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Contributed Paper

Petasites japonicus Ethanol Extract Inhibited Collagen-Induced Platelet Aggregation and Thrombus Formation in Rats

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ABSTRACT

Petasites japonicus (*P. japonicus*), a perennial plant of Asteraceae family, has been reported for antioxidant, anti-obesity and anti-allergic effects. However, the anti-platelet and anti-thrombotic activities of this plant were remained unclear. Therefore, this study aimed to portray the anti-platelet and anti-thrombotic properties of *P. japonicus* ethanol extract (PJET) in rats. PJET (31 -500 μg/ml) impaired *in vitro* collagen-stimulated platelet aggregation, $[Ca^{2+}]i$ mobilization and ATP secretion dose dependently. Moreover, PJET attenuated p38 mitogen-activated protein kinase, c-Jun N-terminal kinase 1 (JNK1) and extracellular-signal-regulated protein kinase 2 (ERK2) protein phosphorylations. Likewise, PJET diminished thrombus formation in extracorporeal shunt model. In conclusion, PJET could be a source of potential natural compounds that reduce disseminated intravascular platelet aggregation and thrombus formation.

Keywords: *Petasites japonicus*, platelet aggregation, thrombus formation, mitogen-activated protein kinases

1. INTRODUCTION

Platelets are small anucleate blood cells derived from bone marrow megakaryocytes. Under normal conditions, platelets maintain hemostasis, however, inappropriate platelet activation leads to cardiovascular diseases such as atherosclerosis [1] and thrombosis [2]. At the sites of vascular injury, collagen rich subendothelial matrix is exposed and this triggers platelet activation. Platelets express integrin α2β1 and glycoprotein VI (GPVI) receptors that serve collagen induced platelet activation through the release of granular contents such as intracellular calcium ($[Ca^{2+}]_i$), ATP, ADP [dense granules] and thromboxan A_2 (TXA_2), fibrinogen, P-selectin [alpha-granules]. The release of these granular

contents further amplifies platelet activation and aggregation [3,4].

Mitogen-activated protein kinases (MAPKs), family of serine/threonine kinases, regulate cell proliferation, differentiation, mitosis, survival, and apoptosis. MAPKs are activated by a variety of extracellular stimuli such as growth factors and hormones. Platelet MAPKs, such as extracellular-signaling-regulated kinase 2 (ERK2), $p38^{MAPK}$, and c-Jun NH2-terminal kinase 1 (JNK1) are activated by platelet agonists [5]. While ERK2 plays a role in ADP-induced TXA2 generation; and JNK1 and p38 are mainly involved in collagen-induced-platelet aggregation [6]. On the other hand, natural products that impair MAPK phosphorylations and impede the release of platelet granular contents may possess potential therapeutic value against aberrant platelet activation and aggregation prone to cardiovascular diseases.

Petasites japonicus (*P. japonicus*), also known as butterbur, is herbaceous perennial plant belonging to the *Asteraceae* family. Extracts from *Petasites* have been reported for therapy against migraines, gastric ulcer, asthma, and oxidant stress [7-10]. However, the effect of *P. japonicus* extracts on agonist induced platelet aggregation and thrombus formation haven't been evaluated. Therefore, this study aimed to investigate the anti-platelet activity of *P. japonicus* ethanol extract (PJET) on collagen stimulated rat platelets *in vitro* and thrombus formation *in vivo*.

2. MATERIALS AND METHODS

2.1 Materials

Fura-2/AM was obtained from Sigma Co. (St. Louis, USA). Collagen was purchased from Chronolog (Havertown, USA). Antibodies to total ERK1/2, phospho-ERK1/2, total p38 MAPK, phospho-p38 MAPK, total JNK1, and phospho-JNK1 were from Cell Signaling (Beverly, USA). ATP assay kit from Biomedical Research Service Center (Buffalo, USA). All other chemicals were of reagent grade.

2.2 Sample Preparation

Petasites japonicus (*P. japonicus*) extract was acquired from whole plants of *P. japonicus* using 70% ethanol. Whole plants of *P. japonicus* were collected at the Seodae area of Chubu, Keumsan, Korea and standard extracts were obtained from wet butterbur stem. The plants were dried and powdered (100g), and then extracted with 70% ethanol (3×0.5 L) for 2 days at room temperature. The combined liquid extracts were concentrated under reduced pressure. A small portion from this extracts (5 mg) was further analyzed by GC-MS with temperatures of GC 70°C→300°C (10 min) at a rate of 7°C / min, injector (200°C) and detector (300°C) in a helium carrier gas at a flow rate of 1.0 ml/min. PJET comprised of phenol (14.7%), catechin (10.6%), palmitic acid (10.4%), xylitol (5.1%) and bakkenolide (4.5%) (Table1).

Table 1. GC-MS analysis of PJET chemical components PJET (5 mg) was analyzed by GC-MS in a helium carrier gas at a flow rate of 1.0 ml/min. PJET major components such as phenol, catechin, palmitic acid, xylitol and bakkenolide were identified.

Name of compound	Peak area (%)
Phenol	14.7
Catechin	10.6
Palmitic acid	10.4
Xylitol	5.1
Bakkenolide	4.5

2.3 Animals

Male SD rats (240 to 250 g) were obtained from Orient Co. (Pusan, Korea). The SD rats were maintained in a standard animal facility with free access to feed and water *ad libitum*. They were acclimated for

at least one week before experiments. The experiments were performed in accordance to internationally accepted guidelines on the use of laboratory animals. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Kyungpook National University.

2.4 Platelet Preparation

Platelet preparation was performed as previously described [11]. Briefly, rat blood was obtained using the heart puncture and transferred to a 15 ml test tube containing anticoagulant (ACD, 85 mM trisodium citrate, 83 mM dextrose, and 21 mM citric acid). The platelet-rich plasma (PRP) was obtained by centrifugation at 170 × g for 7 min. The platelets were precipitated and isolated by centrifugation (350 × g, 10 min) and then adjusted to the proper number (10^8/ml) in Tyrode buffer (137 mM NaCl, 12 mM $NaHCO_3$, 5.5 mM glucose, 2 mM KCl, 1 mM $MgCl_2$, 0.3 mM NaHPO4, pH 7.4). All above procedures were conducted at room temperature.

2.5 Platelet Aggregation Assay

Platelet aggregation assay was carried out as reported earlier [12]. In brief, platelet aggregation was determined using light transmission aggregometer (Chronolog, Havertown, USA). Washed platelets were pre-incubated at 37%C for 2 min with PJET and then further incubated for 5 min with collagen before aggregation was determined. In order to exclude the artificial effect of vehicle (i.e., DMSO), the vehicle concentration kept under 0.05%.

2.6 $[Ca^{2+}]_i$ and ATP Release Assays

$[Ca^{2+}]_i$ was determined with fura-2/AM as described previously [13]. Briefly, the PRP was incubated with 5 mM of fura-2/AM for 60 min at 37°C. The fura-2-loaded washed platelets (10^8/ml) were stimulated by collagen. Fura-2 fluorescence was measured in spectrofluorometer (F-2500, Hitachi, Japan) with an excitation wavelength that ranges from 340 nm to 380 nm, changing every 0.5 sec, and with the emission wavelength of 510 nm. The $[Ca^{2+}]_i$ was calculated as described previously [14]. For ATP release assay, washed platelets were pre-incubated for 2 min at 37°C with PJET and then stimulated with collagen for 5 min. After centrifugation at 12,000 rpm for 2 min, supernatants were used to determine ATP release in a luminometer (GloMax 20/20, Promega, USA).

2.7 Immunoblotting

After pre-treatment of washed platelets with PJET or vehicle, the platelets were stimulated with collagen and incubated for 5 min in an aggregometer. Platelet preparation and immunobloting were described as previously described [11]. Briefly, protein concentration was determined using PRO-MEASURE (iNtRON Biotechnology, Seoul, Korea). Equal volume proteins were resolved in 10% SDS-PAGE and transferred to nitrocellulose membrane in a transfer buffer (25 mM Tris (pH 8.5), 0.2 M glycine, and 20% methanol). Immunoblots were blocked with TBS-T containing 5% nonfat dry milk and incubated with primary antibody diluted in a blocking solution. The immunoblots were again incubated with HRP secondary antibody and the membranes were visualized using enhanced chemiluminescence, ECL (iNtRON Biotechnology, Korea).

2.8 Evaluating Thrombus Formation in Extracorporeal Shunts

The *in vivo* antithrombotic activity of PJET was evaluated in a rat extracorporeal shunt model by the method of Umetsu and

Sanai [15] with a slight modification [16]. Briefly, 1 hr after administration of PJET (100-500 mg/kg, p.o.) or vehicle, non-fasted male rats were anaesthetized with urethane (1.75 g/kg, i.p.) and an incision was made over the trachea. The right jugular vein and left carotid artery were exposed and the two ends of the extracorporeal shunt were inserted into them. The shunt consisted of two 12 cm lengths of polyethylene tubing (0.81 mm and 0.58 mm external and internal diameter, respectively) connected by 5 mm silicone rubber plugs to a 6 cm length of polyvinyl tubing (3 mm internal diameter). A 6 cm length of cotton thread was secured between the two plugs so that it remained longitudinally orientated in the blood flow through the cannula. The shunt was left in place for 15 min after the extracorporeal circulation was started. The flow was then stopped, the thread removed and the formed thrombus weighed.

2.9 Statistical Analysis

Data were analyzed by a one-way analysis of variance, followed by a post hoc Dunnett's test in order to determine the statistical significance of the differences using Statistical Analysis Software (SAS Institute Inc., NC, USA). All data are presented as means ± SEM. p values of 0.05 or less were considered to be statistically significant.

3. Results

3.1 PJET Impaired Collagen-Induced Platelet Aggregation

Collagen at a concentration of 1 μg/ml induced strong platelet aggregation, however, this was dose dependently inhibited by pretreatment of platelets with PJET at a concentration range of 31-500 μg/ml with a marked effect observed at the highest concentration (Figure 1).

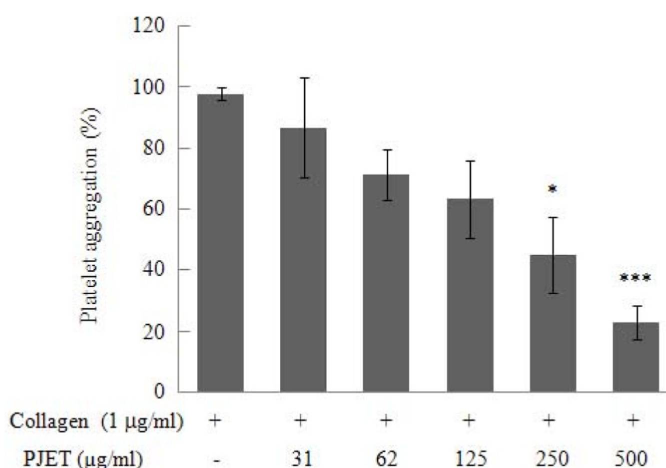

Figure 1. Inhibitory Effect of PJET on platelet aggregation. Platelets (10^8/ml) were pre-incubated with either PJET or vehicle in the presence of 1 mM $CaCl_2$ for 2 min at 37°C and then platelet aggregation was induced by 1 μg/ml collagen. The aggregation reaction was terminated at 5 min and the percent aggregation rate was determined. Each graph shows mean ± SEM of at least 3 independent experiments performed. * $P < 0.05$, *** $P < 0.001$ vs. collagen treatment.

3.2 PJET Inhibited Intracellular Calcium Mobilization and ATP Release

It has been established that intracellular calcium mobilization plays an important role in platelet aggregation. Here, therefore, we investigated whether pretreatment of platelets with PJET diminishes $[Ca^{2+}]_i$ mobilization in collagen stimulated rat platelets. Our finding revealed that pretreatment of platelets with PJET (125-500 μg/ml) showed a marked decrease in $[Ca^{2+}]_i$ mobilization in a concentration dependent pattern (Figure 2 A). At the highest concentration (500 μg/ml), the inhibitory effect of PJET on $[Ca^{2+}]_i$ mobilization was similar to that of the basal level. Likewise, PJET at the same concentrations moderately attenuated ATP release (Figure 2B).

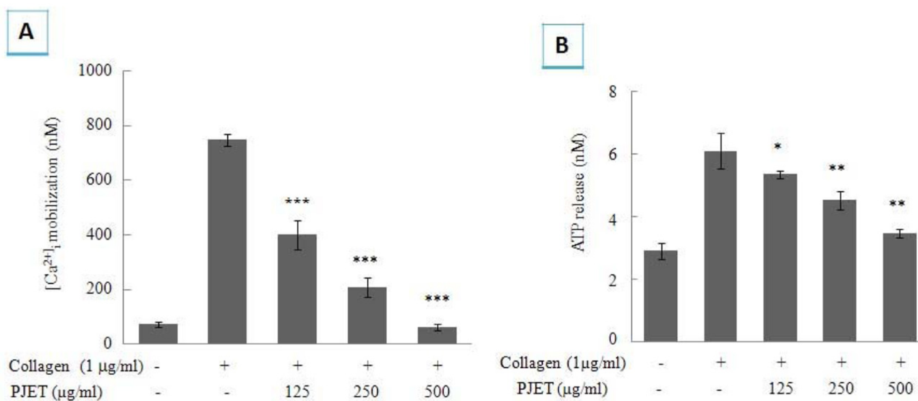

Figure 2. Inhibitory effect of PJET on $[Ca^{2+}]i$ mobilization and ATP release. Washed platelets were incubated with calcium fluorophore (Fura-2/AM) and stimulated with collagen to determine $[Ca^{2+}]_i$ mobilization (A). For ATP release assay (B), washed platelets were pre-incubated with PJET at the indicated concentrations, stirred in an aggregometer for 2 min prior to collagen stimulation for 5 min, and then the reactions were terminated. Results indicate 4 independent experiments performed and bar graphs are presented as mean ± SEM. * $P<0.05$, ** $P<0.01$, *** $P < 0.001$ vs. collagen treatment.

3.3 PJET Attenuated Phosphorylations of ERK, JNK and $P38^{MAPK}$ Proteins in Collagen Stimulated Platelets.

It has been reported that MAPK proteins such as ERK, JNK and p38 are activated when platelets are exposed to agonists such as collagen. It is therefore plausible to investigate whether PJET attenuated phosphorylations of the aforementioned kinases in rat platelets stimulated with collagen *in vitro*. PJET (125 -500 μg/ml) attenuated p-ERK, p-JNK1 and p-p38 protein expressions with a strong inhibitory effect observed on p-ERK and p-JNK1 (Figure 3A &B) in contrast to the moderate inhibitory effect observed on p-p38 (Figure 3C).

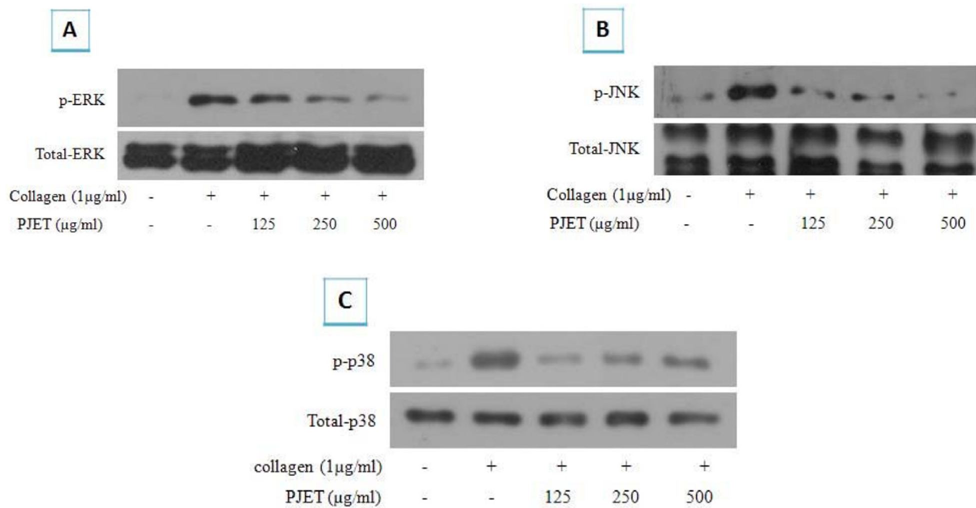

Figure 3. The Effects of PJET on collagen induced MAPK phosphorylations. Washed platelets were stirred in aggregometer with vehicle or PJET for 3 min prior to the addition of collagen for 5 min and then reactions were terminated. Platelet proteins were extracted, separated by SDS-PAGE, blotted onto a PVDF membrane and probed against MAPK antibodies (A, B, C). Images are representatives of 4 independent experiments.

3.4 PJET Reduced Thrombus Formation in Arterio-venous Shunt Model.

To verify whether the anti-platelet activity of PJET *in vitro* in collagen induced platelets also in line with anti-platelet activity *in vivo*, we carried out arterio-venous shunt model in rats orally administered with PJET. Using an arterio-venous shunt model, we showed that PJET at 250 and 500 mg/kg reduced thrombotic mass with a maximal reduction of 30% observed at the highest concentration used (Figure 4), indicating that PJET attenuates platelet aggregation *in vitro* too.

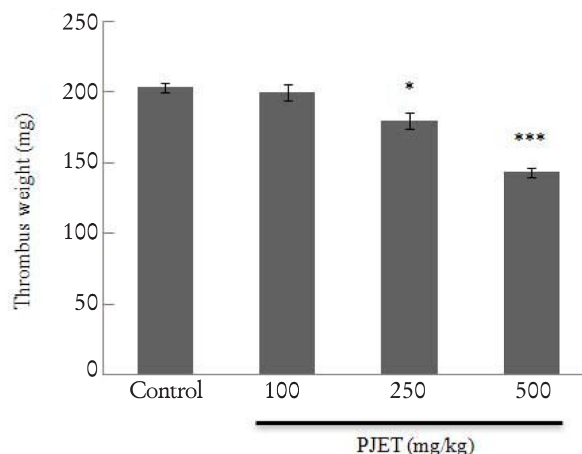

Figure 4. The effect of PJET on thrombus formation in rats. PJET or vehicle was given orally 1 h before thrombus induction. Blood circulation in the cannula shunt was carried out for 15 min and thrombus weight was immediately determined. Bar graphs show mean ± SEM of at least 3 independent experiments performed. * $P < 0.05$ *** $P < 0.001$ vs. control

4. Discussion

Platelet activation and aggregation take a critical role in the development of cardiovascular diseases such as atherosclerosis, thrombosis, and stroke. Platelets have well developed granules that are the storage sites of various mediators involved in platelet activation. Upon agonist binding, platelets start to spread and assume a pseudopodial shape with subsequent release of bioactive granular substances involved in platelet activation and aggregation. Thus, blocking of aberrant platelet activity using agents that inhibit ligand-induced platelet activation and aggregation has therapeutic or preventive merit against cardiovascular diseases.

Current antithrombotic drugs such as aspirin and ticlopidine have been widely prescribed, however, they have reported unwanted side effects such as bleeding disorders [17-19]. As such, efforts have been made to look for novel antithrombotic agents using medicinal plants with little side effects. In this regard, we investigated whether PJET hinders platelet aggregation *in vitro* and thrombus formation *in vivo*. We found that PJET dose-dependently inhibited collagen-induced platelet aggregation, $[Ca^{2+}]_I$ mobilization, ATP release, and MAPK phosphorylations. All these effects were further supported by anti-thrombotic activity with an arterio-venous shunt model *in vivo*.

It is well known that intracellular calcium ion release is enhanced when platelet receptors are stimulated with various agonists [20-22]. This ligand-binding to their cognate receptors activates phospholipase C (PLC) proteins that can cleave phosphatidylinositol 4, 5-bisphosphate into diacylglycerol and inositol 1,4,5-trisphosphate (IP3). IP3 is subsequently binds to its receptor on a ligand-gated Ca^{2+} channel in dense granules, resulting in the calcium mobilization [23, 24]. This rise in intracellular calcium activates multiple target molecules, such as Ca^{2+}/calmodulin kinase, NO synthases, and protein kinase C [25-27] causing platelet activation and aggregation. Although the precise target PJET remained unknown, we speculated that it might be through inhibition of PLC pathway.

MAPKs (ERK1/2, $p38^{MAPK}$ and JNK) are constitutively expressed and activated in platelets by agonists such as thrombin, collagen and ADP [5, 28, 29]. Despite the contentious role of MAPKs in the signal transduction pathways in platelets, they have been the recent spotlight of growing research in various platelet signal transductions [5]. It is interesting that MAPK phosphorylations were restrained by PJET pre-treatment, suggesting that the anti-platelet and anti-thrombotic activities of this extract were partly mediated through the inhibition of MAPK and all these effects could be due to potent antioxidants present in PJET such as phenol and catechins, which have been reported to have anti-platelet activities [30-33] and cardiovascular benefits [34, 35]. Moreover, Liao et al (1997) reported that bakkenolide G, extracted from *Petasites formosanus* inhibited platelet-activating factor (PAF)-induced platelet aggregation and ATP release [36], suggesting the additional role Bakkenolide G in the current anti-platelet activity of PJET.

5. Conclusion

Taken together, our finding showed that PJET inhibited collagen induced *in vitro* platelet aggregation, $[Ca^{2+}]_i$ mobilization, ATP release, and MAPK phosphoryations. Moreover, PJET reduced *in vivo* arterovenous thrombus formation. Thus, extracts from *Petasites japonicus* could be considered as relevant anti-platelet agents against aberrant platelet activation and aggregation.

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