Original Research Article

In-vitro dose response effect of bergenin on platelet aggregation by inhibiting inducers (collagen and ADP) and lipoxygenase

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ABSTRACT

In this study, we investigated the anti-platelet activity of bergenin on platelet aggregation induced by collagen and ADP on blood sample of healthy volunteers (18–40 years). Blood sample was centrifuge and Platelet Rich Plasma (PRP) was carefully collected in tube. Lipoxygenase inhibition activity of bergenin was conducted by spectrophotometric method. In the absence of bergenin about 84 % platelet aggregations showed in PRP, while in its presences 100 % inhibition of platelet aggregation at 6.25 mM induced by collagen. While with the same concentration of bergenin, inhibited platelet aggregation was 91.88 % ± 5.14 induce by ADP. The IC_{50} values of antiplatelet effect of bergenin induced by collagen and ADP were 3.07mM ± 0.22 and 3.32mM ± 0.10, respectively. The study indicates that bergenin has potent antiplatelet activity to collagen and ADP induced aggregation.

***Keywords*:** Platelets, inhibition, aggregation, thrombosis

INTRODUCTION

Platelets are known to play a significant role in thrombosis, haemostasis, wound healing, inflammation, atherosclerosis and immunity [1-3]. In response to injury, the main function of platelets is preventing blood loss, but they are also responsible for dysregulated

thrombus formation causes acute coronary syndrome, myocardial infraction and ischemia [4]. The process of platelets activation is mediated by multiple agonist (Arachidonic Acid (AA), Adenosine Diphosphate (ADP), Collagen, Platelet Activating Factor, Thromboxane A_2 (TXA_2) and Thrombin) [5]. In several cardiovascular disease states, for reduces of serious ischemic events, aspirin has been used for platelet hyperactivity due to elevated TXA_2 synthesis [6]. However, during long term follow-up, 10 – 20 % patients using aspirin as a secondary prophylaxis have a recurrent thrombotic event. This inability of aspirin is due to aspirin resistance [7-8]. The use of currently available antiplatelet drugs including acetylsalicylic, P_2Y_{12} antagonist, phosphodiesterase inhibitors and antagonist of major platelets integrin $\alpha IIb\beta_3$ are associated with complications, and have limitation in their mode of action [9-10]. While those drugs which are extracted from the natural compounds having minimum side effects [11]. Thus there is need to further improved the efficacy of these drugs and investigate the safer, more potent novel non aspirin antiplatelet inhibitors. So now a days, many compounds from the natural sources in traditional medicine are special focus to evaluate antiplatelet activity [6].

Lipoxygenases (LOs) are non-heme iron dioxygenases that catalyze the polyunsaturated fatty acid (linoleic acid or arachidonic acid) by oxidation, yielding cis, trans-conjugated diene hydroperoxides [12]. In the mid 1970s the transformation of AA to 12(S)-hydroxy-5, 8, 10, 14-eicosatetraenoic acid (12(S)-HETE) was first evaluated in human and bovine platelets [13]. Specific cells and tissues show significant species of Lipoxygenases and their metabolites. According to their cell type three isoforms of 12(S)-Lipoxygenases (12-LOX) have been evaluated; epidermis, leucocytes and platelets [14]. Oxidation of AA either by COX-1 or 12-LOX produces bioactive metabolites [13], in leukocytes Prostaglandins E_2 (PGE_2) and leukotriene B_4 (LTB_4) produces, while in platelets TXA2 and 12-HETE are produced [15,16]. For the secretion of dense granules in the platelets and normal platelet aggregation and adhesion, activation of 12-LOX is important. 12-LOX also play a role in regulating calcium mobilization. Baicalein was first described in mid of 1990s for the classical 12-LOX inhibitor in platelets, inhibition of 12-LOX, AA stimulation resulted in significant attenuation of thrombin-induce calcium and aggregation [17].

Bergenin is a polyphenol, chemically is a C-glucoside of methyl gallic acid. It is most

widely used as an active ingredient in herbal and Ayurvedic medication in Asia [18,19]. It is present in the solid state having no hygroscopic properties and showing the stability against heat and humidity [20]. Bergenin was reported having dose dependent reductions in the carrageenin induced rat paw oedema [21]. Hyperlipidemic effect of bergenin on rats showed extremely reduces the serum cholesterol, triglycerides, low-density lipoprotein-cholesterol levels, while elevates the serum high-density-cholesterol level [22]. Bergenin notably oppose arrhythmias after ligation and reperfusion of the coronary artery, and restoring sinus rhythm by decreasing the duration of ventricular premature beat, tachycardia and fibrillation in $BaCl_2$ induced arrhythmias rats [23]. 11–O –(4–O–methylgalloyl) – bergenin from methanol extract of *Crassula cv. 'Himaturi'* was studied for inhibitory effect in arachidonic acid induced platelet aggregation on Platelet rich plasma of mice, which showed more potent effect than acetylsalicylic acid. Until recently the antiplatelet effect of bergenin mechanism has not been investigated [24]. In present study, we examined *in-vitro* antiplatelet effect of bergenin at the PRP of healthy human volunteers induced by collagen and ADP.

MATERIALS AND METHODS

Bergenin was obtained from the Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi. Collagen and ADP were purchased from Chrono-Log Corp. (Haver-town, PA, USA). All other reagents or solvents such as (sodium citrate 3.8%, sodium hydroxide, phosphate buffer solution and dimethyl sulfoxide (DMSO)) were of analytical grade. Preparation of Platelet rich plasma was performed on Centrifuge Machine (Eppendrof Centrifuge 5810R) and platelet poor plasma was performed on Centrifuge Machine (Mini-Spin Eppendrof AG-22331 Hamburg, Germany). The concentration of platelet aggregation was measured on a dual channel platelets aggregometer (Model No: 5490 – 2D Chrono – Log Corporation, USA) which was interlinked with a personal computer.

Human Subjects

Fifty healthy volunteers (male or female, aged 18–40), who had not taken any drug from at least two previous weeks that might affect the function of platelets, and without history of any haematological disease were recruited in this study. Ethical committee of The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of

Karachi was approved the experimental protocol.

Preparation of PRP and PPP

30ml venous blood sample was drawn from healthy volunteer (after signed informed consent) by 21G butterfly needle into a disposable syringe and immediately transfer into polypropylene tubes containing 3.8 % sodium citrate (1:9 V/V). The samples were homogenized gently by slow inversion and allowed to stand for 5 minutes. The citrated blood tubes were centrifuged (Eppendrof centrifuge 5810R) at 1400 rpm for 15 min to obtained PRP. Platelet poor plasma (PPP) was obtained by further centrifugation of PRP at 13000 rpm for 15 minutes.

Platelets Aggregation Assay

Platelets aggregation responses were measured by light transmission method according to Born and Cross, 1963 [25] using a lumi-aggregometer model (5490–2D) (Chrono – Log Havertown, PA, USA). Prior to the experiment 100 % of light transmittance was observed with PPP respectively. 350 µl of PRP was incubated in an aggregometry sample cuvettes with continuous stirring at 1200rpm. After 1 min of incubation bergenin (1.25 mM, 2.5 mM, 3.75 mM, 5 mM, 6.25 mM and 7.5 mM) was added respectively and further incubated for 5 min. Then threshold concentration of agonist Collagen (10 µg/ml), ADP (5 µM) was added to induce platelet aggregation. The extent of platelet aggregation was measured for 6 min. The results of platelet aggregation was expressed as percentage of light transmission in PPP, as increases platelet aggregation, the turbidity of platelet containing sample decreases due to the platelet clearance in PRP, and percentage of light transmission increases. Percentage of platelet aggregation inhibition was measured by using the formula:

$$\text{Percentage inhibition of platelet aggregation} = \frac{A \times B}{A} \times 100$$

A = maximum aggregation recorded by control sample.

B= aggregation recorded after bergenin added.

Lipoxygenase Inhibition Assay

The lipoxygenase inhibition activity was measured by modifying the spectrophotometric method developed by the Tappel [26]. Lipoxygenase solution was prepared in sodium phosphate buffer in such concentration that the enzyme gives 0.05 absorbance/min in reaction mixture. Test compound solution (Bergenin) of various concentrations ranging from 12.5-500 µM in volume of 10 µl was added in each well

labeled as test containing sodium phosphate buffer (pH 8.0; 160 µL; 100 mM). Lipoxygenase (LOX) solution (20 µl), was then added, mixed and incubated for 10 min at 25 °C. The reaction was initiated by the addition of 10 ml substrate solution (linoleic acid, 0.5 mM, 0.12 % w/v tween 20 in ratio of 1:2) in each well and the absorbance was measured after 15 min at 234 nm. The test compound concentration that inhibited the 50 % lipoxygenase activity (IC_{50}) was calculated by EZ-Fit enzyme software by Pellera Scientific Inc. Amherst, U.S.A.

RESULTS

The inhibitory effect of bergenin on platelet aggregation induced by collagen and ADP are presented in the Figures 1 and 2. The results showed that bergenin presented marked significant inhibitory effect on platelet aggregation caused by two inducers collagen and ADP. Bergenin (1.25 mM, 2.5 mM, 3.75 mM, 5 mM, 6.25 mM and 7.5 mM) inhibited human platelet aggregation stimulated by collagen and ADP in concentration dependent manner. The IC_{50} value of platelet aggregation inhibition for bergenin induced by collagen and ADP were 3.07 ± 0.22 mM and 3.32 ± 0.10 mM respectively (Tables 1 and 2).

When *in-vitro* studies for lipoxygenase inhibition activity were performed for bergenin (4-O-methyl gallic acid), it showed significant inhibition at the concentrations ranging from 50 µM to 500 µM in dose dependent manner. The IC_{50} value of bergenin was found to be 23.5 µM which is equivalent to that of standard Baicalein (IC_{50} = 22.6 µM) used in the assay (Figures 3 and 4).

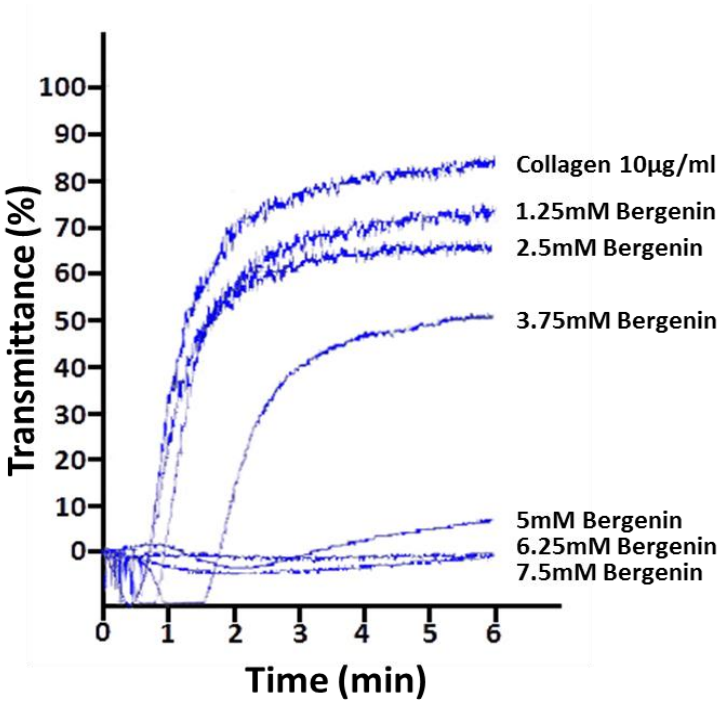

Figure 1. Dose response curve of bergenin (1.25, 2.5, 3.75, 5, 6.25 and 7.5 mM) on platelet aggregation induced by collagen (10 µg/ml).

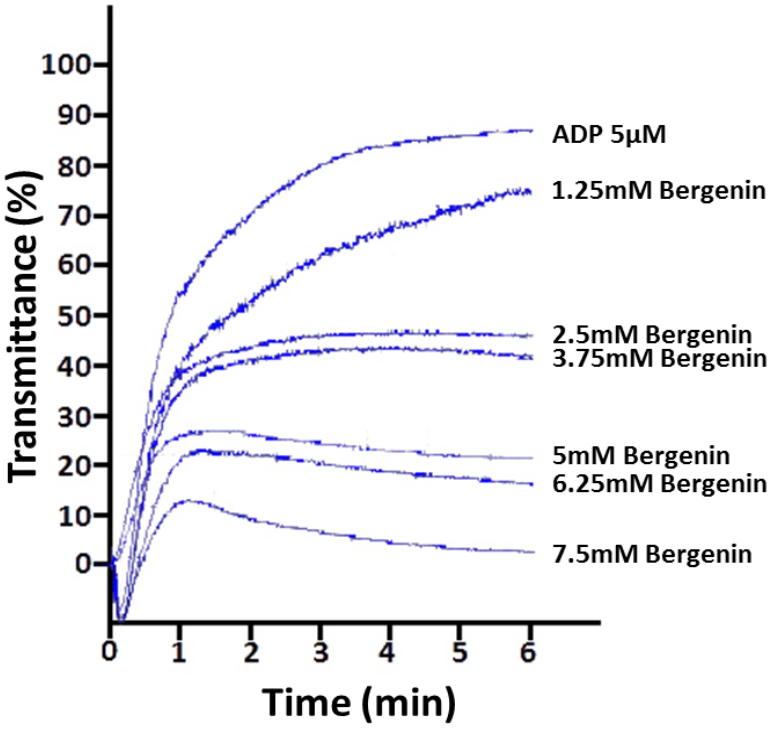

Figure 2. Dose response curve of bergenin (1.25, 2.5, 3.75, 5, 6.25 and 7.5 mM) on platelet aggregation induced by ADP (5 µM).

Table: 1. IC_{50} and inhibitory effect of bergenin on platelet aggregation induced by collagen

Collagen (10 µg/ml)			
Compound	**Concentration (mM)**	**% Inhibition**	**IC_{50} (mM)**
Bergenin	1.25	6.62 ± 3.23	3.07 ± 0.22
	2.5	15.58 ± 3.21	
	3.75	39.94 ± 5.00	
	5	82.02 ± 5.78	
	6.25	100 ± 0.0	
	7.5	100 ± 0.0	

* Values are reported as mean ± standard error of mean (SEM), n = 3.

Table: 2. IC_{50} and inhibitory effect of bergenin on platelet aggregation induced by ADP

ADP (5µM)			
Compound	**Concentration (mM)**	**% Inhibition**	**IC_{50} (mM)**
Bergenin	1.25	18.48 ± 3.26	3.32 ± 0.10
	2.5	33 ± 6.98	
	3.75	45.19 ± 5.53	
	5	76.66 ± 1.74	
	6.25	91.88 ± 5.14	
	7.5	99.23 ± 0.76	

* Values are reported as mean ± standard error of mean (SEM), n = 3.

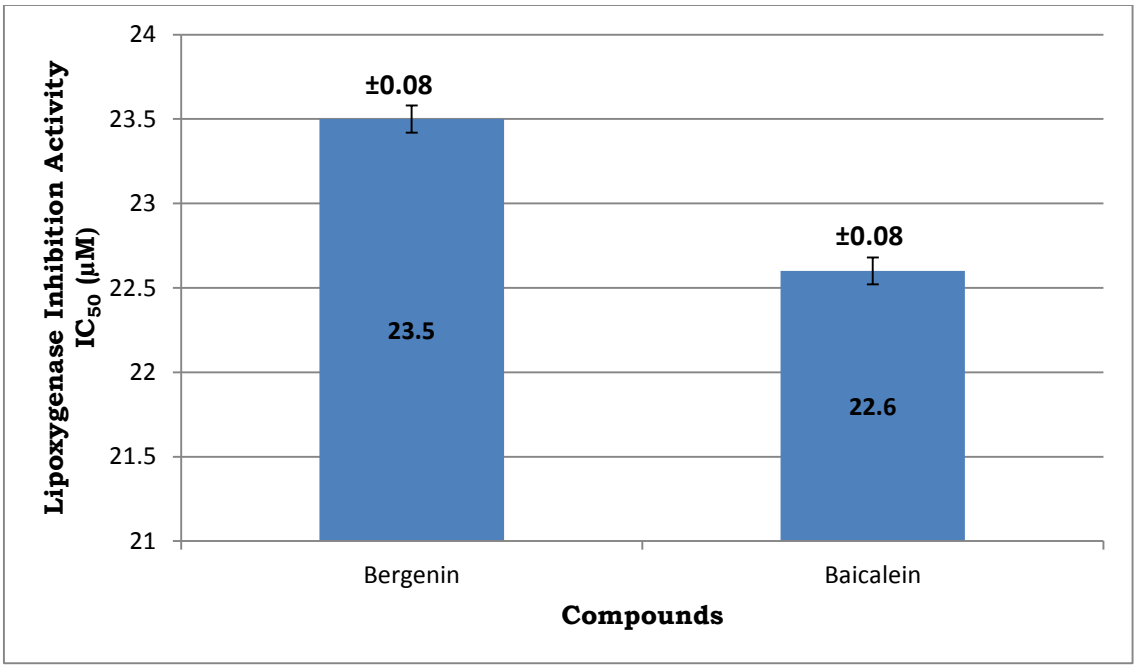

Figure 3. Comparison of IC_{50} values between bergenin and standard compound (baicalein)

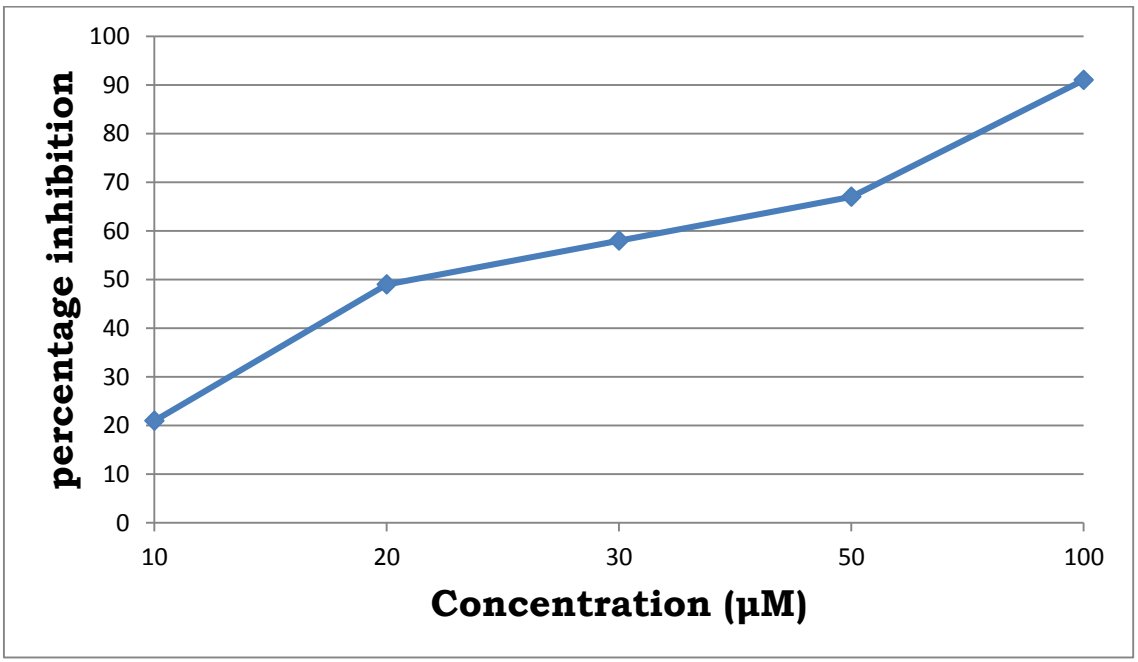

Figure 4. Inhibitory effect of bergenin on lipoxygenase enzyme.

DISCUSSION

Diseases caused by blood clot or thrombus due to activation of platelet and other proteins, such as heart diseases and stroke are the leading cause of death in the world. A number of drugs are available for this treatment. But the researcher continuously worked on different compounds for improving the efficacy and safety profile of the patient by mean of side effects.

Currently aspirin with other synthetic drugs such as clopidogral, ticlopidine and abciximab are commercially being used as a first line drug of choice by cardiologist to prevent the formation of thrombus by inhibiting platelet aggregation and decreasing the chance of many life threatening diseases like myocardial infraction, stroke and atherosclerosis. Such drugs resulted in various adverse effects like bleeding and can develop drug resistance [27].

Plant origin natural products, with antiplatelet activity can be a source of an important compound for therapeutic activity with significant efficacy and limited side effect. Aspirin is most commonly used antiplatelet agent for the prophylaxis of vascular events at the dose of 75–150 mg [7]. Even at low doses aspirin causes side effects, gastric erosions and gastrointestinal haemorrhage [28]. Therefore, novel therapeutic drugs are required for targeting the particular level of platelet activation with fewer side effects. Light transmission aggregometry is the technique used for the determination of collagen and ADP effect on platelet aggregation agonist with reliability and reproducibility [29]. In the current study antiplatelet effect of bergenin induced by collagen showed its moderate activity at 3.75 mM (Figure 1). In case of ADP it showed moderate activity at 3.75mM (Figure 2). We have previously reported that at PRP of rats, bergenin showed significant effect at sodium AA induce platelet aggregation and more potent than the aspirin [24]. So it can be concluded that bergenin may performed antiplatelet activity by inhibiting the cyclooxygenase and lipoxygenase pathway of platelet aggregation.

Cardiovascular Diseases (CVD) are the leading cause of death globally and the current approaches of pharmacological therapy cause by blood clot in cardiovascular diseases such as stroke, angina and MI decreases the morbidity profile of the CVD patient either alone

therapy or in combination such as aspirin and clopidogrel. These therapies some time not extremely useful in decreasing the mortality rate in such patient may be due to long duration of action and themselves causing bleeding complications [30,31]. Bleeding are the major adverse effect and primary concern of currently used medication in CVD therapy against platelet activation, especially before and at the time of surgical procedure [32]. Thus, substitute approaches are warranted which would arrest the platelet activation while attenuate the bleeding side effect. For anti-platelet therapy human 12-Lipoxygenase (12-LOX) may be a novel target in the treatment of patients related to cardiovascular diseases. A comprehensive knowledge about pathophysiological, biochemical and 12-LOX metabolites role among vascular system must be understood in order to establish a potential 12-LOX inhibitors. Although the prior evidence presents the ability and advantages of 12-LOX inhibition on the platelet in order to treating human diseases, meanwhile, current awareness about this enzyme and related oxidized products among platelets and other tissues is still restricted.

Lipoxygenase may be a viable targeting approach for antiplatelet therapy in future with the inhibition of targets such as COX-1 and P_2Y_{12}. This approach may be attenuating the platelets aggregation process without the increasing risk of bleeding. In our studies bergenin showed a significant inhibition of lipoxygenase enzyme with IC_{50} 23.5 μM when compared with the standard compound baicalein with IC_{50} 22.6 μM, which was reported as a selective inhibitor of 12-LOX in human platelets [33] without effecting the cycloxygeanse activity [34]. The role of the AA oxidized metabolite in platelets, 12-HETE, is not well understood. 12-HpETE has also been shown to stimulate 12-LOX but inhibit COX-1 in lysed platelets [35] and one report indicated that 12(S)-HETE acts as an inhibitor of platelet and neutrophil PLA_2 activity [36]. 12-HPEPE and 12-HEPE are other eicosanoid products originating from 12-LOX oxidation of EPA, thought to bring out an inhibitory effect on platelet aggregation [37,38]. In addition to their effects on aggregation, 12-HPEPE and 12-HEPE have been shown to attenuate serotonin (5-HT) release mediated by AA and collagen in a dose dependent manner [39].

Despite the fact that targeting enzymes like COX-1 or surface receptors like PAR-1, P_2Y_{12} and integrin receptor αIIb_3 has been extremely successful in reducing MI-related morbidity, but these therapies are

ineffective to significantly reduce mortality in these patients. This might be expected to some extent to the way that anti-platelet drugs do not completely inhibit platelet activation, may have a prolong onset and duration of action, and may cause substantial morbidity due to bleeding complications [30,31].

Therefore, new therapeutic approaches are required that targeting the level of platelet activation in vessel occlusion and stroke without themselves bleeding problems. This problem may be solved by inhibiting the secondary pathway of platelets activation which further inhibiting the clot formation without altering the bleeding profile following vascular insult as observed with in secondary pathway inhibition of COX-1.

CONCLUSION

Our finding suggest that may be bergenin prevent the aggregation of platelet by inhibiting the secondary pathway of aggregation such as cyclooxygenase and lipoxygenase pathway of platelet aggregation, although the exact mechanism is unknown. On the basis of these finding further studies are required with other platelet aggregation inducing agents and studies on molecular level to investigate the broader approaches in the treatment of these debilitating medical conditions. We conclude that bergenin could be used as a platelet inhibitor at defined concentrations in cardiovascular disorders, after extensive further investigations in the future.

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