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**Original Research Article**

# Anti-platelet aggregation of mixtures of betulinic oleanolic and maslinic acids and derivatives from medicinal plants

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## Abstract

***Purpose:*** *To evaluate the antiplatelet aggregation and cytotoxic potential of betulinic acid (BA), oleanolic acid (OA), maslinic acid (MA) and their derivatives (3-β-acetyloleanolic acid (OAA) and 3-β-acetylbeutulinic (BAA) from medicinal plants.*

***Methods:*** *The compounds were characterized by nuclear magnetic resonance (NMR, both carbon 13 and hydrogen 1) (NMR), infra-red (FTIR) and mass spectroscopy (MS). The platelet aggregation inhibitory activities of the compounds (1, 3, 5 and 10 mg/ml) were investigated separately on adenosine diphosphate (ADP) and thrombin-induced rat platelet aggregation. Cytotoxicity studies were carried out on human embryonic kidney (HEK293) and hepatocellular carcinoma (HEPG2) cell lines using 3, 4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazoliumbromide assay.*

***Results:*** *The compounds significantly ($p < 0.05$) inhibited platelet aggregation in a dose-dependent manner on thrombin and ADP agonist. BAA/OAA showed the highest activity on both agonists with $IC_{50}$ of 2.86 and 3.05 mg/mL respectively. BAA/OAA also showed better antiplatelet activity than aspirin ($IC_{50}$ of 6.45 and 7.36 mg/mL, respectively). In addition the compound (BA/OA, BAA/OAA and MA/OA) exhibited low cytotoxic effect on both HEK293 cells ($IC_{50}$: 724.43, 269.08 and 407.89 mg/mL respectively) and HEPG2 ($IC_{50}$: 585.38, 499.78 and 499.78 mg/mL, respectively).*

***Conclusion:*** *BAA/OAA demonstrate the best antiplatelet potential and low cytotoxicity of in all the tests, and therefore can serve as safer antiplatelet agents.*

***Keywords:*** *Platelet aggregation, Agonist, Aspirin, Betulinic Acid, Oleanolic Acid, Maslinic Acid, Cytotoxicity*

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## INTRODUCTION

Abnormal platelet aggregation is instrumental to the pathogenesis of thromboembolic events such as stroke, deep venous thrombosis, heart attack and pulmonary embolism [1]. Endogenous agonists such as collagen, epinephrine, ADP, and thrombin induce platelet aggregation. These agonists trigger phospholipase (PLC) family that cleave phosphatidylinositol-4, 5- bisphoshate into inositol 1, 4, 5 bisphosphate (IP3) and diacylyglycerol (DG). The IP3 and DG initiate calcium influx and activation of protein kinase respectively [1]. Despite availability the anti-platelet drugs, mortality rate associated with cardiovascular diseases is on the increase. Most

of the current used anti-platelet drugs are with undesirable side effects [2]. Therefore, there is need to search for alternative treatment from natural origin.

*Melaleuca braceteata* belongs to Myrtaceae family and it is indigenous in Australia, commonly called golden bottle brush, punk and white cloud tree [3]. The essential oil from *M. braceteata* has been reported to possess antibacterial, antifungal, antiseptic, insecticide and eliminate genital wart [4].

*Syzygium aromaticum* (clove) belongs to Myrtaceae family and it is indigenous to Indonesia [5]. *S. aromaticum* is commonly used as nutritional spice [5]. *S. aromaticum* has been also demonstrated to possess antimicrobial, antiseptic, aphrodisiac, anti-emetic, anti-inflammatory and carminative properties [5,6].

Triterpenes possess wide spectrum of biological activities such as anti-inflammatory, anti-HIV, antimutagenic antimicrobial, antitumor and anticancer [7]. In this present study, the antiplatelet aggregation activity and cytotoxic effect of the mixture of BA/OA, BAA/OAA as well as MA/OA were investigated.

## EXPERIMENTAL

### Reagents

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich and were of analytical grade.

### Plant materials

Fresh leaves of *Melaleuca braceteata* var. revolution gold were collected in March, 2014 from trees growing within the KwaDlangezwa campus of University of Zululand, South Africa. The plant was authenticated by Dr NR Ntuli of Department of Botany, University of Zululand. A voucher specimen (voucher number FO4) was deposited at the University Herbarium.
*Syzygium aromaticum* (cloves) was purchased from spices market in Durban, South Africa and were authenticated by Mr. Pawn Porum of the Department of Botany, University of KwaZulu-Natal, Westville campus with the voucher specimen (VN/004) which was deposited at the University of KwaZulu-Natal Herbarium.

### Extraction and isolation of BA/OA from *Melaleuca bracetata* var. revolution gold

The method of Habila *et al* [8] was adopted with slight modification. Fresh leaves of *M. braceteata* (600 g) were dehumidified and extracted by maceration in ethyl acetate at room temperature (5 L x 3) for 72 h. The filtrate was concentrated with rotatory evaporator (40 $^{o}$C) to yield 0.8 % of the crude extract. The crude extract was then defatted with n-hexane. A portion of the crude extract (6 g) was subjected to silica gel (60-120 mesh) column chromatography (20 x 5.5 mm) using a gradient of n-hexane and ethylacetate (8:2 to 7:3) as the solvent system. A total of 77 fractions of eluates (20 ml each) were collected and analyzed with thin layer chromatography. Fractions with the desired compounds monitored with TLC were combined and concentrated by rotatory evaporator. This was then recrystallized with methanol to form a white powder. Attempts to further separate the mixture of BA/OA were unsuccessful.

### Synthesis of BAA/OAA from BA/OA

The method described by Andrine *et al* [9] was adopted. The mixture of BA/OA (2 g) isolated from *M. braceteata* var. revolution gold was dissolved in a solution containing a mixture of pyridine (10 ml) and acetic acid anhydride (12 ml). This was refluxed for 8 h until a complete solution result. The reaction was terminated by the addition of distilled water (25 ml) and stirring for another 45 min. The synthesized compounds were rinsed with 12 % HCl to remove excess pyridine and dehumidified at room temperature. The compounds were further purified by silica gel (60 x 120 mesh) column chromatography (20 x 5.5 mm), and eluted with a gradient of n-hexane and ethylacetate (8:2 to 7:3). Forty seven fractions of eluates (20 ml, each) was collected and analysed by TLC. Fractions with desired compounds were combined and concentrated using rotatory evaporator at 40 $^{o}$C. The mixture of BAA/OAA was recrystallized with methanol and air-dried to give a white powder.

### Extraction and isolation of MA/OA from *S. aromaticum* (cloves)

The method of Ibrahim *et al* [10] was used with slight modification. Buds of *S. aromaticum* (400 g) were milled by an industrial blender. The milled samples were extracted with dichloromethane (2 L x 3) for 24 h on a lab com shaker (36 rpm, 37 $^{o}$C) and filtered. The filtrate was concentrated with rotatory evaporator (40 $^{o}$C) to yield 2.0 % of the initial weight of plant samples. The crude extract was de-fatted with n-hexane and a portion (7 g) was subjected to silica gel (60-120 mm) column chromatography (20 x 5.5 mm) using n-hexane and ethyl acetate solvent system ranging from 9:1 to 8:2. Total fractions of (58) eluates (30 ml, each) were

collected and analyzed with TLC. Fractions containing desired compounds were combined and concentrated using rotatory evaporator (40 $^{o}$C) to obtain white amorphous solid.

**Experimental animals**

Adult Sprague Dawley rats (220 – 250 g) of either sex were used. The animals were maintained under light condition (12/12 h light/dark), humidity (55 %), constant temperature (22 ± 2 $^{o}$C) and had access to pellet feeds and safe drinking water. The experiments were approved (UZREC 171110-030 PGD 2014/53) by Research Animal Ethic Committee (RAEC) of the University of Zululand and were conducted based on the guideline for the use of laboratory animals [11].

**Plasma-rich platelet (PRP)**

Plasma-rich platelet (PRP) was prepared according to the method described by Mosa *et al* [12]. The animals were sacrificed by cervical dislocation and blood was collected from punctured heart with surgical needle (5 ml).

The blood was transferred into a 15 ml test tube containing anticoagulant (acid–dextrose, 85 mM trisodium citrate, 83 mM dextrose and 21 mM citric acid). Plasma-rich platelet was obtained through series of centrifugation at 1200 rpm for 15 min and 2200 rpm for 3 min consecutively. The supernatant was further centrifuged at 3200 rpm for 15 min. The residue was re-suspended in washing buffer (5 ml, pH 6.5) while the supernatant was discarded and PRP were suspended in a small volume of suspending buffer (0.4 mM NaCl, 15 mM Tris-HCl, 5 mM glucose, pH 7.4). The platelets were diluted with resuspending buffer (1:10) and were further mixed with calcium chloride (0.4 ml:10 µl $Cacl_2$).

**Anti-platelet aggregation studies**

Anti-platelet aggregation activities of the compounds were determined by the method described by Ibrahim *et al* [13] with slight modification. Anti-platelet aggregation activity was investigated using ADP (5 mM) and thrombin (5 µ/ml) as agonists. PRP (100 µl) were pipetted into 96 wells plate and pre-incubated for 6 minutes at 40 $^{o}$C with various concentration (1, 3, 5 and 10 mg/ml) of the compounds. Platelet aggregation was then induced with separate agonists (20 µl). Anti-platelet aggregation activities of the compounds were measured by Bioteck plate and absorbance read at 415 nm. Aspirin served as the positive control while DMSO (1 %) served as a negative control. Antiplatelet aggregation activity was calculated by the formula. Inhibition % = Ao – A1/ Ao x 100 (where Ao is mean slope of control while A1 is mean slope of tested compound). The value of $IC_{50}$ was calculated using statistical package origin 6.1.

**Cytotoxic assay**

The cytotoxicity of the compounds were determined by MTT (3, 4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazoliumbromide) assay described by Mosaman [14]. Human hepatocellular carcinoma (HepG2) and human embryonic kidney (HEK293) cells lines were used for this assay. The cell line in the tissue culture was grown in flasks (25 $cm^2$) and trypsinized. This was then pipetted into 96 well plate at specific seeding densities and incubated for 24 h at 37 °C. The medium was removed and replaced with fresh medium (MEM + Glutmax + antibiotics). The isolated compounds (50 – 350 µl/ml) were added in triplicate and incubated for 4 h at 37 $^{o}$C. The medium was again removed and replaced with a complete medium (MEM + Glutmax + antibiotics + 10 % fetal bovine serum). This was incubated for 48 h at 37 $^{o}$C for MTT assay. The compounds' cytotoxic were evaluated by regression analysis using QED statistics program and $IC_{50}$ values from the linear equation was used to calculate cells mortality (50 %).

**Statistical analysis**

The experiments were carried out in triplicate and data expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and post-hoc Dunnett's test were used to analyze the results using Graphpad prism software (version 5.03). Statistical significance was set at $p < 0.05$.

## RESULTS

**Compound identification**

***Sample 1 (BA/OA)***

BA/OA (Figure 1) white powder; mp 290–292 $^{o}$C; IR (KBr) $v_{max}$ 3553, 2990, 1739, 1453, 1374, 1239, 1087 $cm^{-1}$; m/z (ESI) 456 ($M^+$, BA/OA); δH (400 MHz, $CDCl_3$ and $CH_3OD$): 4.96 (s, 1H), 4.44 (1H, s), 4.43 (1H, s), 3.11, 2.73, 1.98, 1.43, 1.29, 0.79-1.24 (21H, m); $^{\delta}$C (100 MHz, $CDCl_3$ and $CH_3OD$): see Table 1.

**Sample 2 (BAA/OAA)**

BAA/OAA (Figure 2) white powder; mp 297-299

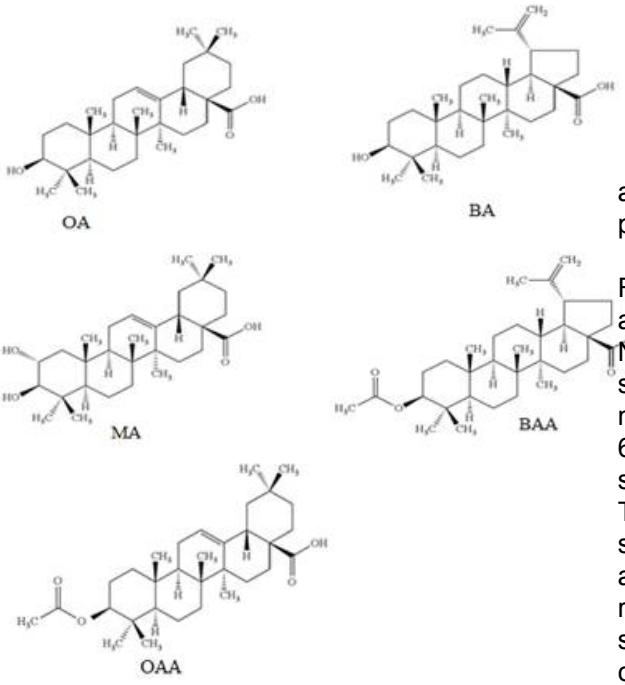


**Figure 1:** Structure of isolated compounds

$^{o}$C; IR (KBr) $\nu_{max}$ 2990, 1739, 1451, 1373, 1235, 1045 cm$^{-1}$; m/z (ESI) 498 ($M^{+}$, BAA) m/z (ESI) 499 ($M^{+}$, OAA); δH (400 MHz, $CDCl_3$): 5.18 (s, 1H), 4.60 (1H, s), 4.47 (1H, s), 4.34 (1H, s), 2.88, 2.11, 1.95, 1.93, 1.81, 1.57, 0.72 – 1.49 (m); $^{\delta}$C (100 MHz, $CDCl_3$): see Table 1.

**Sample 3 (MA/OA)**

The compounds' identification has been reported in our previous research works [12].

The isolation of sample 1 and the formation of sample 2 were confirmed by $^{1}$H, $^{13}$C NMR and IR spectroscopy. The presence of hydroxyl groups in these compounds was indicated by the appearance of an absorption band around 3553 cm$^{-1}$ in the IR Spectra. The C-H stretching frequencies were observed around 2990 cm$^{-1}$, also the bands that are characteristics of the presence of carboxylic acid in a molecule were observed around 1739 cm$^{-1}$ [15]. The IH NMR spectra of samples 1 and 2 both revealed various peak corresponding to the methyl groups at around 0.72-1.49 ppm and terminal methylene protons at 4.34–5.18 ppm.

Further confirmation for the isolation of sample 1 and formation of sample 2 was provided by $^{13}$C NMR spectroscopy. The $^{13}$C NMR spectra of samples 1 and 2 showed that they were mixtures. This is as a result of the appearance of 60 carbons signals in $^{13}$C NMR spectrum of sample 1 (30 each for BA and OA as assigned in Table 1) and 64 carbons signals in $^{13}$C NMR spectrum of sample 2 (32 each for BAA and OAA as assigned in Table 1) which agree with the reported data for related compounds [8,16]. For sample 2 the appearances of four additional carbons in the $^{13}$C spectrum (two each for BAA and OAA) assigned as C-31 and C-32 respectively (Table 1) further confirmed the formation of BAA/OAA. Further evidence for the isolation and formation of BA/OA and BAA/OAA was provided by the ESI-MS spectra which showed intense molecular ions corresponding to $M^{+}$.

**Anti-platelet aggregation**

The compounds significantly ($p < 0.05$) inhibit platelet aggregation induced by ADP in a dose-dependent manner (Figure 3a). BA/OA and BAA/OAA exhibited the highest anti-platelet aggregation activities. BAA/OAA mixture displayed optimal anti-platelet aggregation activity at 5 mg/ml. BA/OA and BAA/OAA showed better $IC_{50}$ value of 3.05 mg/ml and 3.25 mg/ml respectively when compared with aspirin ($IC_{50}$ value of 7.36 mg/ml) the positive control (Table 2). Similarly, the compounds dose-dependent significantly inhibit platelets aggregation in thrombin induced platelet aggregation (Figure 3b). BAA/OAA possessed the highest anti-platelet aggregation activity.

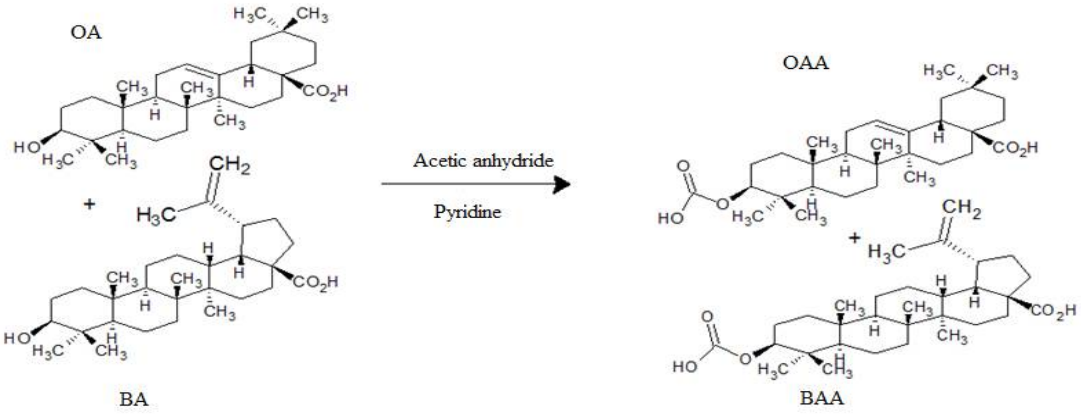


**Figure 2:** Schematic representation of the synthesis of BAA/OAA from BA/OA

**Table 1:** $^{13}$C-NMR (100 MHz) spectral data for sample **1** and **2**

| Position | Sample 1 | Sample 2 |
|---|---|---|
| 1 | 38.5 (BA) 38.7 (OA) ($CH_2$) | 38.1 (BAA) 38.2 (OAA) |
| 2 | 27.3 (BA) 29.2 (OA) ($CH_2$) | 27.7 (BAA) 29.5 (OAA) |
| 3 | 78.2 (BA) 78.2 (OA) (CH) | 81.2 (BAA) 81.2 (OAA) |
| 4 | 38.7 (BA) 40.2 (OA) (C) | 38.2 (BAA) 40.5 (OAA) |
| 5 | 55.0 (BA) 55.9 (OA) (CH) | 55.1 (BAA) 55.2 (OAA) |
| 6 | 17.8 (BA) 18.6 (OA) ($CH_2$) | 18.0 (BAA) 19.0 (OAA) |
| 7 | 33.9 (BA) 32.6 (OA) ($CH_2$) | 34.0 (BAA) 32.1 (OAA) |
| 8 | 40.2 (BA) 39.0 (OA) (C) | 40.5 (BAA) 38.2 (OAA) |
| 9 | 50.1 (BA) 48.9 (OA) (CH) | 50.3 (BAA) 49.9 (OAA) |
| 10 | 36.7 (BA) 37.8 (OA) (C) | 37.0 (BAA) 38.1 (OAA) |
| 11 | 20.5 (BA) 23.7 (OA) ($CH_2$) | 20.7 (BAA) 23.5 (OAA) |
| 12 | 25.1 (BA) 125.10 (OA) ($CH_2$) | 25.3 (BAA) 125.0 (OAA) |
| 13 | 37.9 (BA) 137.8 (OA) (CH) | 37.6 (BAA) 143.0 (OAA) |
| 14 | 42.0 (BA) 42.1 (OA) (C) | 42.2 (BAA) 42.3 (OAA) |
| 15 | 30.2(BA) 27.6(OA) ($CH_2$) | 30.4(BAA) 27.7 (OAA) |
| 16 | 31.8 (BA) 23.7 (OA) ($CH_2$) | 32.1 (BAA) 23.5 (OAA) |
| 17 | 55.8 (BA) 46.7 (OA) (C) | 56.0 (BAA) 46.8 (OAA) |
| 18 | 46.6 (BA) 42.0 (OA) (CH) | 46.8 (BAA) 42.2 (OAA) |
| 19 | 48.8 (BA) 46.7 (OA) (CH) | 49.0 (BAA) 46.8 (OAA) |
| 20 | 150.2 (BA) 31.8 (OA) (C) | 150.5 (BAA) 32.1 (OAA) |
| 21 | 29.2 (BA) 33.9 (OA) ($CH_2$) | 29.5 (BAA) 34.0 (OAA) |
| 22 | 36.5 (BA) 32.6 (OA) ($CH_2$) | 36.9 (BAA) 32.1 (OAA) |
| 23 | 27.5 (BA) 29.2 (OA) ($CH_3$) | 27.7 (BAA) 29.5 (OAA) |
| 24 | 14.8 (BA) 16.4 (OA) ($CH_3$) | 14.4 (BAA) 16.2 (OAA) |
| 25 | 15.1 (BA) 15.6 (OA) ($CH_3$) | 15.7 (BAA) 15.9 (OAA) |
| 26 | 15.3 (BA) 17.8 (OA) ($CH_3$) | 15.6 (BAA) 18.0(OAA) |
| 27 | 14.1 (BA) 26.2 (OA) ($CH_3$) | 14.4 (BAA) 25.3 (OAA) |
| 28 | 178.6 (BA) 180.2 (OA) (C) | 178.9 (BAA) 178.9 (OAA) |
| 29 | 18.6 (BA) 33.9 (OA) ($CH_3$) | 19.0(BAA) 34.0 (OAA) |
| 30 | 108.9 (BA) 23.7 (OA) ($CH_2$) | 109.3 (BAA) 23.5 (OAA) |
| 31 | - | 171.6 (BAA) 171.6 (OAA) (C) |
| 32 | - | 23.5 (BAA) 23.5 (OAA) ($CH_3$) |

Data reported in ppm. BA, OA, BAA and OAA

**Table 2:** $IC_{50}$ values of the compounds on platelet aggregation inhibition

| Compound | $IC_{50}$ (mg/ml) | |
|---|---|---|
| | Thrombin | ADP |
| **BA/OA** | 9.71 | 3.25 |
| **BAA/OAA** | 2.86 | 3.05 |
| **MA/OA** | 9.30 | 5.50 |
| **Aspirin** | 6.45 | 7.36 |

BAA/OAA also showed better $IC_{50}$ value of 2.86 mg/ml than BA/OA and MA/OA mixture with $IC_{50}$ value of 9.71 mg/mL, 9.30 mg/ml respectively. BAA/OAA ($IC_{50}$ value of 2.86 mg/ml) was observed to possess twice the antiplatelet activity of aspirin ($IC_{50}$ of 6.45 mg/ml).

### Cytotoxicity

The result of cytotoxic assay revealed that all the compounds displayed poor cytotoxic effects on HEK293 and HEPG2 cells (Table 3). The compounds also showed better cytotoxic effects on HEPG2 than HEK293 cells, with BAA/OAA showing highest cytotoxic effect.

**Table 3:** $IC_{50}$ (µg/ml) of BA/OA, BAA/OAA and MA/OA acid on HEK293 and HEPG2 cells

| Compound | $IC_{50}$ (µg/ml) | |
|---|---|---|
| | HEK 293 | HEPG2 |
| **BA/OA** | 724.43 | 585.38 |
| **BAA/OAA** | 499.78 | 269.08 |
| **MA/OA** | 499.78 | 407.89 |

## DISCUSSION

Platelet aggregation plays important role in thrombus formation [1]. Therefore, targeting abnormal platelet activation may be a promising approach to reduce cardiovascular mortality. Medicinal plants are currently the source of diverse bioactive compounds which are used for treatment of diseases. The multi-therapeutic properties of plant-derived triterpenes have made them the potential candidates for synthesis of new drugs. The present study revealed that the plant-derived triterpenes and derivatives possess anti-platelet aggregation activities regardless of the agonists. Anti-platelet aggregation activities of some triterpenes have previously been reported [17,18].

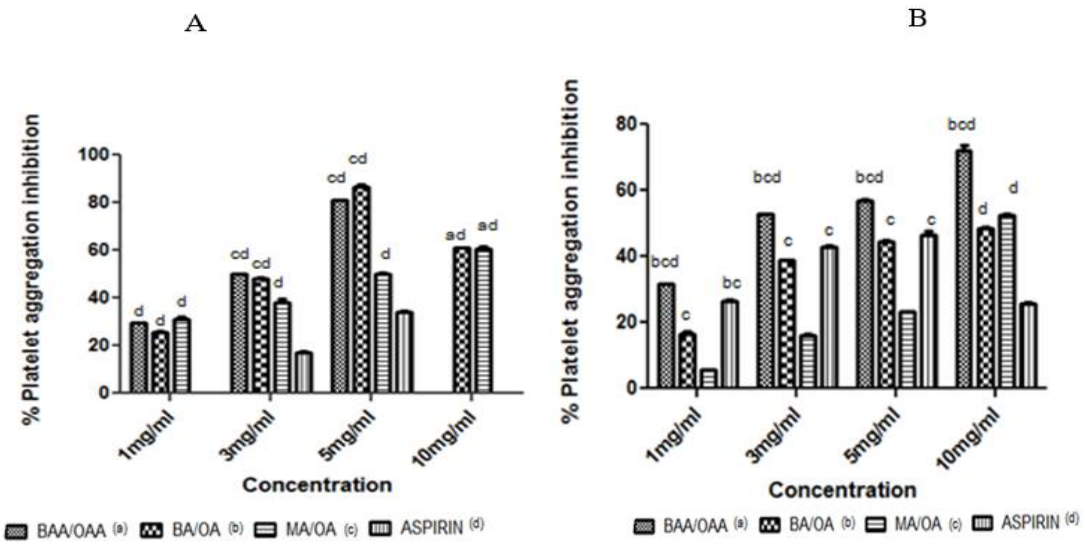


**Figure 3:** Percentage Inhibitory activity of (a) ADP induced by platelet aggregation. (b) Thrombin induced by platelet aggregation. Data expressed as mean ± SD. Values of different letters in graph are significant ($p < 0.05$).

The anti-platelet aggregation activity of BA/OA mixture isolated from the leaves of *Callisteum viminalis* has also been reported, and further separation of BA/OA mixture using open column silica gel chromatography has been unsuccessful [13]. The highest antiplatelet aggregation activity of BAA/OAA mixture could be attributed to the modification of hydroxyl moiety at C3 position with acetyl group. Targeting of carbon positions 3 and 28 of compounds are the new pharmacophores to enhance biological activities [19]. It is worth noting that aspirin, a potent antithrombin agent has acetyl moiety as its major functional group [20]. To the best of our knowledge, this is the first time the BAA/OAA mixture was investigated as antiplatelet aggregation agent.

In the search for potent and clinically safe antiplatelet drugs, there is need to evaluate their toxicities and safety margins [10]. The American National Cancer Institute guidelines consider compounds with $IC_{50}$ < 30 µg/ml as cytotoxic [21]. The present study revealed that the compounds possessed weak cytotoxic properties in Table 3 on both normal cells (HEK293) and cancerous cell (HEPG2). This is contrary to previous reports of some triterpenes with strong cytotoxic effects [22,23]. Despite the weak cytotoxic effect of the compounds, they displayed better cytotoxic effects on HEPG2 than HEK293. This implies that the compounds could inhibit proliferation of cancer cell. Betulinic acid has previously been reported to selectively inhibit tumour cells [24]. Betulinic acid and derivatives isolated from *Melaleuca cajuput* have also been reported to selectively inhibit myeloid leukemia (HL-60) cell line [25].

## CONCLUSION

This study reveals that the test compounds possess antiplatelet potential regardless of the agonists. Structural modifications of the compounds further enhances their activities, but reduced cytotoxic effects on normal cell line. Therefore, these compounds can serve as candidates for safer antiplatelet agents. However, there is need to ascertain the mechanisms of action of the compounds in further studies.

## DECLARATIONS

### *Acknowledgement*

The authors thank the University of Zululand Research Committee for her financial support.

### *Conflict of Interest*

No conflict of interest associated with this work.

### *Contribution of Authors*

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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