academicJournals

7(43), pp. 2881-2886, 29 November, 2013
DOI: 10.5897/AJPP2013.3851
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Inhibitory effect of betulinic acid and 3β-acetoxybetulinic acid on rat platelet aggregation

Habila A. J[1]*, Habila J. D[4], Shode F. O[2], Opoku A. R[3], Atawodi S. E[1] and Umar I. A[1]

[1]Department of Biochemistry, Ahmadu Bello University Zaria-Nigeria.
[2]School of Chemistry, University of Zululand, South Africa.
[3]Department of Biochemistry and Microbiology, University of Zululand, South Africa.
[4]Department of Chemistry, Ahmadu Bello University Zaria-Nigeria.

Accepted 21 November, 2013

Platelet aggregation is one of the major causes of cardiovascular diseases. Our search for bioactive molecules from nature, led to the isolation of betulinic acid (BA) and structural modification of BA to 3β-acetoxybetulinic acid (BAA). Both inhibited blood platelet aggregation induced by thrombin, adenosine diphosphate (ADP) and epinephrine. BAA showed an enhance inhibition of platelet aggregation, in the thrombin-induced platelet aggregation (54.5±0.01 at 1 mg/ml, 63.5±0.17 at 3 mg/ml and 73.5±0.15 at 10 mg/ml; IC_{50} 0.81 mg/ml) which was observed to be significantly ($p<0.05$) similar to that of the standard aspirin (65.4±0.07 at 1 mg/ml, 72.1±0.03 at 3 mg/ml and 76.5±1.22 at 10 mg/ml; IC_{50} 0.33 mg/ml). The results clearly shows that functional group modification of BA to give BAA led to enhanced activity, hence BAA provides a better option as lead in the search for anti-platelet aggregation agents from nature.

Key words: Anti-platelet aggregation, aggregation inducer, Betulinic acid, acetoxybetulinic acid, *Melalueca bracteata*.

INTRODUCTION

Platelets are cells in the blood that help in the formation of clot. They play an important role in the hemostasis and in pathophysiological processess such as thrombosis (Shattil et al., 1998; Stouffer and Smyth, 2003). Intravascular thrombosis is central to the development of a wide variety of cardiovascular diseases (Grenache et al., 2003; Huo and Ley, 2004). Platelet over aggregation is usually the main cause of internal blood clot formation and if not checked, can be fatal leading to artherothrombotic diseases such as strokes and heart attack (Valko et al., 2005). Thrombin, adenosine diphosphate (ADP), epinephrine, arachidonic acid, collagen and other risk factors such as free radicals, inflammation, stress and hypercholesterolemia significantly contribute to platelet dysfunction (Ambrosio et al., 1997; Davi and Patrono, 2007; Bakdash and Williams, 2008; Verhamme and Hoylaaert, 2009). The activation of platelet by thrombin is mediated through two protease activating receptors PAR-1 and PAR-4, in the activation of platelets; these receptors work cooperatively (Fabre and Gurney, 2010). ADP acts through G-protein coupled receptors P2Y1 and P2Y12, they activates phospholipase C and thus resulting in the elevation of intracellular calcium concentration (Davi and Patrono, 2007). Epinephrine is a weak platelet agonist and exerts its effect on human platelets through α_2-adrenergic receptors (A2AR) and potentiates the aggregation potency of other inducers (Choi, 2002).

Antiplatelet drugs help stop blood clot formation and this significantly contributes to the management of pathogennesis of cardiovascular diseases. Among the many drugs used in the management of the condition is aspirin,

*Corresponding author. E-mail: lauro_1999@yahoo.com. Tel: (981) 8119800/73006. Fax: (981) 8119800/73002.

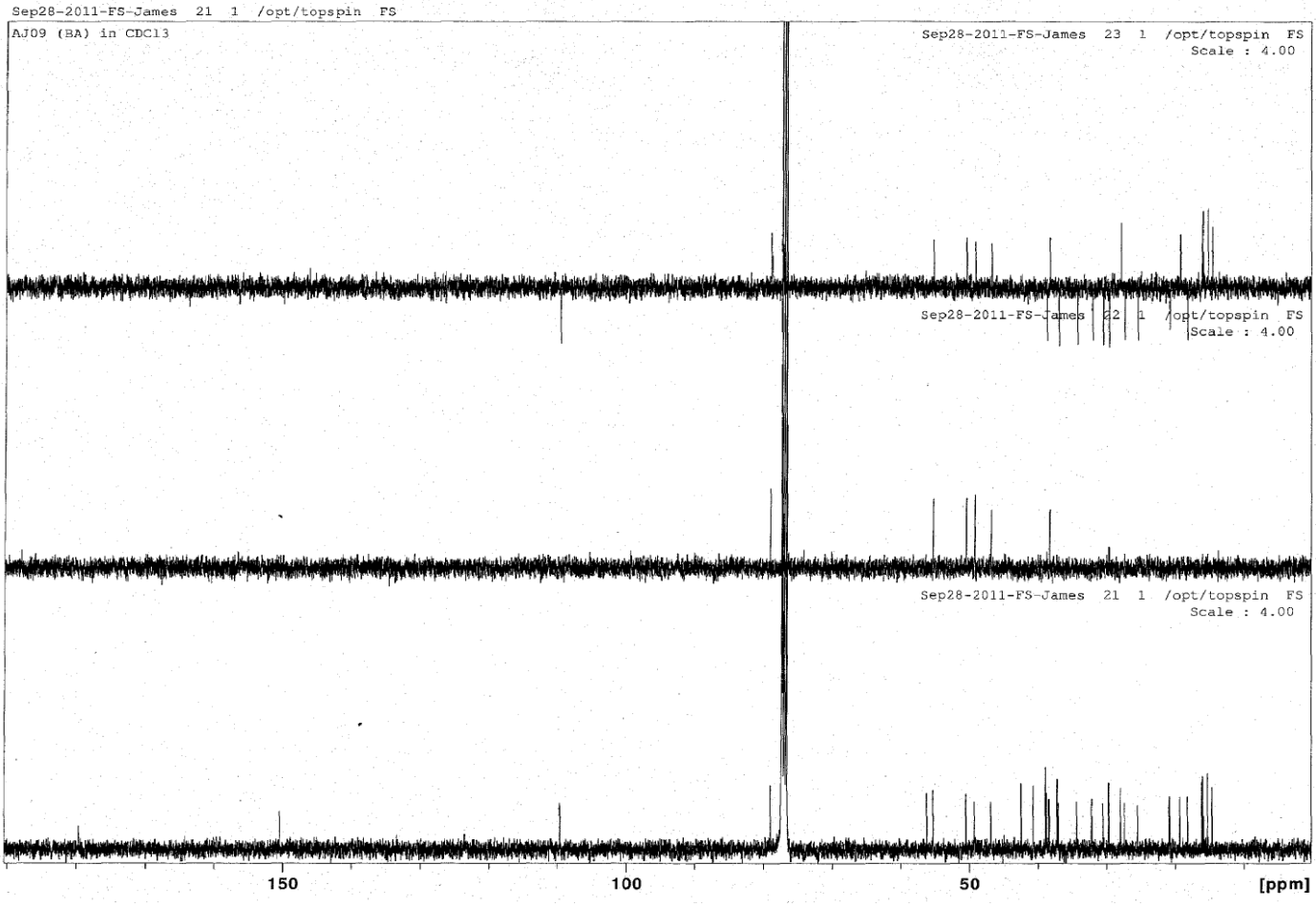

Figure 1. C-NMR, DEPT 90 and DEPT 135 spectrum of BA.

but these drugs are not without side effect, fuelling the search for more effective drugs from natural origin that will help in overcoming the challenges of toxicity and other undesirable side-effects.

The triterpene betulinic acid (BA) is one of the major components of *Malaleuca bracteata*, a plant native to Australia. The essential oil from the leaves of the plant has antiseptic, germicidal and insecticidal properties (Cribb and Cribb, 1981; Yatagai, 1997). The leaves and their volatile oils are locally used in West Africa in the treatment of skin infection (Oliver, 1960; Irvine, 1961; Howes, 1974; Goldstein et al., 1990; Belousova and Denisova 1992). BA possesses diverse biological functions, which includes, but not limited to anticancer (Amico et al., 2006; Huang et al., 2007), HIV-1 maturation inhibition (Fukoja et al., 1994) and antibacterial (Chandramu et al., 2003). The present study reports our findings on blood platelet aggregation (BPA) inhibition of BA and the effect of change in the functionality of BA on BPA.

MATERIALS AND METHODS

Plant

Melaleuca bracteata was supplied by Prof. F. O. Shode of the School of Chemistry, University of KwaZulu-Natal, Durban, South Africa. The plant was identified and a voucher specimen (Glow 001) was prepared and deposited in the Herbarium, School of Biological Sciences, University of KwaZulu-Natal, Durban, South Africa.

Extraction and isolation of BA from *M. bracteata*

The pulverized plant material (2.5 kg) was extracted by cold maceration in dichloromethane (5 L×2) for 48 h, filtered and concentrated under reduced pressure at 40°C using rotary evaporator and allowed to dry under room temperature. The dried residue was defatted with n-hexane to yield a solid mass (9.8 g). The solid mass (5 g) was subjected to column chromatography using ethyl acetate:hexane (8:2) as solvent of elution to give a white amorphous powder which was identified as betulinic acid (BA). The structure was confirmed by nuclear magnetic resonance (NMR) spectra (Figure 1) and comparison with literature values (Mahato and Kundu, 1994).

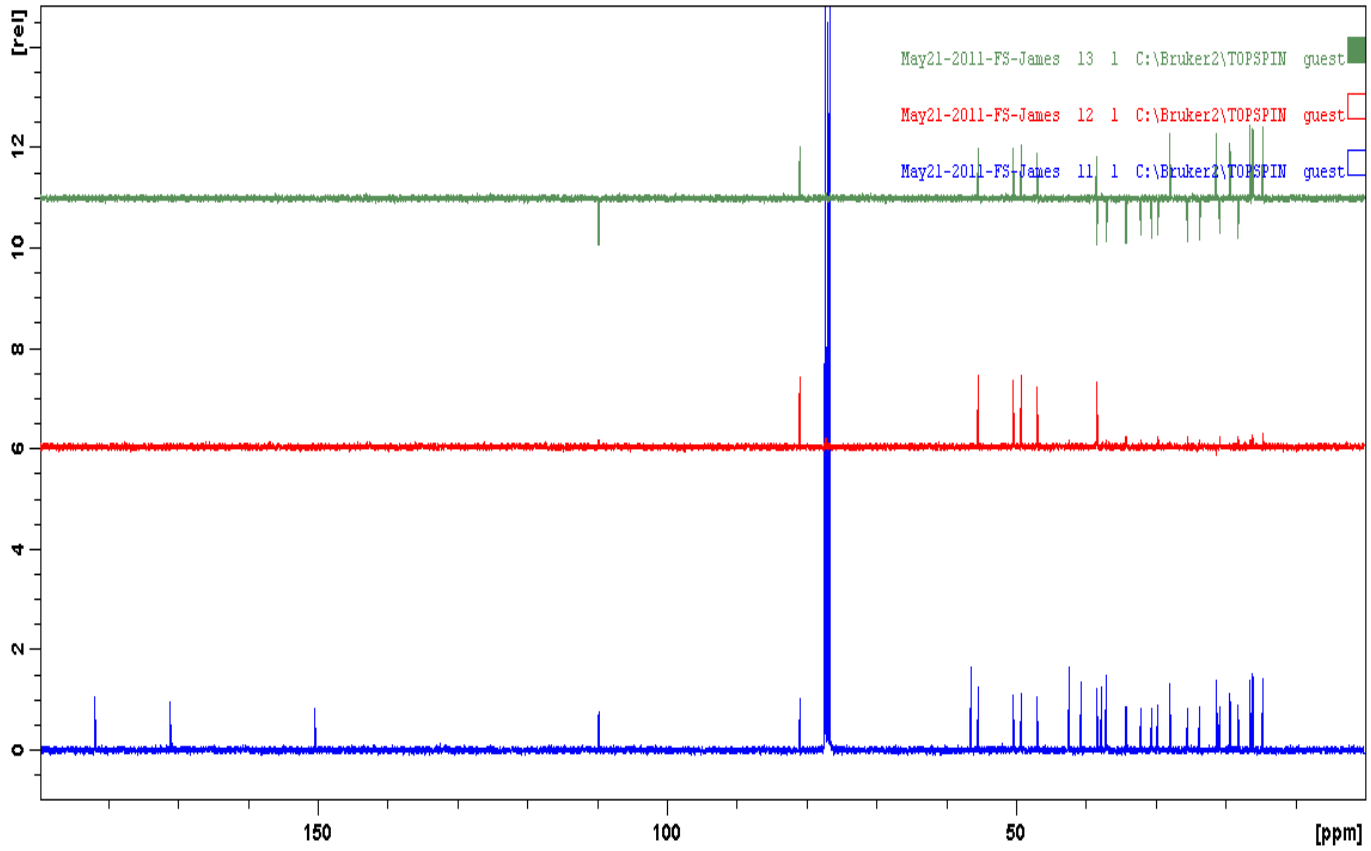

Figure 2. C-NMR, DEPT 90 and DEPT 135 spectrum of BAA.

Preparation of 3β-acetoxy betulinic acid (BAA)

BA (2 g) in a round bottom flask was added acetic anhydride (5 ml) and pyridine (5 ml), the mixture was then refluxed for 2 h. Then stirred at room temperature for 24 h, after which water (10 ml) was added and stirring continued for another 30 min. The mixture was then filtered under suction and washed thoroughly with 10% hydrochloric acid to give a white powder. NMR analysis and comparison with literature (Mahato and Kundu, 1994) confirmed the structure of BAA (Figure 2).

Animals

Ethical clearance for the use of animals in this study was obtained from the research animal ethics committee of the University of Zululand, South Africa. Adult rats (Sprague-Dawley) of either sex were collected from the animal house in the Department of Biochemistry and Microbiology, University of Zululand, South Africa. The animals were maintained under standard conditions and had free access to standard pellet feed and drinking water, for a minimum of 7 days before use.

Preparation of blood platelets

The blood platelet was prepared according to the method described by Tomita et al. (1983). Briefly, blood was surgically collected from the heart immediately after the rat has been rendered unconscious. The blood was transferred into a centrifuge tube and mixed (5:1 v/v) with an anticoagulant, acid-dextrose-anticoagulant (0.085 M trisodium citrate, 0.065 M citric acid, 2% dextrose). The platelets were obtained by series of centrifugation at 1200 rpm for 15 min and at 2200 rpm for 3 min consecutively, the supernatant was collected and discarded and the sediment (platelets) was re-suspended in 5 ml washing buffer (pH 6.5). This was centrifuged again at 300 rpm for 15 min after which the supernatant was discarded and the platelets were finally suspended in a (pH 7.4; containing 0.14 M NaCl, 15 mM Tris-HCl, 5 mM glucose buffer). The platelet was diluted in the re-suspending buffer (1:10), and the resulting solution was mixed with calcium chloride (0.4 ml; 10 µl $CaCl_2$).

Anti-platelet aggregation study

The method of Mekhfi et al. (2004) was adopted with some modifications. Various concentrations (1, 3 and 10 mg/ml) of the test compounds were made, by separately dissolving the compounds in dimethyl sulfoxide (DMSO) and making up the volume with a buffer (pH 7.4) solution. The antiplatelet aggregation activity of the compounds were separately tested on thrombin (5 U/ml), ADP (5 mM) and epinephrine (10 mM) induced platelet aggregation; the platelets (150 µl) were incubated in a 96-well micro-titre plate at 37°C for 5 min, with 50 µl of different concentrations of the compounds (1, 3, and 10 mg/ml) and an aggregation inducer (20 µl)

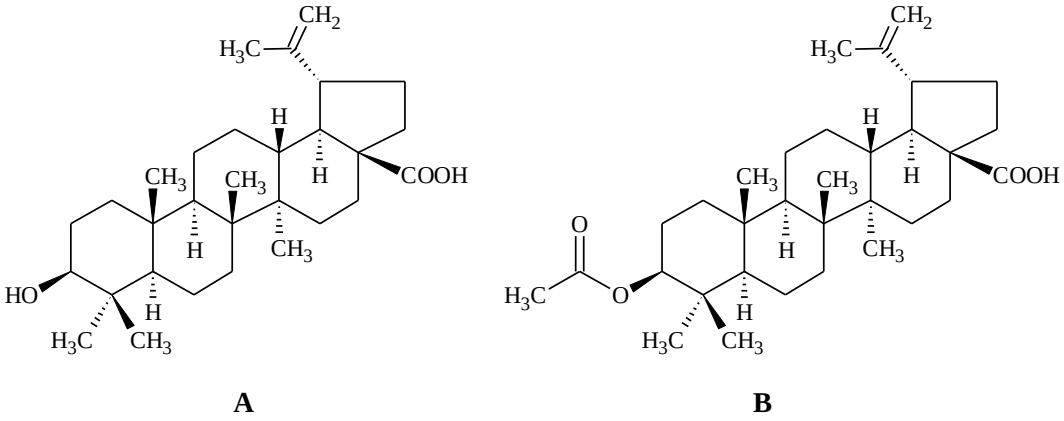

Figure 3. Structure of isolated and modified compounds: (A) Betulinic acid; (B) 3-β acetoxy betulinic acid.

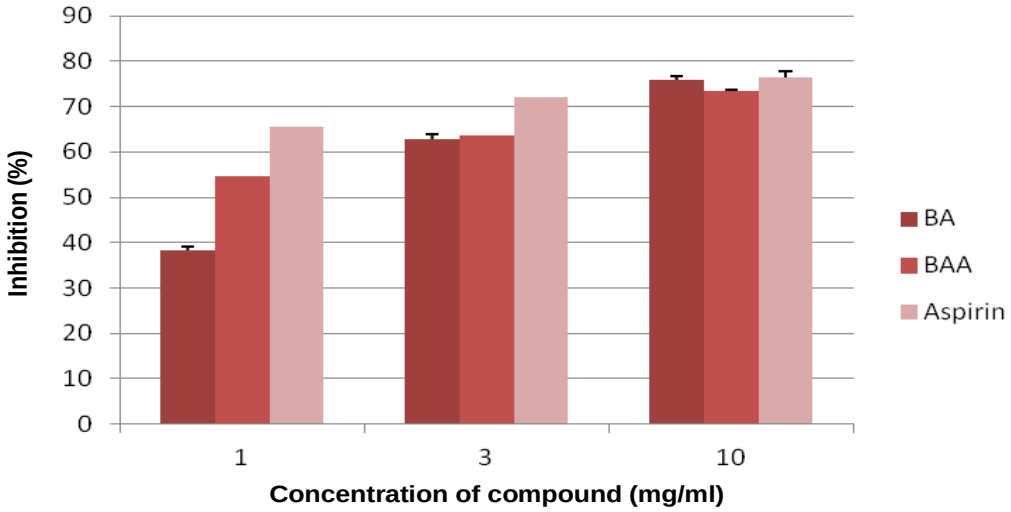

Figure 4. Percentage inhibitory activity of test compounds on thrombin-induced platelet aggregation.

was introduced to the mixtures. Aggregation was determined with the Biotek plate reader using Gen5 software by following change in absorbance at 415 nm for 20 min at 30 s intervals. DMSO (1%) and aggregation inducer were used as negative control; aspirin was used as positive control.

The inhibitory effects of the compounds on each agonist were calculated as:

Inhibition (%) = {(1-(At/Ac) × 100}

The inhibitory concentration providing 50% inhibition (IC_{50}) was determined using statistical package (Origin 6.1), where At is the absorbance of the mixture containing the compounds and Ac is absorbance of the control without the test compounds.

Statistical analysis

All assays were performed in triplicates and the results are expressed as mean ± standard deviation (SD). $P \leq 0.05$ were considered to be statistically significant.

RESULTS

The results of the NMR analysis, ^{13}C, DEPT-90 and DEPT-135 for both BA and BAA are shown in Figures 1 and 2, respectively, were in agreement with those reported by Mahato and Kundu (1994). The results presented in Table 1 showed the inhibitory concentration providing 50% inhibition (IC_{50}) of the test compounds and standard, on platelet aggregation for all the three agonist used (thrombin, ADP and epinephrine). While the results of the percentage inhibition of platelet aggregation of the various concentration of the test compounds on each of the agonist are shown in Figures 3, 4 and 5.

DISCUSSION

Platelet aggregation is one of the major causes of

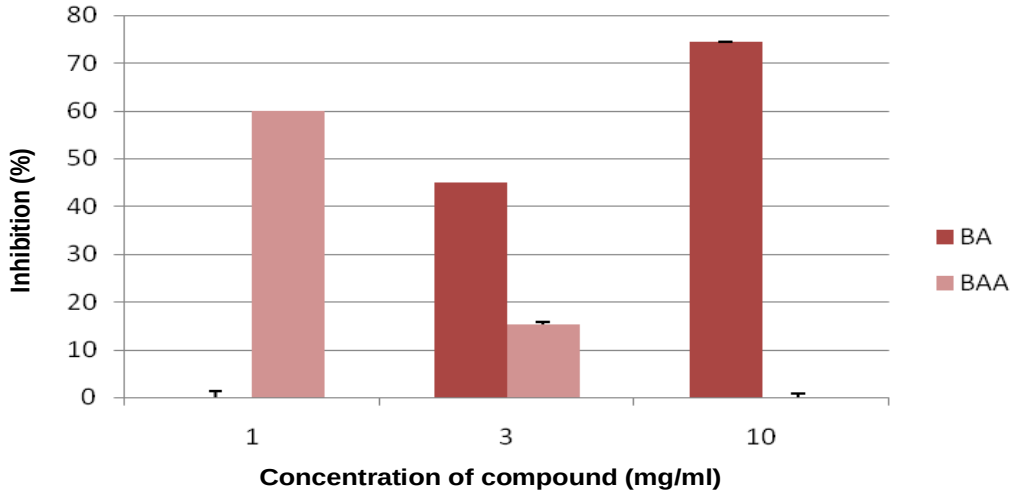

Figure 5. Percentage inhibitory activity of test compounds on ADP-induced platelet aggregation.

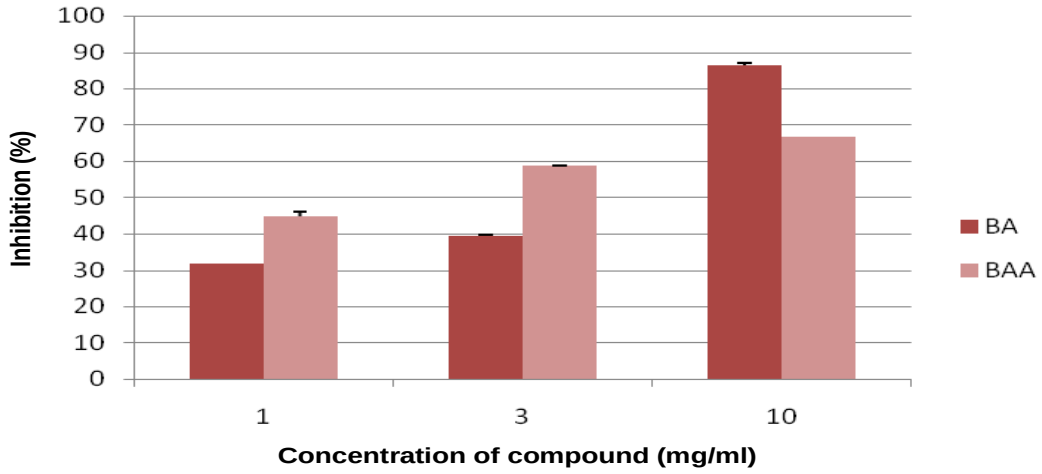

Figure 6. Percentage inhibitory activity of test compounds on epinephrine-induced platelet aggregation.

Table 1. IC_{50} values (mg/ml) of the isolated and modified compound on rat platelet aggregation.

Agonist/Compound	BA	BAA	Aspirin
Thrombin	1.99	0.81	0.33
ADP	4.20	1.39	-
Epinephrine	4.63	1.81	-

artherothrombotic dysfunction, and the commonly used drugs for the treatment of these disorders are becoming undesirable, because of their associated side-effects. This has fuelled the search for new class of effective anti-platelet agents from nature. In this study, the effect of functional group modification of BA to BAA led to enhanced anti-platelet aggregation activity against thrombin, ADP and epinephrine induced rat platelet aggregation.

The spectral data defined BA and BAA as triterpenoids, triterpenes and their various derivatives have been shown to inhibit platelet aggregation induced by thrombin, ADP and epinephrine (Jin et al., 2004; Yang et al., 2009; Sankaranarayanan et al., 2010; Habila et al., 2011; Mosa et al., 2011). The results of our investigation showed that anti-platelet aggregation inhibitions of BA and BAA against thrombin, ADP and epinephrine-induced platelet aggregation (Figures 3 to 5) showed a dose dependent increase in percentage inhibition, with increase in concentration. BA and BAA were more potent in the thrombin-induced platelet aggregation with IC_{50} of 1.99 and 0.81 mg/ml, respectively, which were comparable to that of aspirin (0.33 mg/ml). BAA showed more enhance

activity in all the three agonist used (IC_{50}: thrombin, 0.81 mg/ml; ADP, 1.39 mg/ml; epinephrine, 1.81 mg/ml), as compared to BA (IC_{50}: thrombin, 1.99 mg/ml; ADP, 4.20 mg/ml; epinephrine, 4.63 mg/ml) the parent nucleus. The observed activity was attributed to the modification of the 3β-hydroxyl (-OH) of BA to 3β-acetyl ($OCOCH_3$) functional group. The overall result of the anti-platelet aggregation studies reveals that both compounds, BA and BAA inhibit platelet aggregation at a significant difference ($p<0.05$) between the different concentrations used in all the three agonist. Our findings are in agreement with Tzakos et al. (2012) who reported the potency of betulinic acid to inhibit human platelet aggregation. The results of this study showed that the isolated (BA) and modified (BAA) compounds are good candidates in the search for anti-platelet aggregation agents from nature.

Conclusion

The results showed that structural modification of the isolated compound (BA) to mimic the functionality of aspirin, led to enhanced platelet aggregation inhibition, induced by thrombin, ADP and epinephrine. The findings suggest that BA and BAA may be considered as lead candidates, in the search for anti-platelet aggregation agents from nature that may offer a better alternative to aspirin.

REFERENCES

Ambrosio G, Tritto I, Golino P (1997). Reactive Oxygen Metabolites and arterial thrombosis (Review). Cardiovasc. Res. 34:445-452.

Amico V, Barresi V, Condorelli D, Spatafora C, Tringali C (2006). Antiproliferative terpenoids from almond hulls (*Prunus dulcis*): Identification and structure-activity relationships. J. Agric. Food Chem. 54:810-814.

Bakdash N, Williams MS (2008). Spatially distinct production of reactive oxygen species regulates platelet activation. Free Rad. Biol. Med. 45(2):158-166

Belousova LS, Denisova LV (1992). Rare Plants of the World. A.A. Balkema Publishers, U.S.A. P 254

Chandramu C, Manohar RD, Krupadanam DG, Dashavantha RV (2003). Isolation, characterization and biological activity of betulinic acid and ursolic acid from *Vitex negundo.* Phytother. Res. 17:129.

Choi JW (2002). Incidence of nonresponsiveness to epinephrine in platelets from healthy humans. Acta Haematologica. 108(2):106-108.

Cribb AB, Cribb JW (1981). Useful Wild Plants in Australia. William Collins Pty Ltd. Sidney. ISBN 000-216441

Davi G, Patrono C (2007). Platelet activation and atherothrombosis. New Eng. J. Med. 13;357(24):2482-2494

Fabre J, Gurney ME (2010). Limitations of current therapies to prevent thrombosis: A need for novel strategies. Mol. BioSys. 6(2):305-315.

Fukoja T, Kashiwada Y, Kilkushi RE, Consentino LM, Ballas LM, Jiang JB, Janzen WP, Chen IS, Lee KH (1994). Betulinic acid and platonic acid as anti-HIV principles from *Syzigium clarifbrum* and the anti-HIV activity of structurally related triterpenoids. J. Nat. Prod. 57:243.

Goldstein M, Simonetti G, Watschruger M (1990). Complete Guide to Trees and their Identification. Macdonald and Co. Ltd., London. pp.219-221, 231.

Grenache DG, Coleman T, Semenkovich CF, Santoro SA, Zutter MM (2003). Alpha2beta1 integrin and development of atherosclerosis in a mouse model: assessment of risk. Arterioscler. Thromb. Vasc. Biol. 23:2104-2109.

Habila JD, Shode FO, Opoku AR (2011). Triterpenoids from *Eucalyptus grandis* Hill ex Maiden inhibits platelet aggregation. Afr. J. Microbiol. Res. 5(26):4646-4651.

Howes FN (1974). A Dictionary of Useful and Every Day Plants and their Common Names. Cambridge University Press, London, p.161

Huang L, Ho P, Chen CH (2007). Activation and Inhibition of the proteasome by betulinic acid and its derivatives. FEBS Lett. 581:4955-4959.

Huo Y, Ley KF (2004). Role of platelets in the development of atherosclerosis. Trends Cardiovasc. Med. 14:18-22.

Irvine FR (1961). Woody Plants of Ghana. Oxford University Press, London. P 765.

Jin JL, Lee YY, Heo JE, Lee S, Kim JM, Yun-Choi HS (2004). Antiplatelet pentacyclic triterpenoids from leaves of *Campsis grandiflora*. *A*rch. Pharm. Res. 27(4):376-380.

Mahato SB, Kundu AP (1994). ^{13C} NMR Spectra of pentacyclic triterpentenoids-A compilation and Salient Features. Phytochemistry 37(6):1517-1575.

Mekhfi H, Haouari ME, Legssyer A, Bnouham M, Aziz M, Atmani F, Remmal A, Ziyyat A (2004). Platelet anti-aggregant property of some Moroccan medicinal plants. J. Ethnopharmacol. 94:317-322.

Mosa RA, Oyedeji OA, Shode FO, Singh M, Opoku AR (2011). Triterpenes from the stem bark of *Protorhus longifolia* exhibit anti-platelet aggregation activity. Afr. J. Pharm. Pharmacol. 5(24):2698-2714.

Oliver B (1960). Medicinal Plants in Nigeria. Nigeria College of Arts, Science and Technology, Ibadan. p.71

Sankaranarayanan S, Bama P, Ramachandran J, Jayasimman R, Kalaichelvan PT, Deccaraman M, Vijayalakshimi M, Visveswaran M, Chitibabu CV (2010). *In vitro* platelet aggregation inhibitory effect of triterpenoid compound from the leaf of *Elephantopus scaber* Linn. Int. J. Pharm. Pharm. Sci. 2(2):49-51.

Shattil SJ, Kashiwagi H, Pampori N (1998). Integrin signaling: the platelet paradigm. Blood. 91:2645-2657.

Stouffer GA, Smyth SS (2003). Effects of thrombin on interactions between beta3-integrins and extracellular matrix in platelets and vascular cells. Arterioscler. Thromb. Vasc. Biol. 23:1971-1978.

Tomita T, Umegaki K, Hayashi E (1983). Basic aggregation properties of washed rat platelets: Correlation between aggregation, phospholipid degradation, malondialdehyde, and thromboxane formation. J. Pharm. Methods. 10(1):31-44

Tzakos AG, Kontoqianni VG, Tsoymani M, Kyriakou E, Hwa J, Rodriques FA, Tselepis AD (2012). Exploration of the antiplatelet activity profile of betulinic acid on human platelets. J. Agric. Food Chem. 60(28):6977-6983.

Valko M, Morris H, Cronin MTD (2005). Metals, Toxicity and Oxidative Stress. Curr. Med. Chem. 12:1161-1208.

Verhamme P, Hoylaerts MF (2009). Haemostasis and inflammation: two of kind. Thromb. J. 7(15):764-755.

Yang C, An Q, Xiong Z, Song Y, Yu K, Li F (2009). Triterpenes from *Acanthopanax sessiliflorus* fruits and their antiplatelet aggregation activities. Planta Med. 75(6):656-659.

Yatagai M (1997). Miticidal activities of triterpenes. Curr. Topics Phytochem. 1:85-97.