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Platelet-Aggregation Inhibitory Activity of Oleanolic Acid, Ursolic Acid, Betulinic Acid, and Maslinic Acid

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Platelet aggregation is the process by which platelets adhere to each other at sites of vascular injury. This process has long been recognized as critical for hemostatic plug formation and thrombosis. Until relatively recently, platelet aggregation was considered a straightforward process involving the non-covalent bridging of integrin αIIbβ3 receptors on the platelet surface by the dimeric adhesive protein fibrinogen. Naturally occurring triterpenes are endowed with a broad range of useful pharmacological properties. In our search for new and potent ethnopharmaceuticals, oleanolic acid **(OA) (1)**, ursolic acid **(UA) (2)** betulinic acid **(BA) (3)**, and maslinic acid **(MA) (4)** isolated from Syzygium aromaticum, Eucalyptus grandis, Callistemon viminalis, and Syzygium aromaticum, respectively, were evaluated in vitro for anti-platelet aggregation activity on thrombin, adenosine diphosphate (ADP), and epinephrine-induced rat platelet aggregation. The triterpenes exhibited a dose dependent inhibitory activity on platelet aggregation induced by the three platelet agonists. The compounds exhibited more potency mostly at the highest concentration (10.0 mg/ml) except for **UA (2)** on thrombin-induced platelet aggregation. The percentage inhibitory activity of **UA (2)** on throbine-induced platelet aggregation was found to decrease with increase in concentration (86.8±1.23, 53.0±0.43, 46.2±0.23) 1-10.0 mg/ml, this invariably suggests an optimal concentration (≤ 1.0 mg/ml). The IC50s of the compounds are remarkably better than that of heparin (IC50 of 2.80mg/ml). The highest activity by **OA (1)** (IC50 of 0.84 mg/ml) and mixture of **BA/OA** (IC50 of 2.61 mg/ml) was observed on thrombin-induced platelet aggregation. **BA/OA** (IC50 of 2.57 mg/ml) also showed a significant platelet aggregation inhibitory activity on epinephrine-induced platelet aggregation. These preliminary findings show that these pentacyclic triterpenoic acids possess pharmacological activity and could be potential templates for development of new anti-platelet agents

Keyword: Pentacyclic Triterpenes, Anti-Platelet Aggregation, Atherothrombotic Disorders, Ethnopharmaceuticals, Drug Discovery

1. Introduction

Platelets play an important role in hemostasis during tissue injury. They interact with activated plasma clotting factors at the site of blood vessel injury, forming a mechanical plug which blocks the defect and terminates blood loss (Harker & Mann, 1992). Platelet aggregation is induced by the action of endogenous agonists such as arachidonic acid (AA), adenosine diphosphate (ADP), platelet activating factor (PAF), thrombin,

and collagen (Arita et al., 1989). The propensity of platelets to clump together at sites of vascular injury was first recognized more than 100 years ago (Bizzozero, 1882). This phenomenon was described as platelet cohesion although more commonly referred to as platelet aggregation, and it was later identified as important for hemostatic plug formation. It was also well understood at the time that abnormal platelets aggregation played a key role in the development of thrombosis, but, it was not until almost a century later that it became widely accepted that platelets hyperactivity play a pivotal role in development of cardiovascular diseases. As a result, inhibitors of platelet aggregation have become increasingly important parts of the armamentarium for the prevention and treatment of many atherothrombotic disorders (Jackson, 2007).

Aspirin has been the drug of choice for long-term treatment of platelet hyperactivity, especially to reduce the risk of serious ischemic events in several cardiovascular disease states including stroke, myocardial infarction, unstable angina and following coronary artery bypass surgery. Aspirin usage is associated with resistance and serious side effects such as gastric hemorrhage (Lloyd &Bochner, 1996).

The search for new generation of effective and safer non-aspirin platelet inhibitors from natural sources has been emphasized (Amrani et al., 2009). It is widely accepted that natural products are proven template for the development of new scaffolds of drugs (Cragg et al., 1997). In recent years, alkaloids of diverse structures, phenolic compounds, prenylflavonoids and a diterpene isolated from different plant sources, have shown potent anti-platelet activity (Jantan et., 2006, Nurtjahja-Tjendraputra et al., 2003, Lin et al., 1993, Shen et al., 2000). Recently a number of naturally occurring triterpenes and their derivatives have been reported to possess remarkable anti-platelet aggregation (Mosa et al., 2011; Habila et al., 2011). This paper reports on the platelet aggregation inhibition of **OA** (**1**), **UA** (**2**), mixture of **OA** (**1**) and **BA** (**3**), as well as **MA** (**4**).

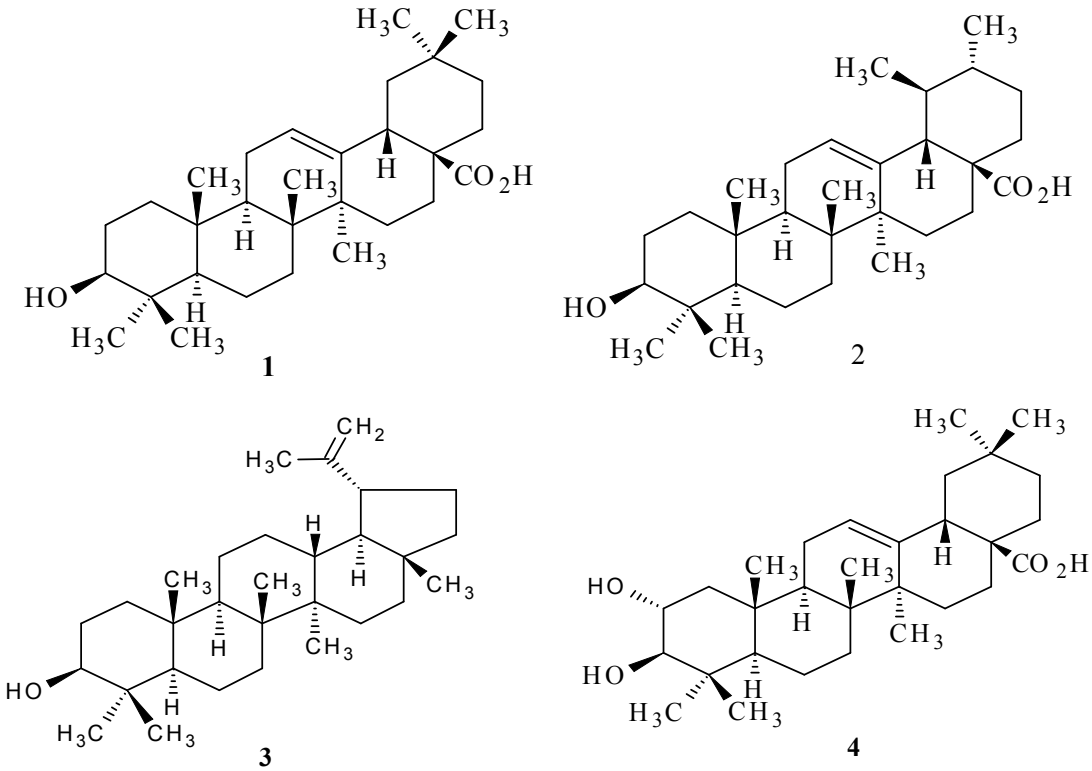

Fig 1: Structure of **OA** (**1**), **UA** (**2**), **BA** (**3**) and **MA** (**4**).

2. Material and Methods

2.1 Plants

The plants used in this study were collected from different areas within KwaZulu-Natal province, Republic of South Africa. The buds of *S. aromaticum* were purchased from the spice market in Durban and were authenticated by Mr. Pravin Poorun a senior plant taxonomist, of the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Westville Campus. A voucher specimen (voucher number OO4) was deposited at the University Herbarium.

Leaves of *Eucalyptus grandis* were collected from Mondi plantation in Empangeni, KwaZulu-Nata Province and authenticated by Mrs NR Ntuli of Botany Department, University of Zululand, a collector's number (Shode/01) was assigned and the leaf sample was kept at the University of Zululand, Herbarium.

Fresh leaf samples of *Callistemon viminalis* were collected in July 2010 from University of Zululand, KwaDlangezwa campus, Empangeni (KwaZulu-Natal Province) in South Africa. The fresh plant materials were authenticated by Dr. S.J. Siebert, a plant taxonomist, of the Department of Botany, University of Zululand, where voucher specimens already existed [OOO 13 (ZULU)].

2.2 Extraction and Isolation of OA (1) and MA (4) from *S. aromaticum*

The buds (500g) were milled using an industrial blender. The milled plant sample was extracted on a Labcom shaker Dichloromethane (2 L) overnight and the solvent removed under reduced pressure to yield 18.4 g (3.8%) of plant extract. The extract was treated with hexane to yield crude mixture of **OA (1)** and **BA (3)**. 5.0 g of the crude extract was subjected to normal phase column chromatography using gradient solvent system of hexane-ethyl acetate (5% stepwise increase). Fractions were collected, like fractions were pooled together, further purification led to isolation of a white amorphous compound coded FS02, and a colourless crystalline substance FS04. These compounds were subjected to NMR spectroscopic analysis for structural elucidation and confirmed to be **OA (1)** and **MA (4)**. The spectra data are in concordant with literature value (Werner et al., 2003).

2.3 Extraction and Isolation UA (2) from *Eucalyptus grandis*

The fresh leaves were dried under shade, pulverized into coarse form (600 g) for extraction using a protocol that is still being developed in our lab. The method selectively extracts various classes of acids including pentacyclic triterpenoic acids**.** The crude extract was chromatographed using gradient elution of hexane–ethyl acetate (5% stepwise increase). A total of 220 fractions (20mL, each) were collected and monitored by TLC. Like fractions were pooled together on the basis of TLC analysis to give a total of 11 fractions (A-K). Fractions 93-105 coded (I) gave light yellowish-green powder which was confirmed spectroscopically using ^{1}H, ^{13}C, DEPT, HSQC and FT IR to be **UA** (**2**).

2.4 Extraction and Isolation of BA/OA Mixture

Fresh matured leaves (500 g) of *Callistemon viminalis* were air-dried and pulverized into coarse form. It was extracted by cold maceration in ethyl acetate (2L x 3) for 60h, filtered and concentrated under reduced pressure at 40 $^{\circ}C$ using rotary evaporator and allowed to dry under air. The concentrated extract was then deffated with n-haxane to afford (21.6 g) of creamy solid material. A portion of the solid material (12.0 g) was subjected to column chromatography using gradient solvent system of hexane-ethyl acetate (8:2) to give a white solid suspected to be mixture of **BA (3)** and **OA (1)**. The structures were confirmed by NMR spectra. Repeated attempts to separate the solid mixture were unsuccessful.

2.5 Biological Studies

Animals

Ethical clearance for the use of animals in this study was obtained from the institutional (University of Zululand) research animal ethics committee. Adult rats (*Sprague-Dawley*) of either sex were collected from the Department of

Biochemistry and Microbiology, University of Zululand. The animals were housed under standard conditions and had free access to standard pellet feed and drinking water *ad libitum*.

2.6 In vitro anti-platelet aggregation study

2.6.1 Preparation of Blood Platelets

The blood platelets were prepared according to the method described by Tomita et al., (1993).The rat was sacrificed by a blow to the head and blood was immediately collected by cardiac puncture. The blood was mixed (5:1 v/v) with an anticoagulant (acid-dextrose-anticoagulant, 0.085 M trisodium citrate, 0.065 citric acid, 2% dextrose).

The platelets were obtained by a series of centrifugation at 1200rpm for 15min and at 2200 rpm for 3min consecutively. The supernatant was collected and centrifuged at 3200rpm for 15 min. The resulting supernatant was discarded and the sediment (platelets) was re-suspended in 5ml washing buffer (pH 6.5). This was centrifuged again at 3000 rpm for 15 min after which the supernatant was discarded and the platelets were finally suspended in a small volume of re-suspending buffer (pH 7.4; containing 0.14 M NaCl, 15 mM Tris–HCl, 5 mM glucose). The platelets were further diluted with the re-suspending buffer (1:10) and the resulting solution was mixed with calcium chloride (0.4 ml: 10 µl $CaCl_2$).

Table1. Percentage (%) Inhibitory activity of the compounds on thrombin- induced platelet aggregation.

Compound	Concentrations			IC_{50} (mg/ml)
	1.0 mg/ml	3.0 mg/ml	10.0 mg/ml	
OA (1)	60.2 ± 0.42	60.2 ± 0.42	88.8 ± 0.42	0.84
UA (2)	86.8 ± 1.23	53.0 ± 0.43	46.2 ± 0.23	2.82
BA/OA	21.0 ± 0.85	56.6 ± 0.39	81.3 ± 0.73	2.61
MA (4)	37.2 ± 0.90	53.0 ± 1.23	69.9 ± 0.35	2.81
Heparin	11.9±0.24	22.1±0.09	66.3±1.01	2.80

Table 2. Percentage (%) Inhibitory activity of OA (1), UA (2), BA/OA, and MA (4) on ADP-induced platelet aggregation.

Compound	Concentrations			IC_{50} (mg/ml)
	1.0 mg/ml	3.0 mg/ml	10.0 mg/ml	
OA (1)	21.1±0.26	27.5±0.31	80.8±0.79	5.98
UA (2)	0.00±0.61	60.1±4.38	76.7±0.76	3.00
BA/OA	0.00±0.85	0.00±0.91	49.0±0.96	ND
MA (4)	0.00± 0.90	0,00±1.23	8.80±0.53	ND

ND- Not determined

Table 3. Percentage (%) inhibitory activity of OA (1), UA (2), BA/OA, and MA (4) on epinephrine-induced platelet aggregation.

Compound	Concentrations			IC_{50} (mg/ml)
	1.0 mg/ml	3.0 mg/ml	10.0 mg/ml	
OA (1)	0.00±0.26	0.00±0.71	47.8.±0.42	ND
UA (2)	0.8±0.68	0.0±0.16	17.4±0.56	ND
BA/OA	27.0 ±0.58	57.9±0.29	67.7±0.50	2.57
MA (4)	0.00± 0.90	35.9±1.32	87.0±1.53	4.99

ND- Not determined

2.6.2 Anti-Platelet Aggregation Evaluation

Anti-platelet aggregation activity was determined by the method described by Mekhfi et al., (2004) with some modifications. The compounds were separately solubilized in dimethyl sulfoxides (DMSO) before making up the volume with 50MmTris–HCl buffer (pH 7.4; containing 7.5 mM ethylenediaminetetra-acetic acid (EDTA) and 175mM NaCl) to a final 1% DMSO concentration. Different concentrations (1, 3 and 10 mg/ml) were used in the assay. The platelet aggregation inhibitory activity of the triterpenes was separately evaluated on thrombin (5 U/ml), ADP (5 mM), and epinephrine (10 mM) induced aggregation. The platelets (100 µl) were pre-incubated for 5 min with different concentrations of the compounds before introduction of platelets agonist (20 µl) to the mixture.

Aggregation was determined with the Biotek plate reader (ELx 808 UI, Biotek Instrument Supplies) using Gen5 software by following change in absorbance at 415 nm. DMSO (1%) was used as negative control and heparin was used as positive control.

Statistical analysis

All assays were done in triplicate and the mean slope (A) ± standard error of mean (SEM) reported. The inhibitory effect of the compounds on each parameter was calculated as: Inhibition (%) = $[(A_o - A_1)/A_o \times 100]$. Where A_o is the mean slope of the control and A_1 is the mean slope of the test compound. IC_{50} values were determined using statistical package origin 6.1

3. Results and Discussion

Aberrant platelet aggregation is central to the development of atherothrombotic disorders. As a result, inhibitors of platelet aggregation have become increasingly important in the prevention and treatment of many atherothrombotic disorders (Jackson, 2007). The dissatisfaction on the efficacy and/or potential undesirable side-effects of the current anti-platelet agents has fuelled the search for new generation of effective agents from natural sources.

A number of naturally occurring triterpenes and their derivatives have been reported to possess remarkable anti-platelet aggregation activity (Mosa et al., 2011; Habila et al., 2011). In this study, the anti-platelet aggregation of four pentacyclic triterpenoic acids namely (**OA**) (**1**), (**UA**) (**2**), (**BA**) (**3**)/**OA** (**1**), **MA** (**4**) was evaluated against thrombin, ADP and epinephrine induced rat platelet aggregation. The triterpenes exhibited a dose dependent inhibitory activity on platelet aggregation induced by the three platelet agonists (Tables 1, 2 and 3). The compounds exhibited more potent activity, mostly at the highest concentration (10.0 mg/ml). The highest activity by **OA (1)** (IC_{50} of 0.84mg/ml) and **BA**/**OA** (IC_{50} of 2.61mg/ml) was observed on thrombin induced platelet aggregation. The IC_{50s} of **OA** (**1**) and **BA**/**OA** mixture were remarkably better than that of heparin (IC_{50} of 2.80mg/ml) (Table 1). A high anti-platelet activity by **BA**/**OA** (IC_{50} of 2.57mg/ml) was also observed on epinephrine induced platelet aggregation (Table 3). The inhibitor activity of **UA** (**2**) on thrombin-induced platelet aggregation was optimal at 1.0 mg/ml, and insignificantly low on epinephrine-induced platelet aggregation. This result is concordant with what was reported in literature (Habila et al., 2011).

The anti-platelet aggregation activity of **UA** (**2**) and **OA** (**1**) from the leaves of *Acanthopanax senticosus* and the fruits of *A. sessiliflorus* has been reported (Jin et al., 2004). Furthermore, the platelet aggregation inhibitory activity of **BA** (**3**) has also been recently explored (Tzakos et al., 2012). Therefore, the potent activity of **BA**/**OA** could be attributed to the synergistic activity of both **BA (3)** and **OA (1)**.

The results obtained from this study revealed that **OA** (**1**), **UA (2)**, **BA**/**OA**, as well as **MA** (**4**) have anti-platelet aggregation properties. The results suggest that these triterpenes as potential templates for development of new anti-platelet agents.

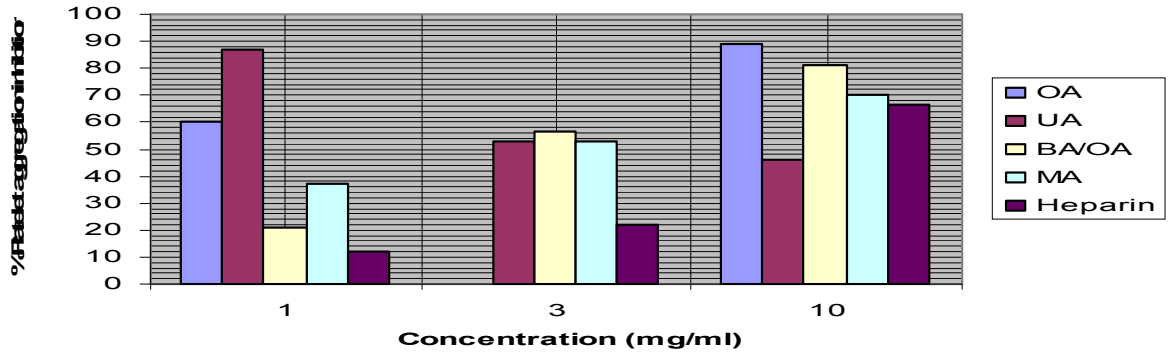

Fig 5. Graphical presentation of Inhibitory activity of the compounds on thrombin-induced rat platelet aggregation

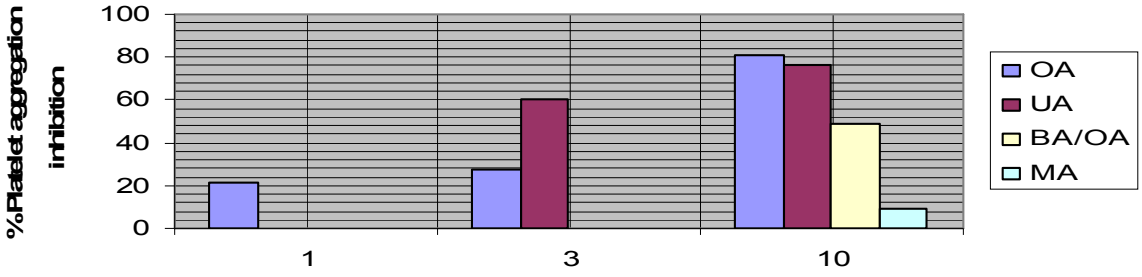

Fig.6 Graphical presenartion of inhibitory activity of the compounds on ADP-induced platelet aggregation

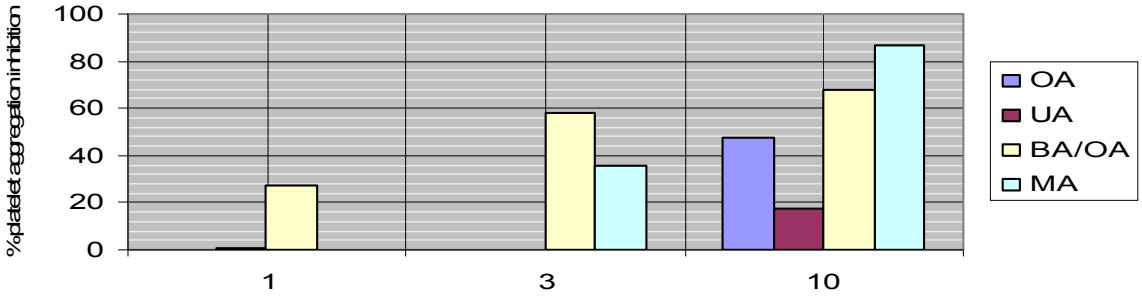

Fig. 7 Graphical presentation of inhibitory activity of the compounds onepinephrine-induced platelet aggregation

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