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Full Length Research Paper

Triterpenoids from *Eucalyptus grandis* Hill ex Maiden inhibits platelet aggregation

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Phytochemical investigation of *Eucalyptus grandis*, a plant with many industrial and traditional applications, led to the isolation of 3β-hydroxyurs-2-en-28-oic acid (FJ-2) and the synthesis of 3β-acetylurs-12-en-28-oic acid (FJ-6), with antiplatelet aggregation activity. Platelet aggregation was induced by thrombin a platelet protease-activated receptors subtype I and IV, adenosine diphosphate (ADP) a potent agonist to platelet G protein-coupled P2Y receptor and epinephrine. The results showed FJ-2 had the highest percentage inhibition (87.8 ± 1.81) which was observed to be significantly ($P < 0.05$) higher than the standards heparin (65.9 ± 0.91) and aspirin (65.4 ± 0.8) at a concentration of 1.0 mg/ml, using thrombin-as agonist. But this percentage inhibition was observed to decrease with increase in the concentration of FJ-2; this implied an optimal concentration (≤ 1.0 mg/ml) for inhibition of platelet aggregation by FJ-2, above which inhibition decreases. FJ-6 showed a dose dependent increase in percentage inhibition (51.4 ± 0.65 at 1.0 mg/ml and 73.8 ± 1.72 at 10 mg/ml). The two compounds differ only in their functionality but behave differently towards platelet aggregation inhibition. This preliminary result suggests that FJ-2 and FJ-6 may be taken as candidate lead natural compounds to be considered in the search for natural products with beneficial effects on aberrant platelet activation mediated cardiovascular disorders.

Key word: Antiplatelet aggregation, blood clot, *Eucalyptus grandis* and platelet agonist.

INTRODUCTION

Platelets are cells in the blood that help to make blood clot. Heart disease risk factors such as smoking, high blood pressure, high blood cholesterol or diabetes, can make platelets clump together more easily and can form a serious blood clot condition. Blood clots can become a problem especially if they are in the heart, brain or other arteries in the body. Platelet hyperactivity and consequent hyper aggregation is the cause of internal clots, if not checked, can be fatal and are indeed the main cause of atherothrombotic diseases such as strokes, heart attack, and pulmonary embolism (Huo and Ley, 2004). Thrombin, adenosine diphosphate (ADP), epinephrine, arachidonic acid, collagen and other risk factors such as free radicals, inflammation, stress and hypercholesterolemia significantly contribute to platelet dysfunctions. The activation of platelet by thrombin is mediated through two protease-activating receptors (PAR), PAR 1 and PAR 4, belonging to G protein-coupled receptors. ADP can mainly bind to G protein-coupled P2Y1 receptor and activates phospholipase C, and thus resulting in the elevation of intracellular calcium concentration [Ca^{2+}] (Kim et al., 2011).

Antiplatelet drugs stop blood clots from forming and this significantly contribute to the management of pathogenesis of cardiovascular diseases (Halt and Chandra, 2002). Among the many drugs used in the management of the condition is aspirin, but these drugs are not without side effect, placing a great pressure in the search for more effective drugs from natural origin (Amrani et al., 2009). Traditional medicinal plants are constantly been investigated for new leads in the search

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for active pharmacological drugs.

Eucalyptus grandis (Myrtaceae) is native to the east coast of Australia; its commonly called rose gum or flooded gum, the bark is thin and deciduous, shedding in strips to expose a smooth surface marked with flowing patterns of silvery white, slaty gray on the trunk (Hall et al., 1970). Rose gum is one of the most important commercial *eucalypts*, with more than one-half million hectares (1.3 million acres) planted in tropical and subtropical areas on four continents. Massive planting programs have been carried out in the Republic of South Africa and Brazil and there are substantial plantings in Angola, Argentina, India, Uruguay, Zaire, Zambia, and Zimbabwe (Jacobs, 1976). The eucalyptus oil extracted from the leaves are used as antiseptics and disinfectant, so find wide application in soap and shampoo production, the boil leaves extract are used to relive flu, bronchitis, pneumonia and respiratory infection (Takasaki et al., 2000). Domingues et al. (2010) reported the presence of triterpene in *E. grandis* and *Eucalyptus urograndis* grown in Brazil and Portugal. In this paper we report our investigation on the antiplatelet aggregation activity of ursolic acid and ursolic acid acetate isolated from South African *E. grandis* specie.

MATERIALS AND METHODS

Plant

Plant material (leaves) was collected around Empangeni area of KwaZulu-Natal, South Africa. The plant was identified by a Botanist from the Department of Biological Sciences University of KwaZulu-Natal, Westville, Durban, South Africa. A voucher specimen (EU01UKZN) was prepared and deposited in the University herbarium. The plant material was air dried and pulverized to coarse powder in preparation for extraction.

Extraction and isolation

The pulverized plant material (500 g) was extracted by cold maceration in ethyl acetate (5 L × 3) for 72 h, filtered and concentrated under reduced pressure at 40°C using rotar y evaporator and allowed to dry under room temperature. The concentrated extract was then defatted with n-hexane to yield a solid mass (35.68 g). The solid mass (10 g) was subjected to column chromatography using ethyl acetate:hexane (8:2) as solvent of elution to give a white powder, which was identified as 3β-hydroxyurs-12-en-28-oic acid (FJ-2). The structure was confirmed by NMR spectra (Figures 1 and 2) comparison with literature values (Mahato and Kundu, 1994).

Preparation of 3β-acetylurs-12-en -28-oic acid (FJ-6)

FJ-2 (1.0 g) in round bottom flask was added acetic anhydride (5 ml) and pyridine (5 ml) the mixture was then refluxed for 2 h, and stirred at room temperature for 24 h. After which water (10 ml) was introduced and stirring continued for another 30 min. The mixture was then filtered under suction and washed thoroughly with 10% HCl to give a white powder (95%). The structure was confirmed by NMR spectra (Figure 1) comparison with literature values (Mahato and Kundu, 1994).

Biological studies

Animals

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

In vitro antiplatelet aggregation study

Preparation of compounds

The compounds were separately dissolved in 1% DMSO and a few drops of Tween 80 for the antiplatelet aggregation study.

Preparation of blood platelets

The blood platelets were collected according to the method described by Tomita et al. (1983). The rat was placed in a container containing diethyl ether, the vapour render the rat unconscious and blood was surgically collected from the heart immediately. 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 1200 rpm for 15 min and at 2200 rpm for 3 min consecutively, the supernatant was collected and centrifuged at 3200 rpm for 15 min. The supernatant was collected and discarded and the sediment (platelets) was resuspended in 5 ml 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 buffer (pH 7.4; containing 0.14 M NaCl, 15 mM Tris-HCl, 5 mM glucose). The platelet is diluted in the resuspending buffer (1:10) the resulting solution was mixed with calcium chloride (0.4 ml: 10 µl $CaCl_2$).

Anti-platelet aggregation evaluation

The method of Mekhfi et al. (2004) was used with some modifications**.** 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 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) 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 second interval. DMSO (1%) and aggregation inducer as negative control, heparin and aspirin were used as positive control.

Statistical analysis

All assays were performed in triplicates and the results are expressed as mean ± SEM. $P \leq 0.05$ were considered to be statistically significant. The inhibitory effects of the compounds on each parameter were calculated as:

% inhibition = {(1-(At/Ac) ×100}

The inhibitory concentration providing 50% inhibition (IC_{50}) was

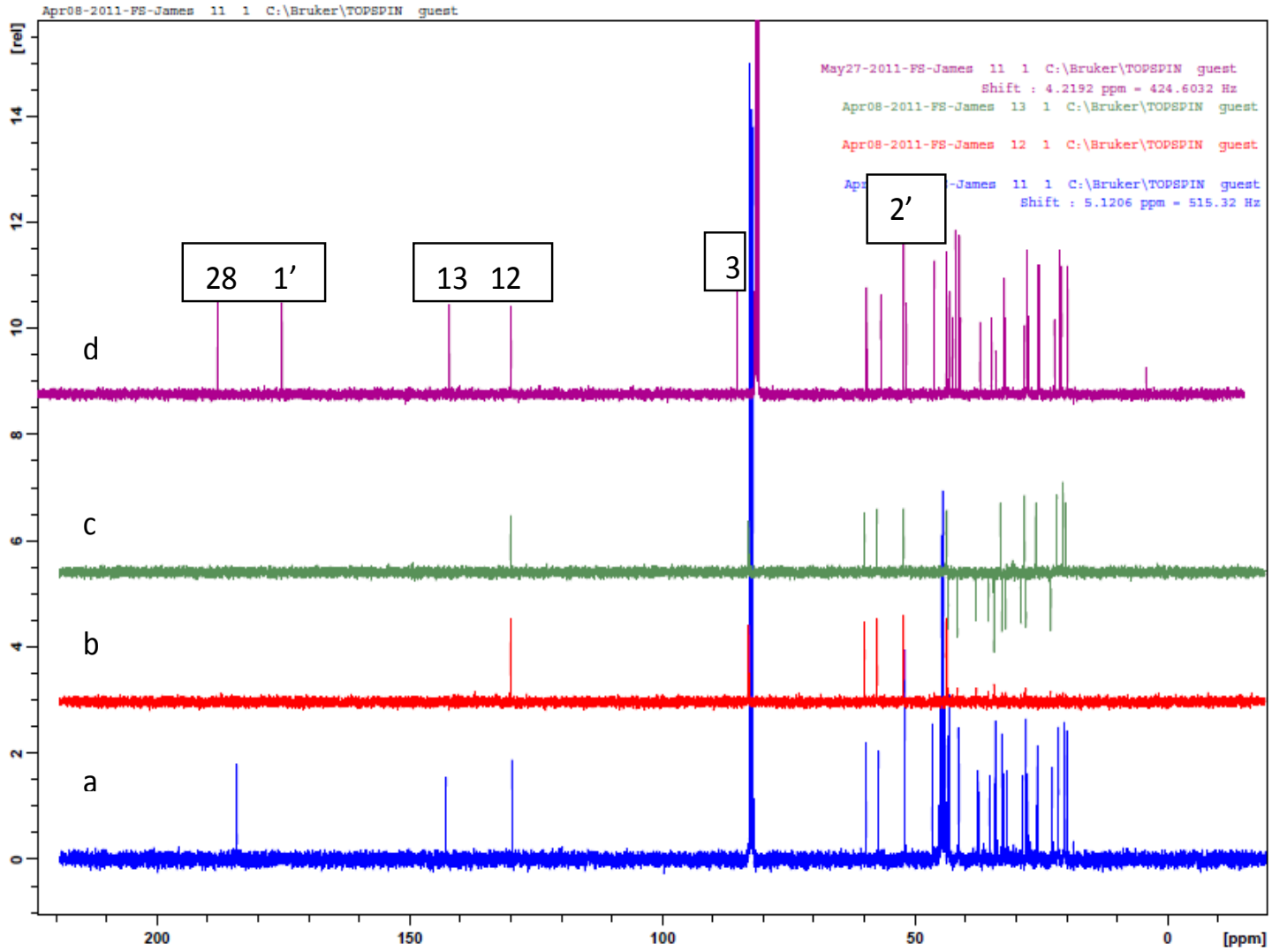

Figure 1. (a, b, c) ^{13}C-NMR, DEPT-90, DEPT-135 spectra of FJ-2, (d) ^{13}C-NMR spectra of FJ-6.

determined using statistical package (Origin 6.1). Where, **At** is the absorbance of the compounds and **Ac** is absorbance of the control.

RESULTS AND DISCUSSION

The results of the carbon-13 NMR spectra of FJ-2 (Figure 1a), DEPT experiment (Figure 1b and c) and FJ-6 (Figure 1d) were in agreement with those reported by Mahato and Kundu (1994). The results of the thrombin-induced platelet aggregation study (Table 1, Figure 3) showed that FJ-2 at a concentration of 1 mg/ml showed the highest percentage inhibition (87.8 ± 1.81, IC_{50} = 3.0 mg/mL) which was observed to be higher than the standards heparin (65.9 ± 0.91, IC_{50} <1.0 mg/ml) and aspirin (65.4 ± 0.8, IC_{50} <1.0 mg/ml) at the same concentration ($P < 0.05$). But a decrease in percentage inhibition was observed as the concentration of FJ-2 increases; this implied that there is an optimal concentration (≤ 1.0 mg/ml) for inhibition of platelet aggregation by FJ-2, above which inhibition decreases. This could be as a result of the influence of FJ-2 on Calcium ion concentration which is needed in aggregation of platelets (Oh et al., 2011), it is possible the compound did not interfered with the cytoplasmic availability of Calcium ion at a concentration greater than 1.0 mg/ml therefore an increase in platelet aggregation was observed with increase in concentration of FJ-2. But a dose dependent increase in percentage inhibition of platelet aggregation was observed for FJ-6 (51.4 ± 0.65 at 1.0 mg/ml to 73.8 ± 1.72 at 10 mg/ml), the IC_{50} was recorded at 0.80 mg/ml. The two compounds are from the same nucleus but because of differences in functionality

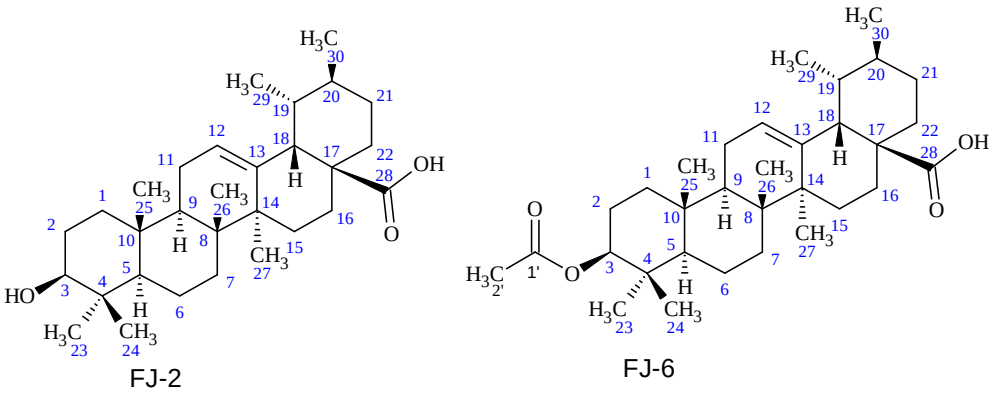

Figure 2. Structures of 3β-hydroxyurs-12-en-28-oic acid (FJ-2) and 3β-acetylurs-12-en-28-oic acid (FJ-6).

Table 1. Percentage inhibitory activity of the isolated compounds on thrombin-induced platelet aggregation.

Compounds	Concentration (mg/ml)			
	1	3	10	IC_{50}
FJ-2	87.8 ± 1.81[a]	50.9 ± 0.86[a]	45.6 ± 0.35[a]	3.005
FJ-6	51.4 ± 0.65[b]	54.9 ± 0.68[a]	73.8 ± 1.72[b]	0.800
Heparin	65.9 ± 0.91[b]	66.9 ± 0.89[b]	70.6 ± 0.09[b]	<1.00
Aspirin	65.4 ± 0.35[b]	72.1 ± 0.96[b]	76.5 ± 1.22[b]	<1.00

Values of different letters along a column are significantly different at 95% probability level (P < 0.05).

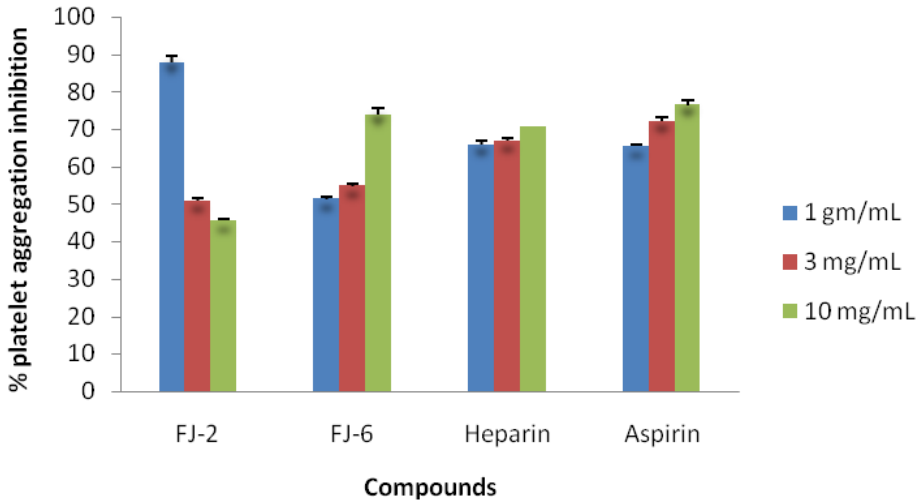

Figure 3. Compounds FJ-2 and FJ-6 inhibited Thrombin induced rat platelet aggregation. Data are presented as mean ± SEM (n=3).

(the presence of acetyl on the C-3 position) behave differently towards platelet aggregation inhibition. When adenosine diphosphate (ADP) was used as the agonist for platelet aggregation, FJ-2 and FJ-6 showed a reversal in behaviour (Table 2 and Figure 4), FJ-2 showed a dose dependent increase in platelet aggregation inhibition

Table 2. Percentage inhibitory activities of the isolated compounds on ADP-induced platelet aggregation.

Compounds	Concentration (mg/ml)			
	1	3	10	IC_{50}
FJ-2	0.0 ± 0.59[a]	59.9 ± 5.26[a]	78.6 ± 0.83[a]	<1.00
FJ-6	63.2 ± 1.02[b]	48.5 ± 0.49[a]	42.4 ± 0.49[b]	<1.00

Values of different letters along a column are significantly different at 95% probability level (P < 0.05).

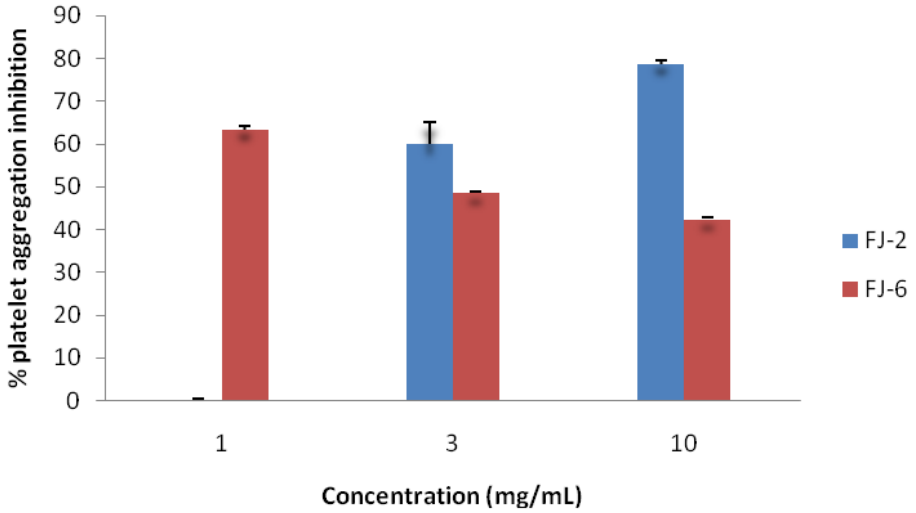

Figure 4. Compounds FJ-2 and FJ-6 inhibited ADP induced rat platelet aggregation. Data are presented as mean ± SEM (n = 3).

Table 3. Percentage inhibitory activities of the isolated compounds on Epinephrine-induced platelet aggregation.

Compounds	Concentration (mg/ml)			
	1	3	10	IC_{50}
FJ-2	0.7 ± 0.74[a]	0.0 ± 0.19[a]	15.1 ± 0.76[a]	>10.0
FJ-6	58.4 ± 0.97[b]	55.2 ± 0.52[b]	22.8 ± 0.19[a]	<1.00

Values of different letters along a column are significantly different at 95% probability level (P < 0.05).

(59.9 ± 5.26 at 3.0 mg/ml and 78.6 ± 0.83 at 10 mg/ml), while FJ-6 showed a decrease in inhibition with increase in concentration (63.2±1.02 at 1.0 mg/ml and 42.4 ± 0.49 at 10 mg/ml). A similar pattern was also observed with epinephrine as agonist (Table 3, Figure 5) while FJ-2 showed a slight increase in percentage inhibition 15.1 ± 0.76 at 10 mg/ml, FJ-6 showed the highest percentage inhibition at 1.0 mg/ml (58.4 ± 0.97) and the lowest at 10 mg/ml (22.8 ± 0.19) decreasing with increase in concentration.

Conclusion

The IC_{50} results of the *in vitro* assays using freshly isolated rat platelets, indicates that FJ-6 showed significant inhibition ($p < 0.05$) of thrombin (0.8 mg/ml), epinephrenine (<1.00 mg/ml) and ADP (<1.00 mg/ml) - induced platelet aggregation as compared to IC_{50} results of FJ-2; thrombin (3.0 mg/ml), ADP (<1.0 mg/ml) and epinephrenine (>10 mg/ml). The main findings of this study suggest that, antiplatelet activity of this plant compounds is dose dependant irrespective of the agonist and functional group modification (FJ-6) has a great influence on platelet aggregation inhibition, therefore these compound may be considered as a lead source candidate in the search of anti-platelet agents.

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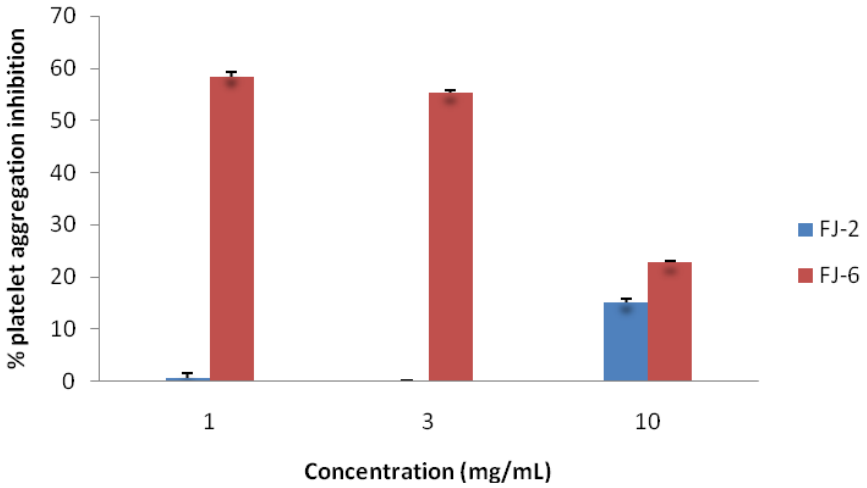

Figure 5. Compounds FJ-2 and FJ-6 inhibited epinephrine induced rat platelet aggregation. Data are presented as mean ± SEM (n = 3).

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