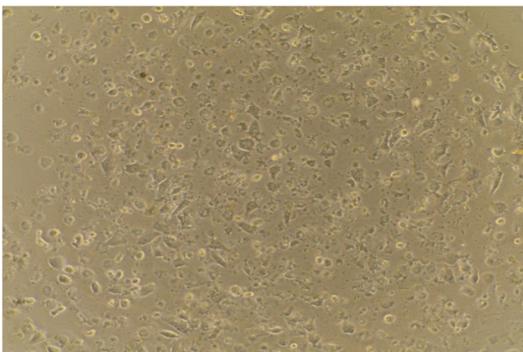
of *R. tomentosa* which had anti-inflammatory activity<sup>34</sup> and lupeol (triterpenoid) which was also isolated from the leaves of *R. tomentosa* had anti-inflammatory and anticancer activities.<sup>35</sup>



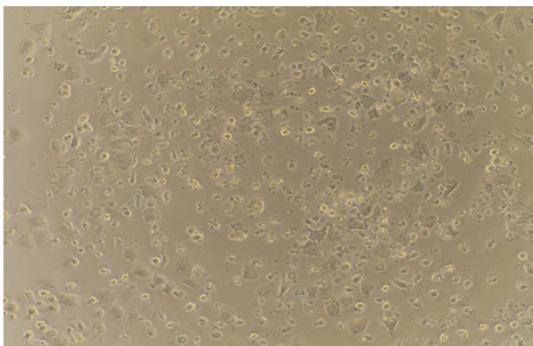
1000  $\mu\text{g}/\text{mL}$



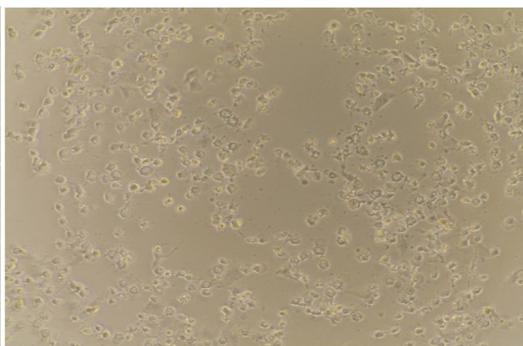
500  $\mu\text{g}/\text{mL}$



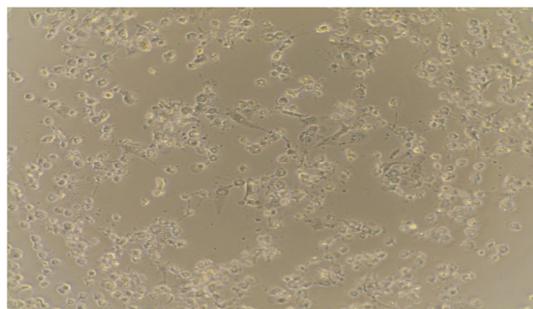
250  $\mu\text{g}/\text{mL}$



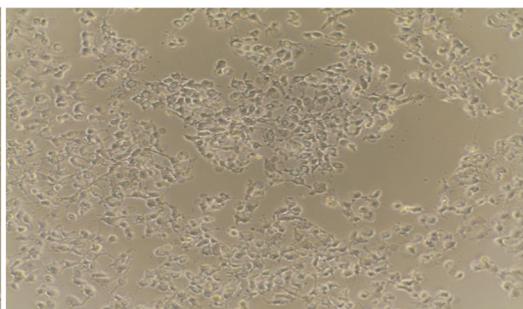
125  $\mu\text{g}/\text{mL}$



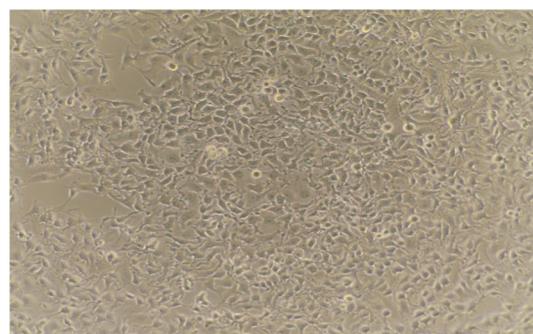
62.5  $\mu\text{g}/\text{mL}$



31.25  $\mu\text{g}/\text{mL}$



15.63  $\mu\text{g}/\text{mL}$



Media + Cell

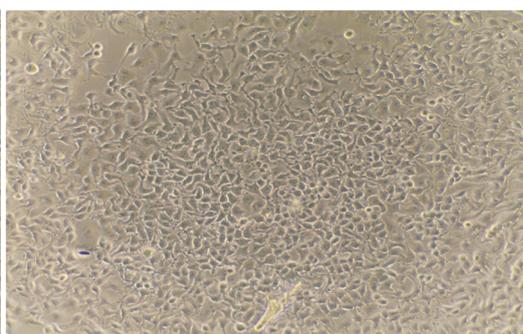


Fig. 2 The morphology of HeLa cells assayed with methanol extract of *R. tomentosa* leaves at various concentrations.



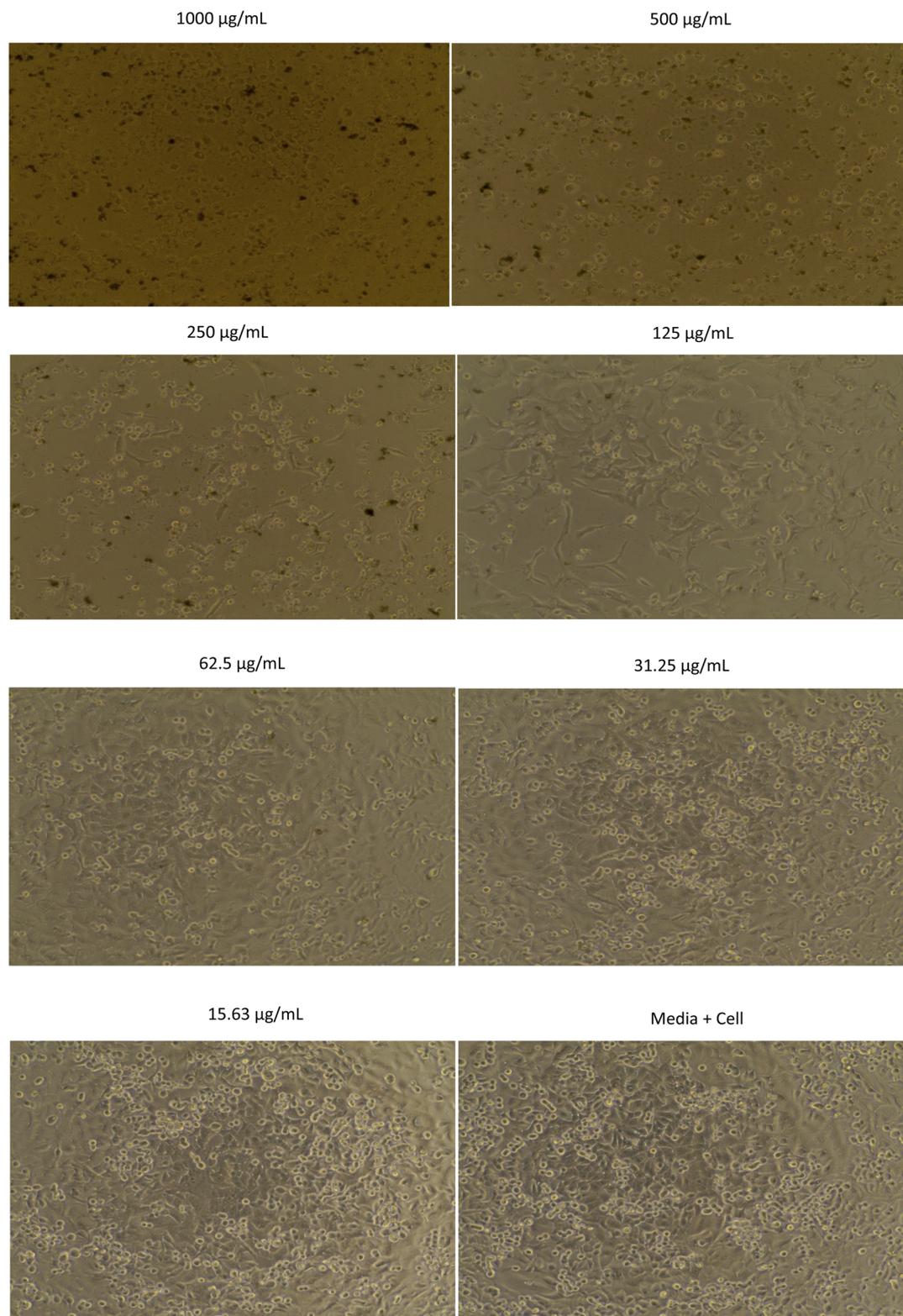


Fig. 3 The morphology of A549 cells assayed with methanol extract of *R. tomentosa* leaves at various concentrations.



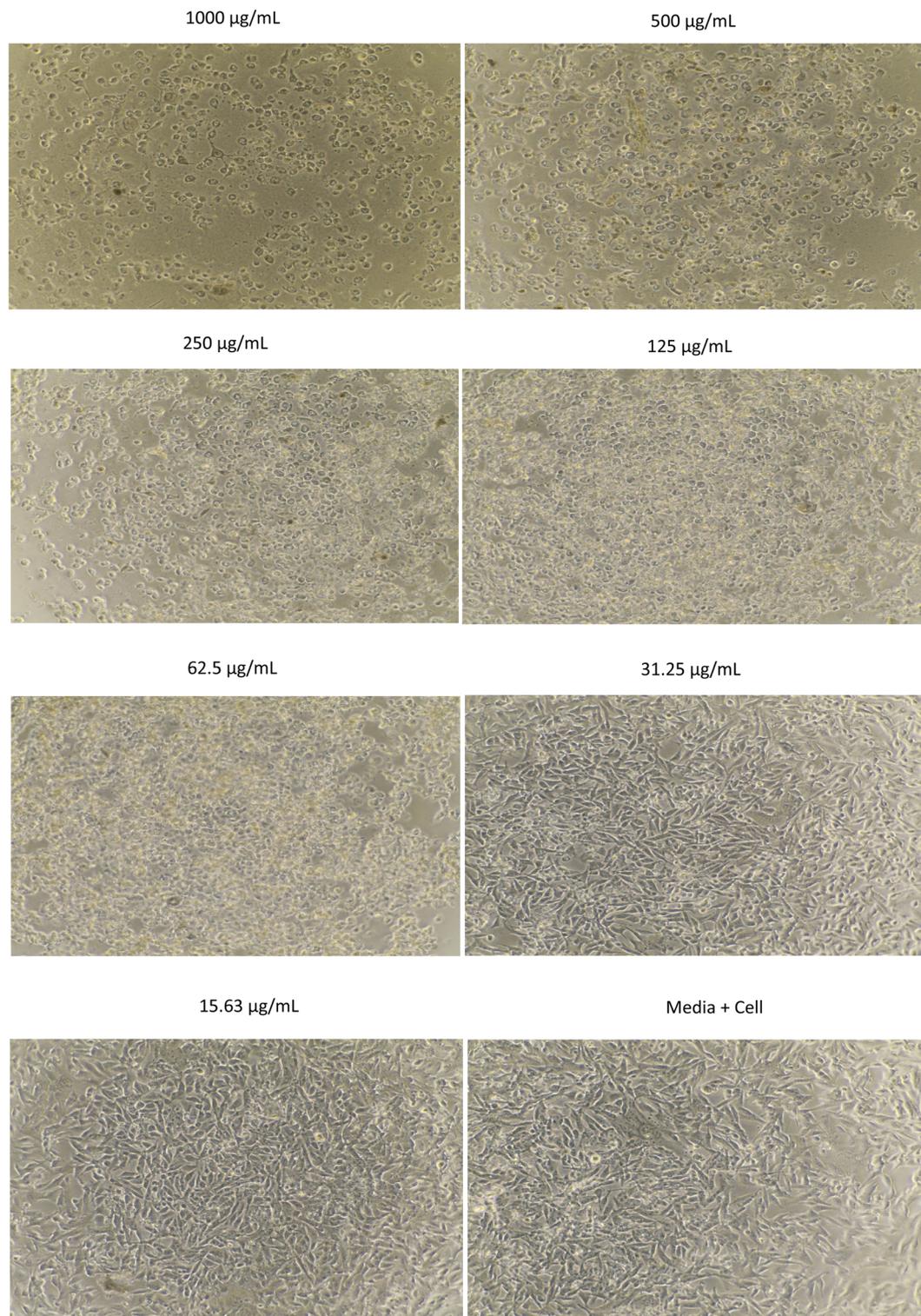


Fig. 4 The morphology of B16 cells assayed with methanol extract of *R. tomentosa* leaves at various concentrations.

This result is a preliminary study which describe the potential of methanolic extract of *R. tomentosa* leaves as anti-oxidant, antidiabetic, and anticancer agents. The phenolic compounds are proposed to be the major constituents in the

extract based on the HPLC profiling. Further study on bioassay-guided fractionation will be conducted to isolate the bioactive components contained in the extract, which might be responsible to those bioactivities.



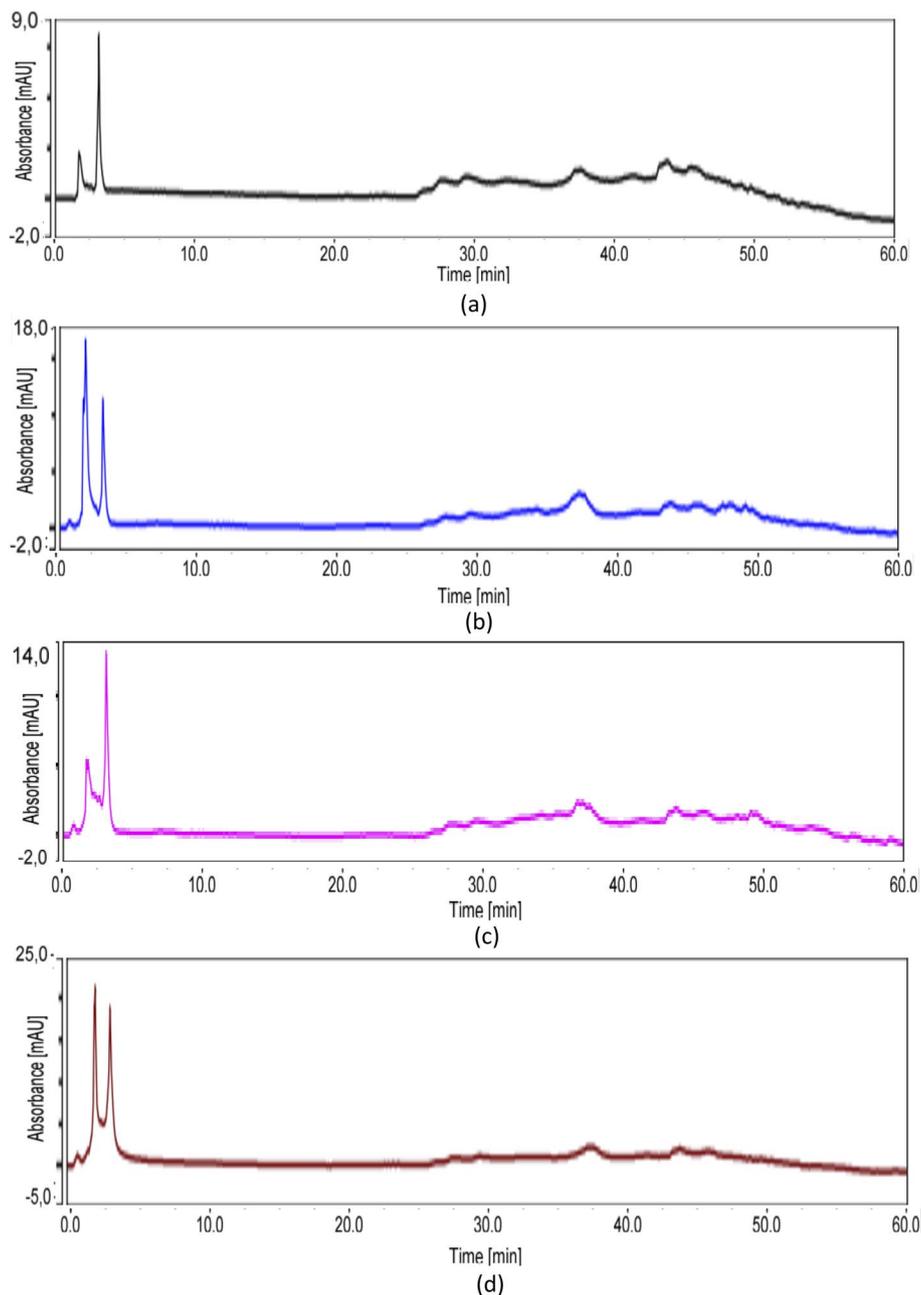


Fig. 5 HPLC chromatogram of *n*-hexane (a),  $\text{CH}_2\text{Cl}_2$  (b), EtOAc (c), and MeOH (d) extracts with methanol :  $\text{H}_2\text{O}$  as a mobile phase and wavelength at 325 nm.

## Conclusion

This study revealed that the MeOH extract of *R. tomentosa* has the highest TPC and TFC values which were in agreement with its significant antioxidant capacity, including DPPH ( $\text{IC}_{50} = 7.79 \pm 0.03 \mu\text{g mL}^{-1}$ ), ABTS ( $\text{IC}_{50} = 4.03 \pm 0.02 \mu\text{g mL}^{-1}$ ), and FRAP ( $64.05 \pm 0.54 \mu\text{M Fe}^{2+} \text{g}^{-1}$ ). The methanol extract was cytotoxic against human MCF-7, HeLa, A549, and B16 cancer cell lines with  $\text{IC}_{50}$  values of in the range of 28.28–168.88  $\mu\text{g mL}^{-1}$ . The *in vitro*  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibitory activities of the methanolic extract were reported for the first time with  $\text{IC}_{50}$  values 5–6 fold

higher than positive control (acarbose). A dose of 400  $\text{mg kg}^{-1}$  body weight of the extract was able to reduce rats blood glucose rate and serum blood glucose by 48.51% and 17.73%, respectively after 15 days treatments. These findings suggested that methanolic extract of *R. tomentosa* leaves can be used as a potential source of antioxidant, anticancer, and antidiabetic agents.

## Author contributions

Muddatstsir Idris: Methodology, investigation, validation, writing – original draft. Sri Fatmawati: Conceptualization,



methodology, validation, supervision, writing – review & editing, resources. Adi Setyo Purnomo: Validation, supervision, writing – review & editing. Edwin Risky Sukandar: Methodology, writing – review & editing. Fahimah Martak: Validation, supervision, writing – review & editing.

## Conflicts of interest

There are no conflicts to declare.

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