Anti-aggregatory effect of boswellic acid in high-fat fed rats: involvement of redox and inflammatory cascades

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Abstract

Introduction: A high-fat diet is one of the main dietary factors promoting platelet aggregation. The present study was conducted to elucidate the involvement of boswellic acid (BA) on the platelet hyperaggregability in HFD-fed rats. As platelet hyperaggregability in HFD rats is closely linked to inflammation and enhanced free radical production, the present study was extended to evaluate the anti-inflammatory and anti-oxidative effect of BA on HFD-promoted platelet aggregation.
Material and methods: Rats were assigned to normal, HFD-fed, aspirin-treated (30 mg/kg), and BA-treated (250 and 500 mg/kg) groups.
Results: Boswellic acid administration in a high dose was effective in attenuating the severity of hyperlipidemia and platelet aggregation, indicated by lower collagen/epinephrine-induced platelet aggregation, as evidenced by the significant increase ($p < 0.05$) in the circulating platelet count and reduction in the number of thrombi in the lungs. Moreover, it attenuated the oxidative stress and the intensity of inflammatory mediators associated with platelet hyperaggregability, as evidenced by the inhibitory effects on interlukin-1β, COX-2 and tumor necrosis factor-α, indicating that the antiplatelet activity of BA is likely a consequence of controlling oxidative stress and inflammation.
Conclusions: The present data suggest that BA shows a promising anti-aggregatory effect by attenuating the enhanced hyperlipidemia, oxidative stress and inflammation associated with HFD.

Key words: boswellic acid, high-fat diet, inflammation, oxidative stress, platelet aggregation, rat.

Introduction

Platelets are essential for normal homeostasis. However, excessive activation of platelets that leads to their aggregation plays a major role in the pathogenesis of cardiovascular disorders such as atherosclerosis, cardiac dysfunction and peripheral artery disease [1]. Metabolic abnormalities as a consequence of a high-polyunsaturated-fat diet (HFD) cause platelet hyperaggregability involving enhanced intraplatelet reactive oxygen species (ROS) production and decreased nitric oxide (NO) bioavailability [2]. The relationship between platelet aggregation and inflammation has been previously discussed [3]. Several studies have shown that animals fed high-fat diets are more likely to develop dyslipidemia [4], insulin resistance [5], hepatic steatosis [6], platelet aggregation [7], inflammation [8] and oxidative stress [9].

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Anti-platelet targets are increasingly used not only for anti-thrombotic prophylaxis, particularly in patients who have had a myocardial infarction or angina pectoris, but also in healthy individuals at increased risk of developing cardiovascular diseases [10, 11]. However, the major limitation to the long-term use of these targets is the increased risk of intracranial hemorrhage and gastrointestinal bleeding [11]. Given the importance of platelet activation in the pathogenesis of cardiovascular diseases, research is oriented towards the discovery of new, naturally occurring anti-platelet medicinal agents with fewer or no adverse effects that can serve as a useful adjuvant to various anti-platelet targets.

Triterpenoids are known for their anti-oxidant and anti-inflammatory properties. Among thousands of triterpenoids found in plants, boswellic acid (BA) is very promising due to its effectiveness and stability [12, 13]. Boswellic acid is extracted from the gum resin of *Boswellia serrata*, exhibits a variety of profound effects such as being anti-inflammatory, antioxidant, cancer drug sensitizing, cardio-protective, insulin resistance lowering, and gastro-protective [13–16].

Considering that platelet hyperaggregability in HFD rats is closely linked to inflammation and enhanced free radical production, together with the fact that BA is known for its antihyperlipidemic, antioxidant and anti-inflammatory properties, this compound was a logical candidate for study as its administration could reverse this scenario. So, the aim of this study is to investigate the role of BA in the platelet hyperaggregability in HFD-fed rats.

Material and methods

Drugs and chemicals

Boswellic acid was purchased from Advance Physician Formulas Inc. (California, USA) in tablet form; it was dissolved in distilled water. Acetyl salicylic acid was kindly provided by Medical Union Pharmaceuticals (MUP, Ismailia, Egypt). Bovine collagen and epinephrine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cholesterol was purchased from GFS chemicals (Texas, USA) and bile salts were purchased from SAS Chemicals Co. (Mumbai, India). All the used solvents were of analytical grade and were supplied by Al-Nasr Company for Chemical Industries (Cairo, Egypt).

Animals

The experiment was performed using 80 healthy male Wistar albino rats with a weight of 170 ±30 g purchased from the Egyptian Organization for Biological Products and Vaccines. Rats were housed in clean, well-ventilated stainless steel cages, maintained on a normal light-dark cycle and temperature 25 ±3°C throughout the experiment. Standard rodent chow and water ad libitum were provided to the rats and they were left 1 week for acclimatization. All experimental procedures were approved by the institutional animal care and use committee at the Suez Canal University, following the National Institutes of Health guide for the care and use of laboratory animals.

Atherogenic diet

The high-fat diet (HFD) was composed of 87.7% standard diet (w/w), 10% pork fat (w/w), 2% cholesterol (w/w) and 0.3% bile salts (w/w) [17].

Experimental design

The experiment was divided into two parts as follows:

Experiment I

Forty male albino rats were randomly allocated to five groups, eight rats in each. Group I: the normal group: rats fed with normal palatable diet. All the other groups were fed with the HFD for 12 weeks followed by a 4-week therapeutic period. Group II: the HFD-fed control rats. Group III: aspirin-treated (30 mg/kg) group [18]. Group IV: BA-treated (250 mg/kg) group. Group V: BA-treated (500 mg/kg) group [19]. In general, aspirin and BA were dissolved in distilled water and administered orally using a gastric tube. Experiment I was conducted to obtain data about all the measured parameters and assay with exception of the collagen-induced platelet consumption assay. At the end of the experiment, rats were anesthetized using thiopental sodium (50 mg/kg) and sacrificed by decapitation. Fresh blood samples were collected for the following assays.

Platelet aggregation assay: Blood was collected into a 3.8% sodium citrate solution (9 : 1 V/V). Then, samples were centrifuged immediately at 160 × g for 15 min at room temperature to prepare platelet-rich plasma (PRP). After that, PRP was transferred into plastic tubes and the remaining blood was centrifuged at 3000 × g for 10 min to obtain the platelet-poor-plasma (PPP). Platelet count in PRP was adjusted to 5×10^8/ml using PPP. Platelet aggregation was performed after addition of 5 μg/ml collagen (Chrono-Log corp.) using a dual channel aggregometer (Clot 2, SEAC- Radim Company, Italy). Results were expressed as a percentage of aggregation; the extent of aggregation was estimated by the change in light transmission [20].

Blood collection and serum separation: Fresh blood samples (1 ml of blood) were collected in a dry centrifuge tube and were allowed to stand for 30 min before centrifugation at 3000 × g for 15 min. Then, sera were separated, collected in

clean tubes and stored at –80°C until used for the following assays.

Determination of serum lipid profile: Serum total cholesterol, triglycerides (TGs), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) were determined using commercial kits, purchased from Bio Diagnostics (Cairo, Egypt). These parameters were determined enzymatically according to the manufacturer's protocol using an ultraviolet-visible spectrophotometer (UV-1601PC, Shimadzu, Kyoto, Japan).
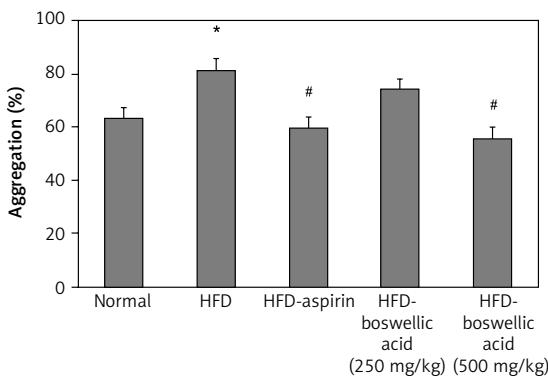
Determination of malondialdehyde (MDA) and reduced glutathione (GSH): Tissues malondialdehyde (MDA) was estimated according to the spectrophotometric method of Ohkawa *et al.* [21] using 1,1,3,3-tetramethoxypropane as a standard. Concentration of reduced glutathione (GSH) was measured spectrophotometrically using commercial kits according to the instructions of the manufacturer [22].

Determination of superoxide dismutase (SOD) and catalase (CATA): The activity of SOD was assessed as described by Marklund [23], and CATA activity was measured according to Aebi [24].

Determination of serum level of IL-1β, COX-2 and TNF-α: Enzyme-linked immunosorbent assay (ELISA) kits for IL-1β, COX-2 and TNF-α were obtained from Glory Science Co., Ltd (Del Rio, TX, USA) and were used to measure these parameters in serum samples. Assays were performed according to the manufacturer's instructions using an automated ELISA reader (Metertech, M960).

Experiment II

In a separate set of experiments, another set of rats (40 rats) – identical in the study groups – were used to develop a rat model of pulmonary thrombo-embolism for thrombogenesis assay. Using these rats, a platelet count was obtained before and af-ter injection of a collagen/epinephrine mixture, and lung specimens were taken for histopathological examination as detailed in the next sections.

Collagen-induced platelet consumption: Collagen was given to induce platelet activation to perform a pulmonary thrombo-embolism model as described previously by Seth *et al.* [25] with minor modifications. A mixture of bovine collagen (1000 µg/kg) plus epinephrine (200 µg/kg) was injected into the rat tail vein.

Platelet count: Platelet count was carried out immediately before and 3 min after injection of the collagen/epinephrine mixture. Blood samples were collected and anticoagulated with a 10% EDTA solution. After mixing, platelets were counted automatically on a Cell-Dyn 1700 instrument (Abbott Laboratories, USA).

Histopathological examination of the lungs: After blood collection, rats were anesthetized with thiopental sodium (50 mg/kg) and killed by decapitation. Then, the chest was opened and the lungs were dissected and fixed in a 10% phosphate-buffered paraformaldehyde solution. Tissues were dehydrated and embedded in paraffin and sectioned at 4-µm and stained with hematoxylin and eosin (H + E). The lung specimens were then examined blindly under a light microscope. The number of thrombi per microscopic field was counted as described by Decrem *et al.* [26] with slight modification. The thrombi were counted at 10× magnification; data are presented as the mean for 10 fields for each rat.
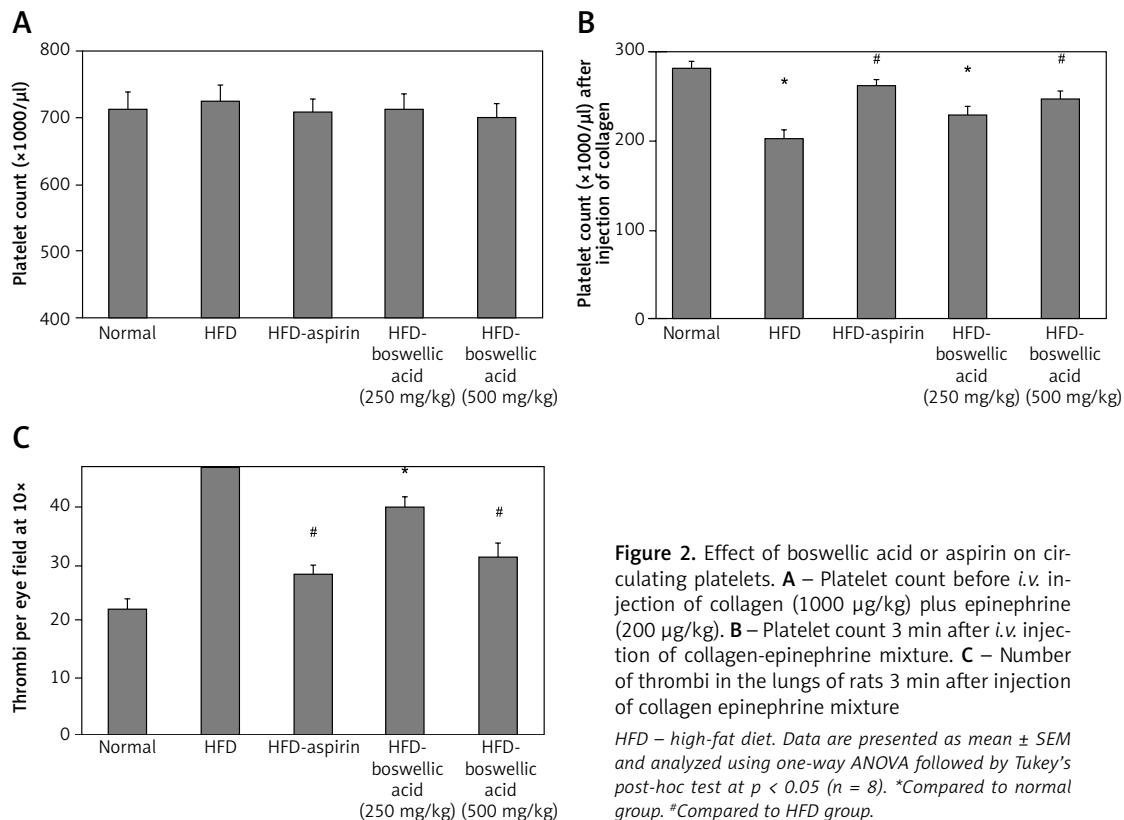
Statistical analysis

All data were tabulated and expressed as mean ± SEM and analyzed employing the Statistical Package for the Social Sciences (SPSS, version 17, Chicago, IL, USA). Comparisons among groups were carried out using one-way ANOVA. The difference of mean values between groups was assessed by Tukey's post-hoc test. All *p*-values reported are two-tailed and a *p*-value < 0.05 was considered to be significant.

Results

Effect of aspirin or BA on HFD-induced platelet aggregation

Figure 1 shows that the percentage of platelet aggregation was significantly higher ($p < 0.05$) in the high-fat diet fed rats compared to the normal control group. Additionally, rats fed with the HFD exhibited higher collagen/epinephrine-induced platelet aggregation, as evidenced by the reduction ($p < 0.05$) in the circulating platelet count (Figure 2 B) and an increase in number of thrombi in the lungs in comparison with those fed with a normal palatable diet ($p < 0.05$, Figure 2 C).

Figure 1. Platelet aggregation assay in the experimental groups. HFD: high-fat diet. Data are presented as mean ± SEM and analyzed using one-way ANOVA followed by Tukey's post-hoc test at $p < 0.05$ ($n = 8$)

Compared to normal group. #Compared to HFD group.

A

B

C

Figure 2. Effect of boswellic acid or aspirin on circulating platelets. **A** – Platelet count before *i.v.* injection of collagen (1000 µg/kg) plus epinephrine (200 µg/kg). **B** – Platelet count 3 min after *i.v.* injection of collagen-epinephrine mixture. **C** – Number of thrombi in the lungs of rats 3 min after injection of collagen epinephrine mixture

*HFD – high-fat diet. Data are presented as mean ± SEM and analyzed using one-way ANOVA followed by Tukey's post-hoc test at $p < 0.05$ (n = 8). *Compared to normal group. #Compared to HFD group.*

Boswellic acid in a high dose was effective in attenuating the severity of HFD-induced platelet aggregation in comparison with the HFD control group ($p < 0.05$, Figure 1). Moreover, BA in a high dose attenuated collagen/epinephrine-induced platelet aggregation, as evidenced by the increase ($p < 0.05$) in the circulating platelet count (Figure 2 B) and a decrease in the number of thrombi in the lungs in comparison with the high-fat fed rats ($p < 0.05$, Figure 2 C).

Effect of aspirin or BA on HFD-induced oxidative stress

In the current study, a high-fat diet induced oxidative stress in the form of a significant increase of MDA concentrations associated with a significant reduction in GSH, SOD activities, and CATA levels in comparison with the normal control group ($p < 0.05$, Figure 3). These deleterious effects associated with HFD were improved ($p < 0.05$) by treatment with either aspirin or BA in comparison with the HFD control group. Notably, BA in a high dose induced a significant improvement in oxidative stress markers compared to aspirin ($p < 0.05$, Figure 3).

Effect of aspirin or BA on HFD-induced increase in inflammatory mediators

Figure 4 shows that the HFD increased ($p < 0.05$) production of the inflammatory mediators

IL-1β, COX-2 and TNF-α compared to the normal control group. These elevations in inflammatory mediators associated with HFD were ($p < 0.05$, Figure 4) ameliorated by treatment with either aspirin or BA, as evidenced by the inhibitory effects on interlukin-1β, COX-2 and TNF-α.

Effect of aspirin or BA on HFD-induced hyperlipidemia

Table I shows that high-fat fed rats exhibited hyperlipidemia, as is evidenced by the significant ($p < 0.05$) elevation in serum total cholesterol, TG and LDL levels and decline in HDL level. The BA in a high dose was effective in attenuating the severity of hyperlipidemia in comparison with the HFD control group ($p < 0.05$, Table I). Notably, the aspirin-treated group exhibited a non-significant improvement in the severity of hyperlipidemia compared to the HFD control group ($p > 0.05$, Table I).

Discussion

Given the importance of platelet activation in the pathogenesis of cardiovascular diseases, research is oriented towards the discovery of anti-platelet agents with improved efficacy for preventing or treating arterial or venous thrombosis [27]. Although a number of anti-platelet targets have been identified to address this issue including COX-1, the P2Y12 receptor, the integrin αIIbβ3,

A

B

C

D
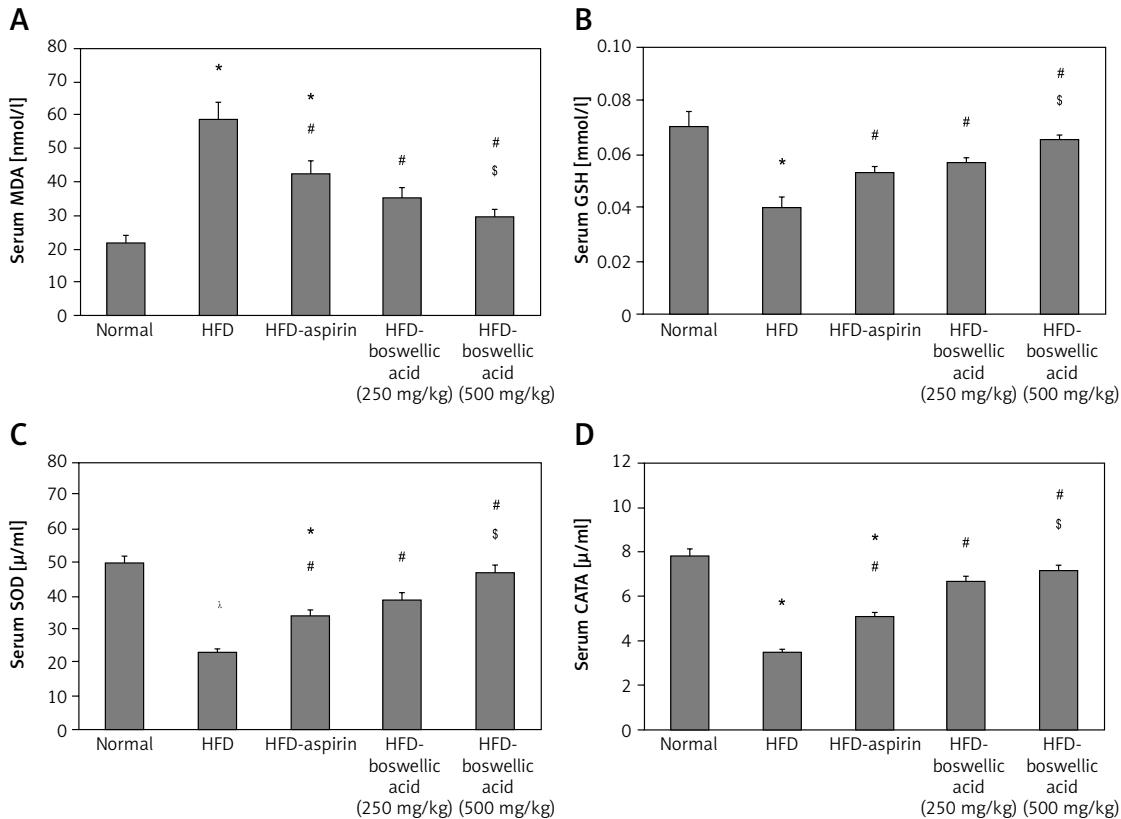
Figure 3. Serum level of MDA (**A**), GSH (**B**), SOD (**C**), CATA (**D**) in the experimental groups

*HFD – high-fat diet. Data are presented as mean ± SEM and analyzed using one-way ANOVA followed by Tukey's post-hoc test at p < 0.05 (n = 8). *Compared to normal group. #Compared to HFD group. $Compared to aspirin.*

A

B

C
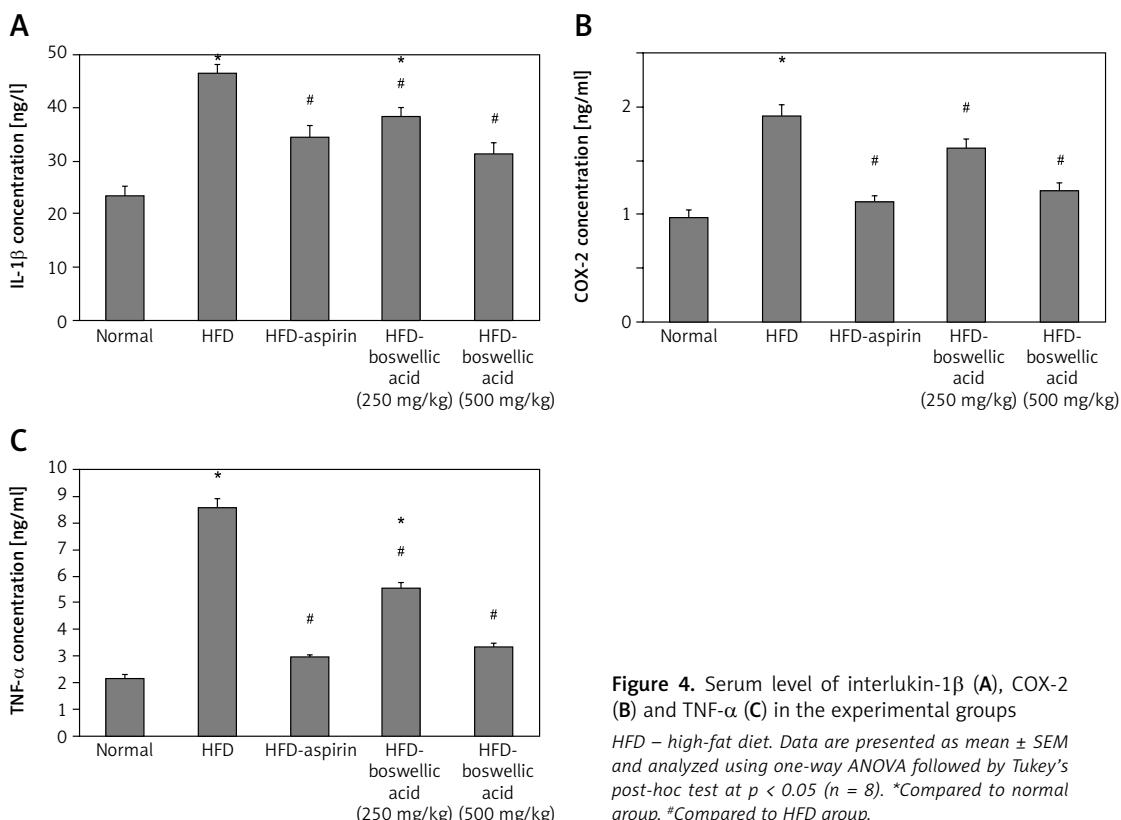
Figure 4. Serum level of interlukin-1β (**A**), COX-2 (**B**) and TNF-α (**C**) in the experimental groups

*HFD – high-fat diet. Data are presented as mean ± SEM and analyzed using one-way ANOVA followed by Tukey's post-hoc test at p < 0.05 (n = 8). *Compared to normal group. #Compared to HFD group.*

Table I. Effect of treatment with boswellic acid (250 or 500 mg/kg) on serum total cholesterol, triglycerides, HDL-C and LDL-C in hyperlipidemic rats

Groups	Total cholesterol [mg/dl]	Triglycerides [mg/dl]	HDL-C [mg/dl]	LDL-C [mg/dl]
Normal	45.2 ±4.2	27.73 ±1.8	32.2 ±2.4	15.3 ±1.2
HFD	79.3 ±6.6*	59.43 ±5.2*	15.5 ±1.8*	39.4 ±3.9*
HFD + aspirin	71.3 ±26.8*	55.32 ±4.3*	19.2 ±17.6*	31.2 ±4.2*
HFD + boswellic acid (250 mg/kg)	69.1 ±21.4*	53.3 ±1.9*	20.12 ±19.2*	33.9 ±4.8*
HFD + boswellic acid (500 mg/kg)	52.0 ±20.4#$	37.0 ±2.6#$	28.12 ±21.6#	26.22 ±5.7#

Rats were fed with a HFD for 12 weeks. HFD – high-fat diet. Data are expressed as mean ± S.E.M. and analyzed using one-way ANOVA followed by Tukey's post-hoc test at p < 0.05. *Compared to normal group at p < 0.05, #compared to HFD group, $compared to aspirin. n = 8.

and more recently the protease-activated receptor-1, these targets often result in a significant increase in risk of bleeding, which may lead to pathologies as serious as the thrombosis they were meant to treat, including intracranial hemorrhage and gastrointestinal bleeding [11, 12]. The interest in finding naturally occurring components with anti-inflammatory and antioxidant properties to serve as a useful anti-aggregator adjuvant to various anti-platelet targets is increasing [13]. One group of such compound is triterpenoids. Among thousands of triterpenoids found in plants, BA is very promising due to its effectiveness and stability [28].

As a high-fat diet is one of the main dietary factors promoting platelet aggregation and involved in the pathogenesis and development of cardiovascular thrombotic complications, the present study was conducted to investigate the involvement of BA in the platelet hyperaggregability in HFD rats. As platelet hyperaggregability in HFD rats is closely linked to inflammation [3] and enhanced ROS production [7], our study was extended to evaluate the anti-inflammatory and anti-oxidative effect of BA on HFD-promoted platelet aggregation. Consistent with many lines of evidence [2, 29–33] that emphasized that platelet aggregation is fully activated and accelerated by a high-fat cholesterol diet, the present results revealed that rats fed a high-fat cholesterol diet exhibited higher collagen/epinephrine-induced platelet aggregation, as evidenced by the decrease in circulating platelet count and the increase in number of thrombi in the lungs. The platelet hyperaggregability during HFD may in part be related to hyperlipidemia, as evident from the elevated serum total cholesterol, TG and LDL levels and the decline in HDL level. Wang et al. [30] and Ito et al. [32] emphasized that platelet sensitivity and aggregation are enhanced with hyperlipidemia.

Another explanation could be attributed to the presence of enhanced ROS production. Trocha et al. [34] emphasized that conditions including dyslipidemia, obesity, and diabetes mellitus are associated with aggravated oxidative stress. Compatible with findings from previous studies [2, 7, 31] linking platelet hyperaggregability in HFD rats with enhanced oxidative stress, a rise in MDA levels and depletion in the antioxidant enzyme pool were observed, as evident from the declined activity of GSH, SOD, and CATA.

As inflammation plays a crucial and modulating role in various diseases [35], the relationship between platelet aggregation and acute inflammation has been previously discussed [3, 8]. In line with this hypothesis, we observed that platelet hyperaggregability was associated with increased production of the inflammatory mediators IL-1β, COX-2 and TNF-α. Ziccardi et al. [36] and Damas et al. [37] emphasized that the pro-inflammatory and pro-oxidant state that accompanies the HFD has been suggested to be involved in endothelial dysfunction and plaque destabilization, providing a connection between inflammation, oxidative stress and platelet hyperaggregability in metabolic abnormalities as a consequence of HFD [2, 38].

The results of the present study also revealed that BA was effective in attenuating hyperlipidemia-promoted platelet aggregation indicated by lower collagen/epinephrine-induced platelet aggregation, as evidenced by the increase in the circulating platelet count and the reduction in the number of thrombi in the lungs. Consistent with findings from previous studies [39, 40], the present results clearly demonstrated an improvement in lipid profile associated with BA administration. Another explanation could be afforded by Ali and Mansour [41], who emphasized that platelet hyperaggregability associated with HFD was prevented by the use of an antioxidant, indicating the critical role for intracellular ROS in this phenomenon. Similarly to the elsewhere published data [42–44], the results of the present study revealed that antioxidants, by altering the oxidant/antioxidant balance, attenuated HFD-induced hyperaggregability in rats.

As increased production of inflammatory mediators results in endothelium dysfunction as well

as alterations in the coagulation system [2, 45], the interest in finding naturally occurring components with potent anti-inflammatory properties is increasing. One potential treatment to reduce inflammatory mediators involves the use of BA, which has shown efficacy as an anti-inflammatory agent [15]. The current results are compatible with those reporting the inhibitory role of BA on serum level of IL-1β [43, 44, 46], COX-2 and TNF-α [44, 47, 48]. It is obvious that the anti-platelet activity of BA is most likely a consequence of COX-2, IL-1β and TNF-α inhibition. The results of this study demonstrated that BA administration in a high dose, by its known anti-oxidant and anti-inflammatory properties [14], exerted an attenuating effect on the extent and severity of ROS production and inflammation, indicating that the anti-platelet activity of BA is likely a consequence of controlling oxidative stress and inflammation.

In conclusion, the present data suggest that BA shows a promising anti-aggregatory effect by attenuating the enhanced hyperlipidemia, oxidative stress and inflammation associated with HFD. Consequently its administration may warrant further attention as a potential adjuvant to existing medications that are used to treat patients with platelet hyperaggregability.

Conflict of interest

The author declares no conflict of interest.

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