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Antiplatelet aggregation and cytotoxicity of BA from *M. bracteata* and its acetyl derivative

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Platelet dysfunctions are implicated in cardiovascular diseases. Management of abnormal platelet aggregations with natural products is a promising approach to the treatment of cardiovascular diseases. In this study, betulinic acid (BA) isolated from *Melaleuca bracteata* leaf extract, and its acetyl derivative (3-β acetylbetulinic acid) (BAA) were investigated for their antiplatelet aggregation and cytotoxic activity. Structures of the compounds were established and confirmed through spectral (nuclear magnetic resonance [NMR], infrared [IR], mass spectroscopy [MS]) data analysis. The antiplatelet aggregation activity of the compounds was separately evaluated on collagen, adenosine diphosphate [ADP], thrombin and epinephrine induced rat platelet aggregations. The 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cytotoxicity assay was used to determine the cytotoxic effect of the compounds against human embryonic kidney (HEK293) and hepatocellular carcinoma (HEPG2) cell lines. The triterpenoids exhibited significant ($p<0.05$) dose dependent antiplatelet aggregation activity. The highest inhibitory activity of BA and BAA was observed on epinephrine induced platelet aggregation with IC_{50} values 0.78 and 0.85 mg/ml, respectively. BA and BAA showed less cytotoxicity effect on both HEK293 cell (IC_{50} 1027 and 1051 µg/ml, respectively) and HEPG2 cells (IC_{50} 448 and 672 mg/ml, respectively). The results suggest that the compounds could serve as potential templates for synthesis of new antiplatelet drugs.

Key words: Antiplatelet aggregation, thrombin, collagen, cytotoxicity, aspirin, triterpenes.

INTRODUCTION

Blood platelets are non-nucleated cells that are important for regulation of hemostasis and repair of damaged

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Figure 1. Picture of *Melaleuca bracetata*.

endothelium cells (Semple et al., 2011). Abnormal platelet aggregation has been implicated as the underlying causes of cardiovascular diseases (stroke, heart attack, pulmonary thrombosis and venous thrombosis). These diseases are among the leading cause of death worldwide (Dickneite et al., 1995). Platelets are activated by various physiological agonists such as thrombin, collagen, epinephrine and adenosine diphosphate (ADP), which result into among others, secretion of the content of platelet granules, adhesions and aggregation. The roles of thrombin in normal platelet functions and coagulation processes showed the link between cellular (platelet) and biomolecules (coagulation) responsible for blood hemostatis (Takahashi et al., 2007).

Various antiplatelet aggregation drugs such as aspirin are currently used to manage abnormal platelet functions. Despite the use of the current antiplatelet agents, the incidences of cardiovascular diseases are still increasing. Also these antiplatelet agents are associated with some adverse effects. Aspirin, a commonly used antiplatelet drug for the treatment of cardiovascular related disease has been implicated in mucosa irritation and gastrointestinal bleeding (Armani et al., 2009). Thus a search and development of new effective antiplatelet drugs with improved safety profile is necessary.

Medicinal plants have traditionally been used for treatments of various chronic diseases with reduced side effects. The therapeutic activity of these plants is attributed to the presence of a wide range of phytochemicals such as flavonoids, phenols, alkaloids, glycosides and terpenoids (George et al., 2001).

Melaleuca bracetata var. revolution gold (Figure 1) commonly known as Johannesburg gold is a myrtle species that is aborigine in Australia. The plant is widely cultivated in South Africa as ornament (Craven, 2008). *M. bracteata* has been reported for the treatment of stroke, heart attack, sickle cell anemia and fungal infection (Habila et al., 2011).

Various plant derived triterpenes such as oleanolic acid, masculic acid, sawamilletin and ursolic acid have been reported to possess wide range of bioactivities including antioxidant, anti-plasmodial, anti-inflammatory, anticoagulant and antiplatelet aggregation activities (Mthokozisi et al., 2013; Aster et al., 2004; Habila et al., 2013). Betulinic acid (BA), a naturally occurring pentacyclic triterpene have also been reported to possess various bioactivities such as antiretroviral (Huang et al., 2006; Qian et al., 2007), anti-angiogenesis (Mukherjee et al., 2004), antioxidant and anti-inflammatory activities (Amico et al., 2006; Huang et al., 2007). Anti-sickling and anticancer activity of 3-β acetylbetulinic acid have been reported (Faujan et al., 2010; Habila et al, 2012). In this study, the antiplatelet aggregation and cytotoxicity of BA from *M. bracteata* and its acetyl derivative have been reported.

MATERIALS AND METHODS

Reagents

Unless otherwise stated, all solvents and reagents were purchased from Sigma-Aldrich and were used as received.

Plant

The leaves of *M. bracetata* var. revolution gold were harvested from the trees growing on and around the University of Zululand Campus, KwaDlangezwa, South Africa. The plant was identified

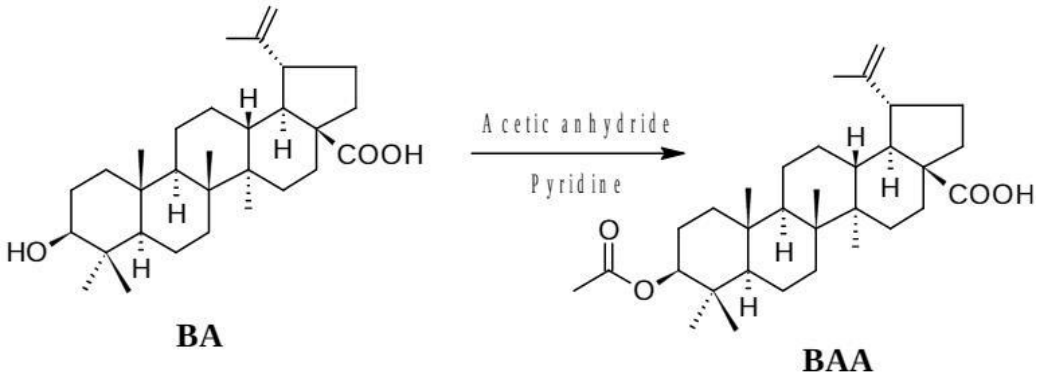

Figure 2. Schematic representation of the synthesis of 3-β acetylbetulinic acid (BAA) from betulinic acid (BA) using acetic anhydride and pyridine.

and confirmed by chief botanist at the Department of Botany, University of Zululand, voucher number 0256 and deposited at the university herbarium.

Extraction and isolation of BA

The method described by Habila et al. (2011) was adopted with some slight modification to extract and isolate the triterpene from the plant material. The fresh leaves of *M. bracetata* (400 g) were extracted with dichloromethane (4 L × 3) for 24 h using mechanical platform shaker (40 rpm, 36°C). The filtrate was concentrated with rotary evaporator at 40°C. The crude extract was air dried at room temperature to yield 5% of the plant extract. The crude extract was then defatted with hexane and 5 g of the extract was subjected to silica gel (60 to 120 Mesh) column chromatograph (20 × 5.5 cm) for isolation of the desired compound, n-hexane and ethylacetate solvent system (8:2 to 7:3) was used to elute the column. A total of 80 fractions (20 ml) were collected and analysed with thin-layer chromatography (TLC). Fractions with similar profile on TLC were combined. The combined fractions with the desired pure compound were concentrated and recrystallized with methanol to give a white amorphous powder.

Synthesis of 3-β acetylbetulinic acid (BAA)

The method of Adrine et al. (2012) was adopted with slight modification to synthesize the acetyl derivative of BA (Figure 2). BA (2 g) isolated from the *M. bracetata* was dissolved in a mixture of pyridine (10 ml) and acetic anhydride (12 ml) in a round bottom flask. The mixture was refluxed for 6 h at room temperature (25°C). The reaction was then terminated with addition of distilled water (25 ml). The mixture was further stirred with magnetic rod for 45 min. The filtrate was washed with HCl (12%) to remove excess pyridine, concentrated by suction and air-dried. The synthesized compound was further subjected to silica gel (60 × 120 mesh) column chromatography (20 × 5.5 mm) for purification, eluted with n-hexane and acetyl acetate solvent system (8:2 to 7:3). A total of 47 fractions (20 ml) were collected and similar fractions on TLC were combined. The combined fraction with desired compound was concentrated *in vacuo* at 40°C. BAA was recrystallized with methanol to obtain a white powder.

Structural elucidation

All NMR experiments were conducted on a 400 MHz Bruker Ultrashield spectrometer. BA was dissolved in a mixture of deuterated chloroform and methan(ol-d_4), whereas BAA was dissolved in deuterated chloroform. Infrared spectra were recorded with a PerkinElmer Spectrum FTIR spectrophotometer. Mass data were run on Agilent 1100 series LC/MSD trap system Electrospray ionization. All solvents and reagents were purchased from Sigma-Aldrich and were used as received. Melting points were recorded on an Electrothermal (thermoscientific) digital melting point apparatus and were uncorrected.

Compound identification

BA (Figure 3) Colourless crystal; mp 315-316°C; IR (KBr) ν_{max} 3456, 2920, 2851, 1724 cm^{-1}; m/z (ESI) 455.2 (M^+-1); δH (400 MHz, $CDCl_3$ and CH_3OD): 4.59 (1H, s), 4.46 (1H, s), 3.10 (2H, d), 2.13 (2H, dd), 1.80 (2H, s), 1.45 (8H, m), 1.38 (11H, m), 0.80-1.17 (21H, m); δC (100 MHz, $CDCl_3$ and CH_3OD) (Table 1).

BAA (Figure 4) white powder; mp 258-260°C; IR (KBr) ν_{max} 3424, 2919, 2851, 1724, , 1692, 1642, 1240 cm^{-1}; m/z (ESI) 496.8 (M^+-1); δH (400 MHz, $CDCl_3$): 4.71 (1H, s), 4.59 (1H, s), 4.45 (1H, m), 2.98 (1H, m), 2.25 (1H, d), 2.15 (1H, d), 1.94 (5H, d), 1.59 (9H, m), 1.43 (3H, s), 1.40 (4H, m), 1.24 (3H, d), 1.17 (2H, s), 1.00 (8H, m), 0.80 (10H, m); δC (100 MHz, $CDCl_3$) (Table 1).

Experimental animals

The ethic clearance (UZREC 171110-030 PGD 2014/53) was obtained from the Research Animal Ethical Clearance Committee (RAEC) of University of Zululand. Sprague Dawley rats (8 weeks, 220 to 250 kg) were collected from the animal house at the Department of Biochemistry and Microbiology, University of Zululand. The animals were acclimatized in the standard laboratory facility and maintained using standard ethic protocol with access to enough clean drinking water and pellet feeds.

Preparation of plasma rich platelet (PRP)

The method described by Tomita et al. (1983) was adopted to prepare and obtain the platelets. The rats were sacrificed by cervical dislocation and blood (5 ml) collected by cardiac puncture. The blood was immediately mixed (5:1 v/v) with an acid-dextrose anticoagulant. The blood was centrifuged at 1200 rpm for 15 minutes and 2200 rpm for 3 min consecutively using Eppendorf centrifuge 5804R. The sediment was discarded and the supernatant was further centrifuged at 3200 rpm for 15 min. The

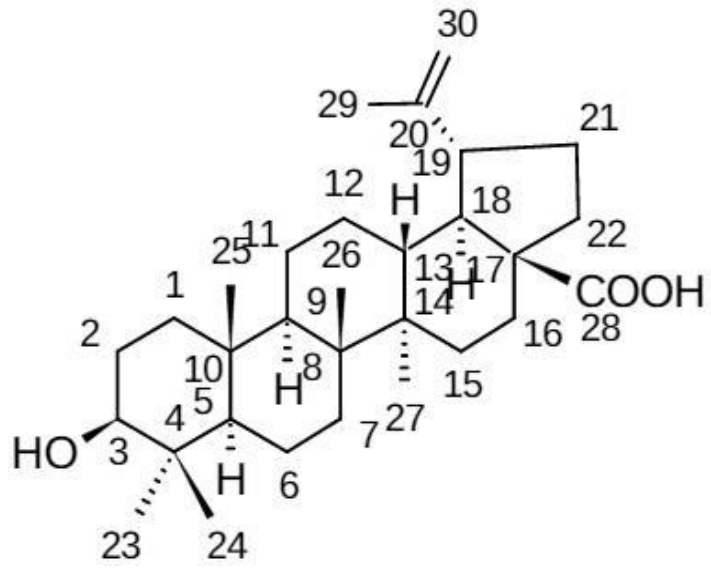

Figure 3. Chemical structure of BA.

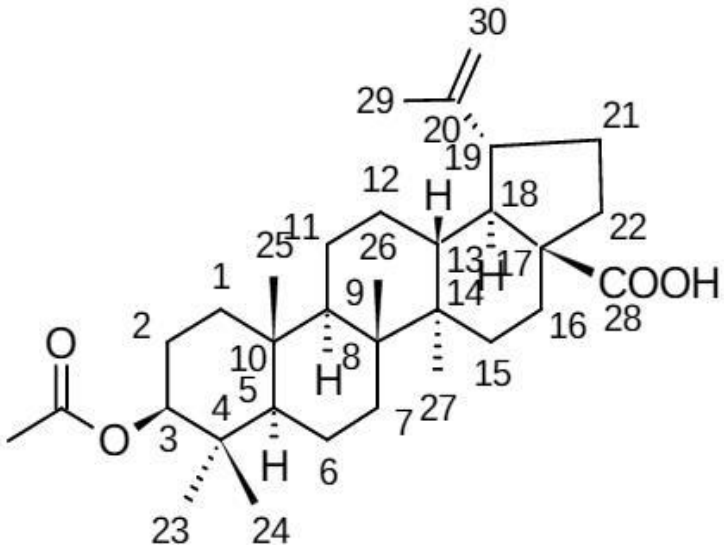

Figure 4. Chemical structure of BAA.

supernatant was then discarded and the sediment (platelets) was suspended in 5 ml of washing buffer (pH 6.5). This was further centrifuged at 300 rpm for 15 min and the supernatant was discarded. The washed platelets were then suspended in a resuspending buffer (0.14 mM NaCl; 15 mM Tris- HCl; 5 mM glucose, pH 7.4). The working solution was prepared by further diluting (1:10) the platelets with the resuspending buffer and supplementing with calcium chloride (0.4 ml: 10 µl $CaCl_2$).

Preparation of compound for the antiplatelet aggregation assay

The compounds were dissolved in 2% tween 20 to make different concentration of 1, 3, 5 and 10 mg/ml.

Evaluation of platelet aggregation inhibition activity

The antiplatelet aggregation activity of the compounds was evaluated following the method described by Mekhfi et al. (2004) with slight modification. Platelet aggregation inhibitory activity of the compound was separately investigated in thrombin (5 µg/ml), collagen (10 µg/ml), ADP (10 µg/ml) and epinephrine (5 µg/ml) induced platelet aggregation. Platelets (200 µl) and 20 µl of various concentration (1, 3 ,5 and 10 mg/ml) of the compound was pipetted into corresponding wells of a 96-wells, mixed well and incubated for 5 min at 37°C. Platelet aggregation was induced by addition of the

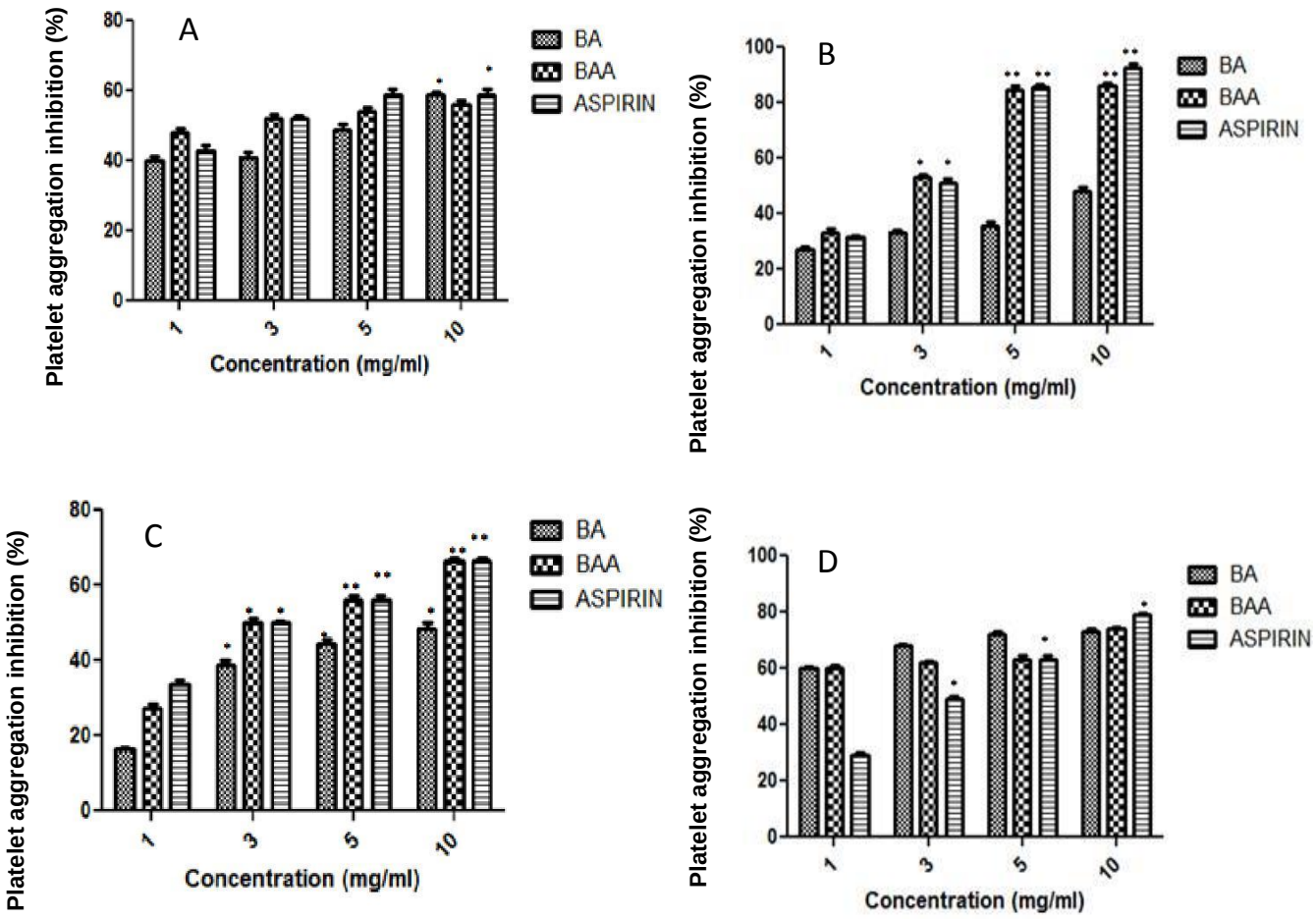

Figure 5. Percentage inhibition activity of the compounds on (a) collagen induced platelet aggregation, (b) ADP induced platelet aggregation, (c) thrombin induced platelet aggregation, and (d) epinephrine induced platelet aggregation. Data were expressed as mean ± SD. *P<0.05, **P<0.01.

agonist (20 µl). The aggregation was read at 415 nm for 20 min at 30 s interval using Biotek plate reader. Aspirin served as the positive control while DMSO (1%) was used as negative control.

Percentage inhibition of platelet aggregation was calculated by the formula:

Inhibition (%) = (Ao – A1/ Ao) 100

where Ao=control, A1=tested sample. The IC_{50} values were calculated using statistical package Origin 6.1.

Determination of cytotoxicity of BA and BAA

The cytotoxicity of the triterpene and its derivative was determined using 3 (4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazoliumbromide (MTT) cytotoxicity proliferation assay (Mosman, 1983). The cytotoxic effect of the compounds was evaluated against normal human embryonic kidney (HEK293) and cancerous human hepatocellular carcinoma (HEPG2) cell lines. The cytotoxicity results were calculated by regression analysis using QED statistics program.

Statistical analysis

Unless otherwise stated, all the experiments were triplicated and values were expressed as mean ± standard deviation. One way analysis of variance (ANOVA) and Post Hoc Dunnett's test were used to analyse the results using Graphpad prism version 5.03. Statistical significance was considered at P<0.05.

RESULTS

The results revealed that the compounds exhibited concentration dependent platelet aggregation inhibitory activity against the four platelet agonists (collagen, ADP, thrombin, epinephrine) (Figure 5a to d). BAA exhibited significantly (p<0.05) higher platelet aggregation inhibition at 10 mg/ml on aggregation induced with ADP and thrombin in comparison with BA (Figure 5b and c). The IC_{50} values (1.72, 2.72, and 2.92 mg/ml, respectively) of BAA on collagen, ADP and thrombin induce platelet

Table 1. ^{13}C-NMR (100 MHz) spectral data for BA and BAA.

Position	BA	BAA
1	38.4 (CH_2)	38.1
2	26.5 (CH_2)	27.6
3	78.4 (CH)	80.7
4	38.5 (C)	37.5
5	55.0 (CH)	55.1
6	17.9 (CH_2)	17.8
7	33.9 (CH_2)	33.9
8	40.2 (C)	40.4
9	50.1 (CH)	50.1
10	36.7 (C)	37.5
11	20.5 (CH_2)	20.5
12	25.1 (CH_2)	25.1
13	37.9 (CH)	38.1
14	42.0 (C)	42.1
15	30.2 (CH_2)	30.3
16	31.9 (CH_2)	31.8
17	55.8 (C)	56.1
18	46.6 (CH)	46.6
19	48.8 (CH)	48.9
20	150.3 (C)	150.1
21	29.2 (CH_2)	29.4
22	36.7 (CH_2)	36.8
23	27.4 (CH_3)	27.6
24	14.9 (CH_3)	15.7
25	15.4 (CH_3)	15.9
26	15.6 (CH_3)	16.1
27	14.2 (CH_3)	14.3
28	178.8 (C)	182.2
29	18.8 (CH_3)	19.0
30	109.3 (CH_2)	109.8
31	-	170.8 (C)
32	-	23.4 (CH_3)

Data reported in ppm.

Table 2. The IC_{50} values of betulinic acid and 3-β acetylbetulinic on platelet aggregation inhibition.

Compound	IC_{50} (mg/ml)			
	Collagen	ADP	Thrombin	Epinephrine
Betulinic acid	5.45	11.1	11.6	0.78
3-β Acetylbetulinic acid	1.72	2.72	2.92	0.85
Aspirin	2.58	2.72	2.72	2.98

aggregation were lower than that of BA (IC_{50}: 5.45, 11.1, and 11.6 mg/ml, respectively) (Table 2). Both BA and BAA showed significant inhibitory activity on epinephrine induced platelet aggregation with IC_{50} of 0.78 and 0.85 mg/ml, respectively when compared to aspirin with IC_{50} of 2.98 mg/ml (Table 2).

The cytotoxicity of the triterpenoids was evaluated and the results are presented in Table 3. BA and BAA showed weak cytotoxic effect on both HEK293 cells (IC_{50} 1027 and 1051 µg/ml, respectively) and HEPG2 cell (IC_{50}

Table 3. The IC_{50} (µg/ml) of betulinic acid and 3-β acetylbetulinic acid on HEK293 and HEPG2 cells.

Compound	IC_{50} (µg/ml)	
	HEK 293	HEPG2
Betulinic acid	1027	448
3-β Acetylbetulinic acid	1051	672

448 and 672 µg/ml, respectively). However, a relatively higher activity was observed on the cancerous HEPG2 cell than on the normal HEK293 cells.

DISCUSSION

The structures of BA and BAA were established and confirmed with ^{1}H and ^{13}C NMR spectroscopy, IR and mass spectrometry. The melting points of the two compounds were also determined which is in agreement with previously reported values (Habila et al., 2013). The presence of hydroxyl groups in the compounds was indicated by the appearance of an absorption band between 3424 to 3456 cm^{-1} in the IR Spectra. The ^{1}H NMR spectrum of BA revealed various peaks corresponding to the methyl groups at around 0.80 to 1.17 ppm and terminal methylene protons at 4.46 to 4.59 ppm, which is indicative of the presence of 48 hydrogen atoms in BA. As expected, the ^{1}H NMR spectrum of BAA showed the presence of 50 hydrogen atoms, which agrees with literature for previously reported values (Habila et al., 2011). In ^{13}C NMR spectra, the appearances of two additional carbons assigned as C-31 and C-32 (Table 1) further confirmed the formation of BAA. The carboxylic acid carbon assigned as C-28 (Table 1) appeared as the most deshielded around 178.8 and 182.2 ppm for both BA and BAA, respectively, which is also in agreement with literature (Habila et al., 2013). Further evidence for the isolation of BA and BAA was provided by the ESI-MS spectra which showed intense molecular ions corresponding to M^{+}-1 at 455.2 and 496.8, respectively.

Blood platelets are crucial for hemostatic system and repair of damaged endothelium. However, aberrant platelet aggregations have been reported as the major cause of cardiovascular diseases (Dickneite et al., 1995). Targeting the abnormal platelet aggregation could be a good strategy to combat the ever increasing cardiovascular events. Medicinal plants have always served as rich sources of diverse bioactive compounds vital to human health. Triterpenes, due to their diverse potential pharmacological activities, are now targets for new drugs development. The results obtained from this study showed that BA and BAA (Figure 5a to d) inhibited platelet aggregation regardless of the agonists (thrombin, collagen, ADP and epinephrine). Antiplatelet aggregation activities of some other pentacyclic triterpenes against the platelet agonists (ADP, thrombin and epinephrine) have previously been reported (Jin et al., 2004; Kim et al., 2010; Xuemei et al., 2010). The higher antiplatelet aggregation activity (Table 2) exhibited by BAA than BA is consistent with the results reported by Habila et al. (2013) on the antiplatelet activity of BA and its derivative. Targeting carbon positions 3 and 28 are new pharmacophores for increasing biological activity (Ban et al., 2010). Thus, the relatively higher antiplatelet aggregation activity of BAA could be attributed to the acetyl modification at carbon-3 (C-3) position. This compound has potential to serve as a template for antiplatelet drug development or synthesis.

Depending on the intended biological activity, a good antiplatelet drug has to be active with no cytotoxic effects on normal cells. While reports of some triterpenes indicate their strong cytotoxicity effect (Lee et al., 2007; Peteros and Uy, 2010), the results from this study (Table 3) indicated weak cytotoxic effect of BA and BAA on normal cells (HEK293) and cancerous cell (HEPG2). The American National Cancer Institute guidelines consider a pure compound as cytototoxic with IC_{50} < 30 µg/ml (Suffness and Pezzuto, 1990). Despite the weak cytotoxic effect exhibited by the two triterpenes, a relatively higher activity on HEPG2 than HEK293 implies the compound could selectively inhibit the proliferation of cancer cells at higher concentration. Betulinic acid has previously been reported to selectively inhibit tumour cells (Pisha et al., 1995). Faujan et al. (2010) reported a selective cytotoxic effect of BA and BAA from *Melaleuca cajuput* on myeloid leukemia (HL-60) cell line. The weaker cytotoxic effect of BAA on the two cells used in this study could also be attributed to the acetyl modification of C-3 position. Therefore, the compound could be potential safer antiplatelet agent.

Conclusion

The present study revealed that betulinic acid and its acetyl derivates (3-β acetylbetulinic acid) have antiplatelet aggregation activity regardless of the agonist. In addition to efficacy, the weak cytotoxic effect showed by the compounds indicated their potential use as templates for synthesis of safe pharmacologically active antiplatelet agents. For further study, elucidation of the

mechanism of action of the compounds is recommended.

ACKNOWLEDGEMENTS

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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