

Original Research Article

Antithrombotic, anticoagulant and antiplatelet activity of betulinic acid and 3 β -acetoxybetulinic acid from *Melaleuca bracteata* 'Revolution Gold' (Myrtaceae) Muell leaf

Foluso O Osunsanmi^{1*}, Godfrey E Zaharare¹, Babatunji E Oyinloye^{2,3}, Rebamang A Mosa², Monisola I Ikhile⁴, Francis O Shode⁵, Idiat B Ogunyinka⁶, Andy R Opoku²

¹Department of Agricultural Science, University of Zululand, Private Bag X1001, KwaDlangezwa 3886, ²Department of Biochemistry and Microbiology, University of Zululand, Private Bag X1001, KwaDlangezwa 3886, ³Department of Biochemistry, College of Sciences, Afe Babalola University, PMB 5454, Ado-Ekiti 360001, Nigeria, ⁴Department of Applied Chemistry, University of Johannesburg, Doornfontein Campus, PO Box 17011, Doornfontein 2028, Johannesburg, ⁵Department of Biotechnology and Food Technology, Durban University of Technology, PO Box 1334, Durban 4000, ⁶Department of Consumer Sciences, University of Zululand, Private Bag X1001, KwaDlangezwa 3886, South Africa

*For correspondence: **Email:** alafin21@yahoo.com; **Tel:** +27 791565341

Sent for review: 16 April 2018

Revised accepted: 20 September 2018

Abstract

Purpose: To investigate the antithrombotic, anticoagulant and antiplatelet activity of betulinic acid (BA) and 3 β -acetoxybetulinic acid (BAA) from *Melaleuca bracteata* 'Revolution Gold'.

Methods: Betulinic acid was isolated from the ethyl acetate extract of *M. bracteata* leaves by column chromatography, from which BAA was subsequently synthesized by acetylation. Structural elucidation of the compounds was conducted using mass spectrometry (MS), infra-red (IR) spectroscopy and nuclear magnetic resonance (NMR). The antithrombotic potential of the compounds was assessed using chromogenic substrate. Anticoagulation studies were carried using bleeding tail time assay in a rat model. Plasma-rich platelets from rats were employed for platelet aggregation studies using light microscope.

Results: The compounds significantly ($p < 0.05$) showed antithrombotic activities in a dose-dependent manner. BAA showed significantly ($p < 0.05$) higher half-maximal concentration (IC_{50}) value of 1.10 ± 0.03 mg/mL than BA (2.36 ± 0.09 mg/mL) and aspirin (2.65 ± 0.01 mg/mL) which served as positive control. The compounds exhibited anticoagulation activity with poor bleeding time, compared to aspirin. Likewise, the compounds attenuated platelets aggregation induced by thrombin.

Conclusion: BAA displays better antithrombotic, antiplatelet, and anticoagulant activities than BA. Therefore, it may be a promising remedy for the management of cardiovascular events.

Keywords: Betulinic acid, Thrombin, Anticoagulation, Antiplatelet, Aspirin, Platelets

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INTRODUCTION

Cardiovascular disease is the leading cause of mortality globally. It has urged burden on the

socio-economic lifestyle and health system [1]. Dysfunctional haemostatic process like endothelium coagulation and platelets is the major culprit of cardiovascular events. This

causes the formation of abnormal clots in veins and arteries which eventually manifested as venous thromboembolism and chronic coronary syndrome [1]. During endothelium damage, platelets are exposed to interact with platelet receptors, glycoproteins and subendothelial matrix proteins. As a result, resting plasma platelets are activated to secrete agonists such as thrombin, serotonin, ADP, adrenaline and thromboxane A₂ from the platelets granules. These platelets agonists further potentiate the binding of glycoprotein 11b/111a to fibrinogen that enhances platelets aggregation [2].

Concurrently, coagulation cascade complex including intrinsic tenase, extrinsic tenase and prothrombinase accelerate the conversion of soluble fibrinogen to insoluble fibrin to mobilize more platelets aggregation. The cumulative effects of these processes enhance thrombus formation [1]. As such, antithrombotic, anticoagulant, and antiplatelet agents have become the focal points in the treatment of cardiovascular events [3].

Current drugs such as: aspirin, prasugrel, ticagrelor, clopidogrel, apixban, and warfarin are beneficial, however with undesirable side effects which include: prolonged bleeding time, purpura, thrombocytopenia and gastrointestinal tracts ulcer [4]. In consideration of efficacy to safety ratio in drug design and selection, there is need to search for effective cardiovascular agents with minimal or no side effects especially in prevention of unacceptable bleeding rate [1]. Medicinal plants are preferable alternative remedies compared to orthodox medicine due to their accessibility, affordability and safety [5]. In previous studies, the antithrombotic, antiplatelet, and anticoagulant activities of some bioactive compounds isolated from medicinal plants have been demonstrated [6].

Melaleuca bracteata 'Revolution Gold' belongs to myrtle family (myrtaceae) and aborigine to Australia. It is widely cultivated in South Africa as ornamental tree and referred to as Johannesburg Gold [8]. The antioxidant, anti-platelet aggregation, anti-inflammatory and anti-microbial activities of crude extracts from *M. bracteata* have been reported [5,8]. In the beneficiation programme of the secondary metabolites of *M. bracteata*. This study focused on isolation of betulinic acid (BA) from *M. bracteata* leaves. Betulinic acid is a pentacyclic triterpene with wide biological properties including antioxidant, anti-platelet aggregation, anti-sickling, anti-ulcer, anti-plasmodial, anticancer, anti-inflammatory, anti-angiogenesis, anticoagulant and antiplatelet aggregation activities [5,8,9,10]. However, there

is little information on the biological activities of its acetylated derivative. Therefore, this study aims at focusing on the anti-thrombotic, anticoagulant and anti-platelets aggregation potential of betulinic acid and 3 β - acetoxybetulinic acid isolated from *M. bracteata* leaves.

EXPERIMENTAL

Reagents

The chemicals used in this study were of analytical grade and purchased from Sigma Chemical Company limited (Saint Louis, MO, USA).

Plant identification

Melaleuca bracteata leaves were harvested at KwaDlangezwa campus of the University of Zululand, South Africa (28.8524° S, 31.8491° E). The leaves were identified by Dr NR Ntuli of the Department of Botany, University of Zululand. The voucher specimen (no. VN 0256) was kept at the University's herbarium.

Preparation of betulinic acid

Betulinic acid was isolated from *M. bracteata* by following the method of Habila *et al* [8]. *M. bracteata* leaves (200 g) were dried at 25 °C and extracted with ethylacetate (5 L x 3; 1: 5 w/v) for one day. The extract was filtered with Whatman filter paper 1, and concentrated using rotary evaporator (40 °C; 60 rpm) and further air dried in fume cupboard yielding 0.6 % of crude extract. The crude extract (5 g) was screened in a column chromatographic with column (20 x 5.5 cm), silica gel (60 - 120 mesh and hexane/ ethyl acetate solvent gradients (8:2 to 7:3) to isolate BA. Sixty collected eluates (20 mL) were monitored using thin-layer chromatography (TLC). Eluate fractions corresponding to the compound of interest on the TLC plate were mixed. The mixture was concentrated with rotary evaporator (60 rpm; 40 °C). The sample was recrystallized with methanol to form white powder.

Preparation of 3 β - acetoxybetulinic acid (BAA)

BAA was synthesized from BA (Figure 1) following the method of Adrine *et al* [25]. BA (3 g) was added to the solution in a round bottom flask (50 ml) consisting of acetic anhydride (12 mL) and pyridine (10 mL). This was refluxed (10 h at 40 °C: 10 hours) in a fume cupboard, and the reaction was terminated by the addition of distilled water (25 mL). This was further stirred

for 45 minutes before being filtered. The excess pyridine was removed from the filtrate by addition of hydrochloric acid (12 %) and the filtrate was air-dried at 25 °C. Crude product (3 g) was screened with column chromatography consisting of columns (20 x 5.5 mm), silica gel (60 x 120 mesh), and n-hexane and acetyl acetate solvent system (8:2 to 7:3) for purification. Fifty eluates (20 mL) were collected and fractions with similar spots on the TLC plate were mixed and concentrated with rotary evaporator. The residual compound was recrystallized using methanol to yield yellowish powder.

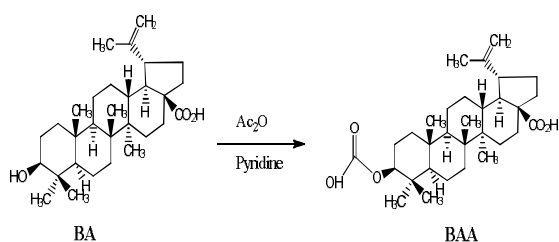


Figure 1: Preparation of BAA from BA

Structural elucidation of BA and BAA

Structural elucidation of the compounds was confirmed using mass spectrometry, IR, ¹H and ¹³C NMR spectroscopy. All NMR experiments were conducted on a 400 MHz Bruker Ultrashield spectrometer and samples were dissolved in an equimolar mixture of deuterated chloroform/methanol and 100 % deuterated chloroform for BA and BAA respectively. All mass data were run on the Waters Alliance 3100 Empower 2154 HPLC-mass spectrometer using Electrospray ionization in positive/negative mode and samples were dissolved in acetonitrile. IR spectra were recorded on a Perkin Elmer FT-IR spectrometer using nujol mulls on a KBr plates.

Evaluation of antithrombin activity

Antithrombotic activity was evaluated following the method of Rob *et al* [26]. BA and BAA were first solubilized in DMSO (5 %) before preparing its various concentrations (1, 3, 5 and 10 mg/mL). Each concentrate was further diluted with 50 µl of Tris-HCl buffer (121.15g Tris-HCl, in 800 ml dilute water at pH 7.4). Thrombin (10 µl) was pipetted into various cells of a 96-well plate containing 50 µL various concentrates. This was then incubated for 10 min at 25 °C, afterward chromogenic substrate (190 µL; 0.76 M) was added. The absorbance (412 nm; 8 min at 1-min intervals) was read using a spectrometer (Biotek ELx 808 UI plate reader). Aspirin served as the positive control. Antithrombin level (B) was computed as in Eq 1.

$$B (\%) = \{(A_o - A_t)/A_o\}100 \dots\dots\dots (1)$$

where A_o and A_t denote the absorbance of negative control and the test drug, respectively.

Animal experiments

Ethical clearance number UZREC 171110-030 PGD 2014/53 was issued by Ethical Committee of University of Zululand for the animal studies which were conducted in accordance with international guidelines for handling of animal [27]. Sprague–Dawley rats (230 - 260 g) were obtained from the animal house of the Department of Biochemistry and Microbiology, University of Zululand. The rats were kept in wire mesh cages under a conducive environment (12 h/12 h light/dark cycle, 25 °C) with access to regular pellet feed and water.

Bleeding time assay (*Ex vivo*)

Bleeding time assay for transected rats tail were determined by the method of Mosa *et al* [12]. The rats (20) were divided into three groups of four each. Groups 1 and 2 received different concentration of the compounds (50 mg/kg and 250 mg/kg) respectively while group 3 received aspirin (40 mg/kg). The rats were anaesthetized with Sodium pentobarbital (50 mg/kg) 2 hours after sample administration. Afterward, the tail of the rats were transected 5 mm from the tail tip, and at every 30 seconds the filtered paper was blotted on the tail until the paper was void of blood stain. The bleeding time was calculated as the period between transecting of the tail and when bleeding stopped.

Preparation of plasma-rich platelets

Plasma rich platelets (PRP) were prepared from the rats' blood [22]. Sprague-Dawley rats (2) were euthanized by cervical dislocation. Blood (5 ml) was siphoned surgically from punctured abdominal aorta and transferred into a tube containing acid-dextrose anticoagulant (0.085 mM citric acid; 0.093 mM dextrose and 0.017 mM Sodium), and mixed (5:1 v/v) thoroughly. The bloods' mixtures were centrifuged (1200 rpm; 15 minutes) and repeatedly centrifuged again at 2200 rpm for another 3 minutes. The resulted supernatant was further centrifuged at 3200 rpm for 15 min. The obtained residues were then transferred into a tube consisting of 5 ml of washing buffer. (4.3 mM K_2HPO_4 ; 5.5 mM glucose; 0.113 M NaCl; 4.3 mM Na_2HPO_4 ; 4; 1 mM EDTA; pH 6.5). This was again centrifuged at 3000 rpm for 15 min. The supernatant was discarded while the residue containing plasma rich platelet (PRP) was preserved. The residue

was diluted to the ratio of 1:10 with resuspension buffer (0.005 M glucose, 0.14 M NaCl; and 15mM Tris-HCl) and mixed with calcium chloride (0.4 mL).

Platelet aggregation study

Plasma rich plate solution (200 μ L) was pipetted into various cells of 96 wells plate consisting of 20 μ L of DMSO (1 %), BA, (10 mg/mL), BAA (10 mg/mL), and aspirin (10 mg/mL) respectively. The 96 wells plate was kept in the incubator (37 °C, 5 min), afterward thrombin (5 μ g/mL; 20 μ L) were pipetted to respective cells to initiate platelet aggregation. After two seconds, 2 μ L of the mixture was pipetted from each cell and dropped on prepared slides for onward viewing under light microscopy (x 1500).

Data analysis

The experiments were carried out in triplicate and the mean \pm standard deviation (SD) of the data was calculated. Post-hoc Dunnett's test and one-way ANOVA of the data were analyzed with GraphPad Prism (version 5.03). Statistical significance was set at $p < 0.05$.

RESULTS

Spectral data for BA and BAA

BA (Figure 1) m/z (ESI) 455.2 ($M^+ - 1$); IR (KBr) ν_{max} 3456, 2920, 2851, 1724 cm^{-1} ; δC (100 MHz, $CDCl_3$ and CH_3OD); 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) mp 315 - 316 °C [5].

BAA (Figure 1) m/z (ESI) 496.8 ($M^+ - 1$); IR (KBr) ν_{max} 3424, 2919, 2851, 1724, 1692, 1642, 1240 cm^{-1} ; δC (100 MHz, $CDCl_3$); 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) mp 258-260 °C [5].

Antithrombin activity

BA and BAA displayed antithrombin activities in dose dependent manner (Figure 2). However, BA showed the highest activity at 10 mg/kg. BAA at 1 mg/kg showed significant ($P < 0.05$) better activity than BA and aspirin. BAA also showed significant better IC_{50} values of 1.10 ± 0.03 mg/ml than BA (2.36 ± 0.09 mg/mL) and aspirin (2.65 ± 0.01 mg/mL) (Table 1).

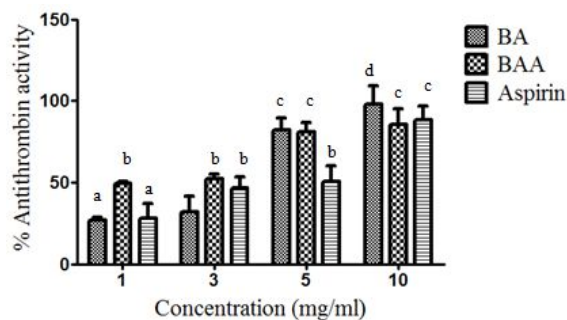


Figure 2: Antithrombotic activity of the compounds and aspirin. Values with different alphabets (a, b, c, d) were considered significant ($p < 0.05$)

Table 1: IC_{50} values of the compounds with antithrombotic activity. Data expressed as mean \pm SD. Values with different alphabets (a, b) in the chart are significant ($p < 0.05$)

Compound	IC_{50} (mg/mL)
BA	2.36 ± 0.09^b
BAA	1.10 ± 0.03^a
Aspirin	2.65 ± 0.01^b

Tail bleeding time

The compounds (BA and BAA) significantly ($P < 0.05$) accelerated bleeding time in dose dependent pattern (Figure 3). Likewise, the compounds at various concentrations exhibited significant shorter bleeding time than aspirin (40 mg/kg) the positive control.

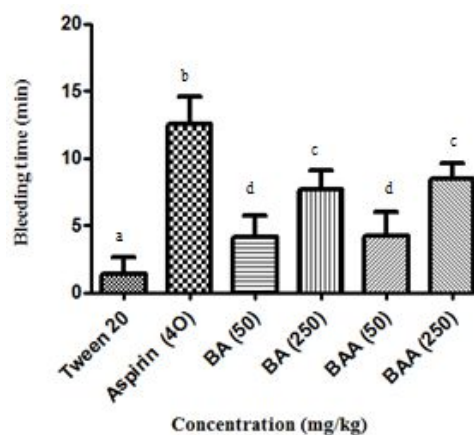


Figure 3: The effect of BA and BAA on tail bleeding time assay. Values with different alphabets (a, b, c, d) were considered significant ($p < 0.05$)

Anti-platelets aggregation

In the untreated group, platelet aggregation was observed to be stimulated by thrombin; the platelets agonist (Figure 4 B). This was attenuated in the treated group despite the presence of thrombin (Figure 4 C and Figure 4 D and Figure 4 E). BAA showed similar platelets

orientation in comparison to aspirin the positive control (Figure 4 D and Figure 4E).

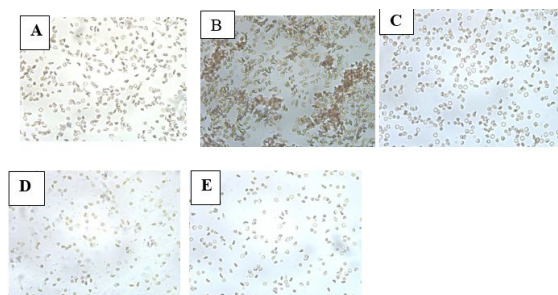


Figure 4: Light microscope pictures of plasma rich platelets with untreated and treated compounds (10 mg/mL) at magnification x 1500. Platelets aggregation was induced by thrombin. Picture (A) Inactivated platelets, (B) Activated platelets (C) Activated platelets and BA, (D) Activated platelets and BAA, (E) Activated platelets and aspirin

DISCUSSION

Medicinal plants have been the mainstay of pharmacotherapy since the era of civilization. It has also contributed immensely to the commercial manufacturing of cardiovascular medications [11]. Recently, research on triterpenes have attracted urgent attention based on its wide biological activities [9]. Previously, triterpenes isolated from *Protorhus longifolia* stem bark were demonstrated to possess anticoagulation, antiplatelet and anti-inflammatory [12]. In this study, BA and BAA were successfully isolated from *M. bracetata* leaves (Figure 1). The structural elucidations of these compounds further confirmed the report of Osunsanmi et al [5]. Similarly, BA was isolated from *Orthosiphon stamineu* [13].

Despite the combined drugs-therapy approach, cardiovascular mortality is still on the increase [4]. This faults the potency of the therapy to tackle the links among thrombin, coagulation cascade and platelets functions [12]. In this study, betulinic acid and 3 β - acetoxybetulinic acid were screened for their anti-thrombotic, anticoagulation and anti-platelet aggregation potential.

Thrombin plays a crucial role in abnormal coagulation formation. It helps to convert soluble fibrinogen into insoluble fibrin during the coagulation cascade. It also activates coagulation factors V, XIII, and XI that triggers mobilization of activated platelets into the blood circulation [14]. These processes further stimulate the arachidonic pathways to release phospholipids which activate more platelets [15]. Thrombin also acts as pro-inflammatory by

enhancing the proliferation and mobilization of smooth muscles cells, which activate more platelets [16]. Inversely, antithrombin attenuates platelet activation by encouraging the secretion of prostaglandin from vasculoendothelial cells. [17]. This study revealed the antithrombin potential of BA and BAA. In a previous study, diterpenes from *Dictyota menstrualis* showed antithrombin activities [18]. The observed higher antithrombin activity of BAA than BA in this study can be attributed to their structural modification where carboxyl group is replaced with acetyl group at carbon-3 (C₃) (Figure 1). Structural alteration of triterpenes at carbon-3 (C-3) and C-28 moiety were reported to boost their biological activities [5,8]. Lower IC₅₀ values of substances imply higher biological activity and vice versa. This explains the better antithrombotic activity of BAA than aspirin (19). The disparity between BAA and aspirin also suggests different mechanism of action. Aspirin attenuated thrombin synthesis by inhibiting factor XII activation, the thrombin-mediated coagulant. Likewise, aspirin promotes fibrinolysis through acetylation of lysine components in fibrinogen [20].

Tail bleeding time assay is used to measure prohemorrhagic properties of anticoagulant drugs [12]. Coagulation cascade abnormalities enhance thromboembolic disorders including heart attack, deep venous thrombosis, stroke and pulmonary emboli [23]. Hypercoagulation was implicated in the onset and progression of various cancers [24]. The findings from this study indicates that BAA is a preferable anticoagulant agent than aspirin because of its shorter bleeding time (Figure 3). In previous studies, aspirin administration was linked to prolonged bleeding time [1]. The finding was in accordance with the report of Mosa et al [12] in which lanosteryl triterpenes from stem bark of *Protorhus longifolia* showed poor bleeding time. This variation in activity could also imply that the compounds displayed different pharmacodynamics and pharmacokinetic from aspirin [1].

Platelets agonists such as ADP, serotonin, thrombin and collagen stimulate platelets aggregation via various receptors. This potentiates the secretion and release of chemical components from platelet granules, thus amplifying platelets aggregation. However, the secreted chemicals triggered coagulation cascade and thrombotic activities [2]. In this study, thrombin was observed to induce platelet aggregation which was attenuated by the isolated compounds and aspirin. This implies that the compounds and aspirin might possess similar mechanism of action against thrombin. Thrombin

induced platelet aggregation via PAR 1 and PAR 2 glycoprotein receptors [21]. This finding agrees with the reports of Mosa *et al* [22], in which triterpenes from *Protorhus longifolia* inhibited platelets aggregation induced by thrombin. This finding further validates the report of Osunsanmi *et al* [5] on *in vitro* anti-platelets aggregation activity of BA and BAA induced by thrombin. However, this is the first report on anti-platelet aggregation studies using light microscopy based on the available information.

CONCLUSION

The findings of the study showed that BA and BAA possess antithrombotic, antiplatelet aggregations and anticoagulants potential. In addition, they displayed poor bleeding time required to counter the side effects associated with other cardiovascular agents. BAA showed higher antithrombotic activity than BA and aspirin. Therefore, BAA is a promising single-dose remedy for the management of cardiovascular events.

DECLARATIONS

Acknowledgement

The authors appreciate the financial support of Research Unit of University of Zululand, South Africa for this project.

Conflict of interest

The authors declare that no conflict of interest is associated with this work.

Contribution of authors

We declare that this study was carried out by the authors named in this article and all liabilities pertaining to claims relating to the contents of this article will be borne by them. F.O Shode and A.R Opoku designed the project; F.O Osunsanmi, B.E Oyinloye, R.A Mosa, M.I Ikhile, I.B Ogunyinka performed the experiments, analyzed the data and wrote the manuscript; F.O Shode and G.E Zaharare perfected the editing. All the authors approved the final draft.

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