

Aqueous Extract of *Tournefortia sarmentosa* Stem Inhibits ADP-induced Platelet Aggregation

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Wang, *et al.*: *T. sarmentosa* Inhibits Platelet Aggregation

Tournefortia sarmentosa is a Chinese herbal medicine used as an antioxidant, detoxicant, and antiinflammatory agent. The present study evaluated the effect of *T. sarmentosa* on adenosine diphosphate-induced platelet aggregation. Our results showed that aqueous stem extract of *T. sarmentosa* inhibited adenosine diphosphate-induced platelet aggregation. In addition, we found components in *T. sarmentosa*, including caffeic acid, rosmarinic acid, salvianolic acid, play important roles in mediating adenosine diphosphate-induced platelet aggregation suppression. The stem extract of *T. sarmentosa* inhibited the adenosine diphosphate receptor P2Y₁₂-mediated cyclic AMP production. Caffeic acid inhibited P2Y₁-induced calcium influx. Furthermore, treatment of platelets with *T. sarmentosa*, or the components in stem extract of *T. sarmentosa*, suppressed adenosine diphosphate-induced release of thromboxane A2 and arachidonic acid and surface PAC-1 expression. These data demonstrate the aqueous stem extract of *T. sarmentosa* significantly suppressed platelet aggregation through P2Y₁ and P2Y₁₂ receptor signal pathways. The antiaggregation properties found in the stem extract of *T. sarmentosa* might help to prevent cardiovascular disease.

Key words: *Tournefortia sarmentosa*, platelet, caffeic acid, rosmarinic acid, salvianolic acid

Tournefortia sarmentosa Lam. (Boraginaceae) is a Chinese herbal medicine with antioxidant activities, detoxicating qualities and antiinflammatory uses^[1-3]. Numerous pharmacological observations have shown therapeutic effects. Lin *et al.* revealed that isolated components from the stems of *T. sarmentosa* decreased Cu²⁺-induced low-density-lipoprotein oxidation^[1]. Teng *et al.* investigated the hepatotoxicity effects of *T. sarmentosa* stem extract and found that it reduced elevated levels of liver function markers, including serum glutamate oxaloacetate transaminase (SGOT), glutamate pyruvate transaminase, and alkaline phosphatase (ALP). It also reduced levels of inflammatory markers, including tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6 in acetaminophen-intoxicated rats^[2]. However, some results measured the inflammatory cytokine expression in cells treated with *T. sarmentosa* stem extract are contradictory. For example, extracts of *T. sarmentosa* enhanced the release of cytokines, including IL-6,

IL-8, and TNF- α ^[4] and increased phagocytosis by macrophages and neutrophils^[4-5].

Various pharmacologically active compounds from aqueous stem extract of *T. sarmentosa* have been identified^[3] and these components play roles in immune or detoxification regulation. For example, salvianolic acid A, isosalvianolic acid C, and rosmarinic acid exhibited roles in reducing reactive oxygen species activity^[1]. Caffeic acid increased the phagocytic ability of macrophages and neutrophils by increasing phosphorylated p38 MAPK or AKT, respectively^[4-5]. Salvianolic acid inhibited phagocytic ability of macrophages by suppressing lipopolysaccharide-induced ERK1/2 phosphorylation^[4]. These results

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suggest that each of the components in aqueous stem extract of *T. sarmentosa* possesses a modulating role in regulating macrophage/neutrophil cells.

The main physiological function of platelets is to stop bleeding by clumping and clotting blood vessel injuries. In addition to coagulation factors (thrombin), collagen and hormones (epinephrine), adenosine diphosphate (ADP) have been well identified as a contributor to the propagation of platelet activation at sites of vascular injury. The receptors that mediate signalling of ADP-induced platelet aggregation have been thoroughly described using whole blood or platelet-rich plasma (PRP)^[6-10]. Previous studies demonstrated that two main G protein-coupled receptors; P2Y₁ and P2Y₁₂, are involved in the ADP receptor signalling of platelet activation^[11-14]. Gq-coupled ADP receptor P2Y₁ leads to the activation of phospholipase C β , and subsequently induces intracellular calcium mobilization and activates protein kinase C. P2Y₁₂ receptor activated by ADP and couples with Gi to inhibit adenylyl cyclase and activate PI3-kinase^[12,15,16]. P2Y₁₂ receptor is essential for the release of arachidonic acid from membrane-bound phospholipids, and subsequent thromboxane A₂ (TXA₂) generation catalysed by cyclooxygenase in platelets^[17]. TXA₂ functions as an amplified mediator in platelet activation^[18,19]. While normal blood clots form a protective seal over an injury, abnormalities of the blood clotting are significant causes of illness and death. The most common form of heart attack occurs when a blood clot blocks the blood flow through the heart muscle. Also, the leading cause of stroke occurs when a blood clot blocks an artery supplying blood to the brain. Antiplatelet drugs are most effective for arterial clots and are useful in preventing cardiovascular diseases.

T. sarmentosa has been reported to function as an antioxidant and enhance inflammatory responses. However, there have been no reports on the action of *T. sarmentosa* against platelet activity. In this study, we investigated the regulatory effects of aqueous stem extract of *T. sarmentosa*, and the major components in stem extract of *T. sarmentosa*, on ADP-induced platelet aggregation.

MATERIALS AND METHODS

The stems of *T. sarmentosa* Lam. were collected from Jhongpu Township, Chiayi County, Taiwan and the extraction of *T. sarmentosa* have been described previously^[4,5]. Briefly, the dried stems of *T. sarmentosa* were grinded into powder and 100 g of *T. sarmentosa*

stem powder were macerated with hot water (95°) for 2 h. The aqueous extract was filtered under vacuum at 70°. The extracted solution was centrifuged at 4° and 9000 rpm for 15 min, and then lyophilized to obtain dry powder and stored at -20° before use. The ADP, forskolin, fibrinogen, caffeic acid, rosmarinic acid, salvianolic A, and salvianolic B were obtained from Sigma-Aldrich (St. Louis, USA). The adenosine 2',5'-diphosphate (A2P5P) and clopidogrel were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of human platelets:

Human blood was obtained by venipuncture from healthy adults and collected in a vacutainer containing sodium citrate. Blood was centrifuged for 20 min at 180×g at room temperature to get PRP. Samples of PRP (2×10⁸ platelets/ml) were preincubated with *T. sarmentosa* stem extract, caffeic acid (100 μM), rosmarinic acid (100 μM), salvianolic A (100 μM), or salvianolic B (100 μM) at 37° for 30 min. PRP were then treated with A2P5P or clopidogrel for 3 min and 10 μM of ADP was added. Alternatively, washed platelets and platelet poor plasma (PPP) were obtained from PRP by centrifugation for 15 min at 1500×g. The supernatant was PPP and the pellet (washed platelets) was resuspended to a density of 2×10⁸ platelets/ml in a modified calcium-free Tyrode buffer (138 mM NaCl; 2.7 mM KCl; 1 mM MgCl₂; 3 mM NaH₂PO₄; 10 mM HEPES; 5 mM glucose; 0.2 % BSA; and 20 μg/ml apyrase, pH 7.4). All subjects signed a consent form for participation in the study and our study was reviewed and approved by Taipei Tzuchi Hospital, the Buddhist Tzuchi Medical Foundation Institutional Review Board.

Measurement of platelet aggregation:

Platelet aggregation was measured in PRP by the turbidimetric method in duplicate, using a light transmittance aggregometer (PAP-8E, Platelet Aggregation Profiler, Bio/Data Corporation, Horsham, PA). Briefly, ADP-induced platelet aggregation was monitored using an aggregometer at 37° under continuous stirring. Baseline optical density was set using the PPP sample. Aggregation was induced by addition of 10 μM ADP and monitored for 6 min by optical aggregometry.

Measurement of intracellular calcium in platelets:

PRP was pre-treated with *T. sarmentosa* stem extract or each compound (100 μM) for 30 min. About 3 μM

Fura-2AM (Molecular Probes, Invitrogen, Carlsbad, CA) was added to the PRP and incubated at 37° for 45 min as mentioned in an aggregometer at 37° with stirring at 900 rpm. Platelets were centrifuged and resuspended in modified calcium-free Tyrode buffer. ADP (10 µM)-induced calcium responses were subsequently measured at 37° in a multi-functional microplate reader (Infinite F200, Tecan, Durham, NC) with fluorescence excitation set at 340 nm and 380 nm, emission was set to 510 nm.

Measurement of cyclic AMP (cAMP) levels and TXA2 in platelets:

PRP was treated with *T. sarmentosa* stem extract or each compound (100 µM) for 30 min. Washed platelets were prepared in modified calcium-free Tyrode buffer and then incubated with 10 µM forskolin and 10 µM ADP at 37° for 5 min as mentioned in an aggregometer at 37° with stirring at 900 rpm. After washing twice with PBS, cells were lysed with 0.1 N HCl, scraped, and collected by centrifugation. Levels of cAMP in the supernatants were determined using a cAMP enzyme immunoassay (EIA) kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

PRP was treated with *T. sarmentosa* stem extract or each compound (100 µM) for 30 min. Washed platelets were prepared and then stimulated with 3 µM fibrinogen and 10 µM ADP at 37° for 3 min as mentioned, in an aggregometer at 37° with stirring at 900 rpm. The reaction was stopped by quickly freezing the sample in a dry ice-ethanol bath. After thawing at room temperature, the samples were centrifuged at 3000×g for 10 min at 4°. The supernatants were collected and the content of thromboxane B2, the stable metabolite of TXA2, was measured using an EIA according to the manufacturer's instructions (Cayman Chemical).

Measurement of arachidonic acid release in platelets:

PRP was treated with *T. sarmentosa* stem extract or each compound (100 µM) for 30 min. Washed platelets were prepared and then activated with 10 µM ADP and 3 µM fibrinogen at 37° for 3 min as mentioned in an aggregometer at 37° with stirring at 900 rpm. Levels of arachidonic acid in the supernatants were determined using a human arachidonic acid ELISA kit (Cusabio Biotech, Wuhan, Hubei, China).

Measurement of platelet activation markers expression:

Whole blood was diluted 1:2 with PBS. The diluted

whole blood (20 µl) was treated with *T. sarmentosa* stem extract or each compound (100 µM) for 30 min. Diluted whole blood was stained with phycoerythrin or fluorescein isothiocyanate-conjugated antibodies targeting CD61 (BD Pharmingen, San Jose, CA) and PAC-1 (BD Pharmingen) along with 20 µM ADP for 20 min at room temperature. The labelled whole blood were immediately fixed with 400 µl of 1 % (W/V) paraformaldehyde at room temperature for 30 min and subjected to flow cytometry analysis (FACScan, Becton Dickinson, Franklin Lakes, NJ).

Statistical analysis:

All values are expressed as mean±SD of at least triplicate samples. Statistical analyses were assessed using one-way ANOVA with Dunnett's post hoc test for comparison of more than two groups. A p-value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

To examine the effect of *T. sarmentosa* on human platelet aggregation, human PRP were treated with various doses of *T. sarmentosa* stem extract for 30 min. After *T. sarmentosa* stem extract treatment, platelet aggregation was examined using an aggregometer. Results shown ADP-induced aggregation was suppressed in a dose-dependent manner, with maximal inhibition (37.8 %) in cells treated with 100 µg/ml *T. sarmentosa* stem extract (fig. 1A and B). To sort out the possibility of contamination of polysaccharides in the extract to exhibit antiaggregative activity, PRP were pre-treated with polymyxin B. We found the suppressive platelet aggregation by *T. sarmentosa* stem extract was not affected by treating platelet with polymyxin B (fig. 1C and D). The results indicate that *T. sarmentosa* stem extract exhibited antiaggregative ability.

We also determined the effects of commercially available compounds, found in aqueous stem extract of *T. sarmentosa* on the aggregative capability of human platelets. Results show when human PRP were treated with *T. sarmentosa* stem extract, and the components studied of *T. sarmentosa* stem extract, ADP-induced platelet aggregation was suppressed. *T. sarmentosa* stem extract, rosmarinic acid, salvianolic acid A and salvianolic B significantly suppressed aggregation by 27.04 % to 61.37 %, and the caffeic acid had the weakest effect (the aggregative ability was decreased by 16.3 %) on ADP-induced platelet aggregation suppression among the major components in stem extract of *T. sarmentosa* tested (fig. 2A and B).

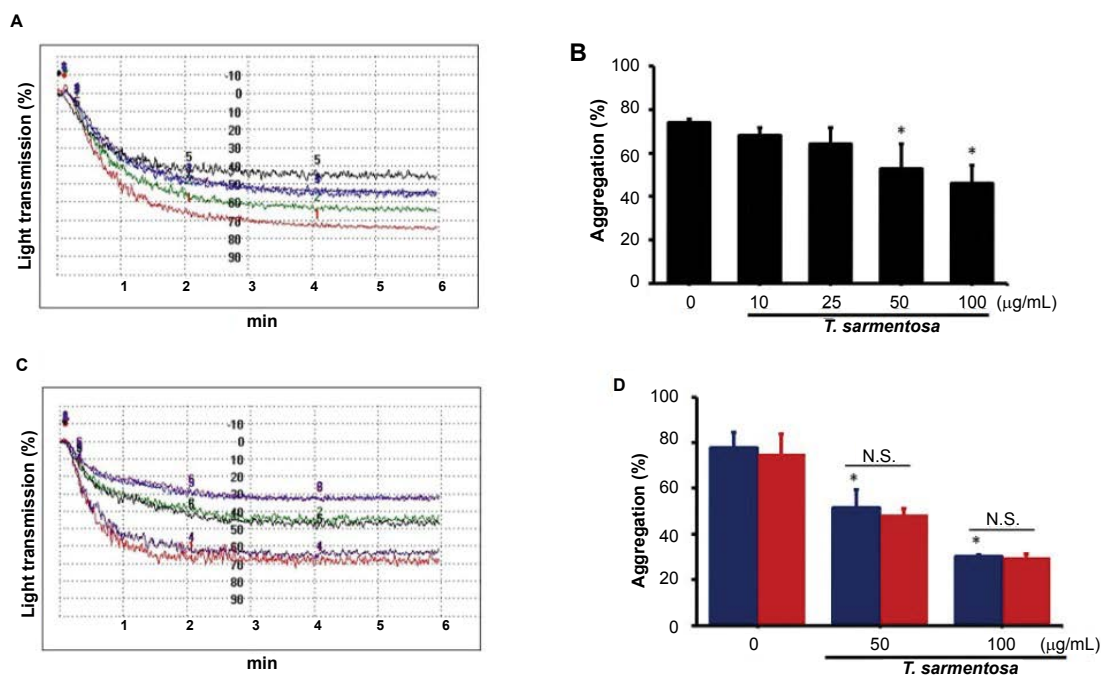


Fig. 1: The effect of *T. sarmentosa* stem extract on ADP-induced platelet aggregation

A. — 100 µg/ml *T. sarmentosa*; — 50 µg/ml *T. sarmentosa*; — 25 µg/ml *T. sarmentosa*; — 10 µg/ml *T. sarmentosa*; — control; C. — 100 µg/ml *T. sarmentosa*+polymyxin B; — 100 µg/ml *T. sarmentosa*; — 50 µg/ml *T. sarmentosa*; — 50 µg/ml *T. sarmentosa*+polymyxin B; — polymyxin B; — control; D. ■ control; ■ polymyxin B

Two G-protein-coupled receptors, P2Y₁ and P2Y₁₂, play a central role in ADP-mediated platelet aggregation. To explore the potential signalling involved in *T. sarmentosa*-suppressed platelet aggregation, we treated PRP with P2 receptor antagonists. As shown in fig. 3A and B, ADP-induced platelet aggregation was blocked by 37 % by the P2Y₁ receptor antagonist A2P5P (1 mM). Pretreatment with 50 µg/ml *T. sarmentosa* stem extract, and the components studied of *T. sarmentosa* stem extract (100 µM), significantly reduced platelet aggregation by 15.5 % to 56 % in platelets incubated with A2P5P. ADP-induced platelet aggregation was blocked by 21.7 % in PRP pretreated with P2Y₁₂ receptor antagonist clopidogrel (30 µM). *T. sarmentosa* stem extract and caffeic acid further blocked platelet aggregation by 34 % in platelets incubated with clopidogrel (fig. 3C and D). The clopidogrel-mediated ADP-induced platelet aggregative suppression was not affected by PRP pretreated with rosmarinic acid, salvianolic A, or salvianolic B (fig. 3C and D). ADP-induced platelet aggregation was decreased by 50.9 % in platelets pretreated with A2P5P and clopidogrel. We found that neither *T. sarmentosa* stem extract nor the components studied of *T. sarmentosa* stem extract affected platelet aggregative ability when PRP pretreated with A2P5P and clopidogrel (fig. 3E and F). These results suggest

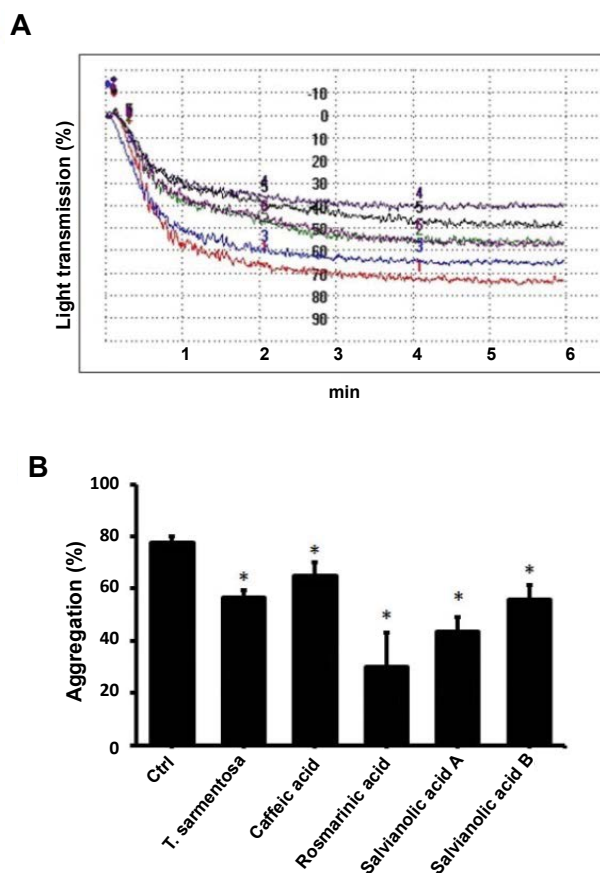


Fig. 2: The effect of constituents derived from aqueous stem extract of *T. sarmentosa* on ADP-induced platelet aggregation

— Salvianolic acid A — rosmarinic acid; — salvianolic acid B; — *T. sarmentosa*; — caffeic acid; — control

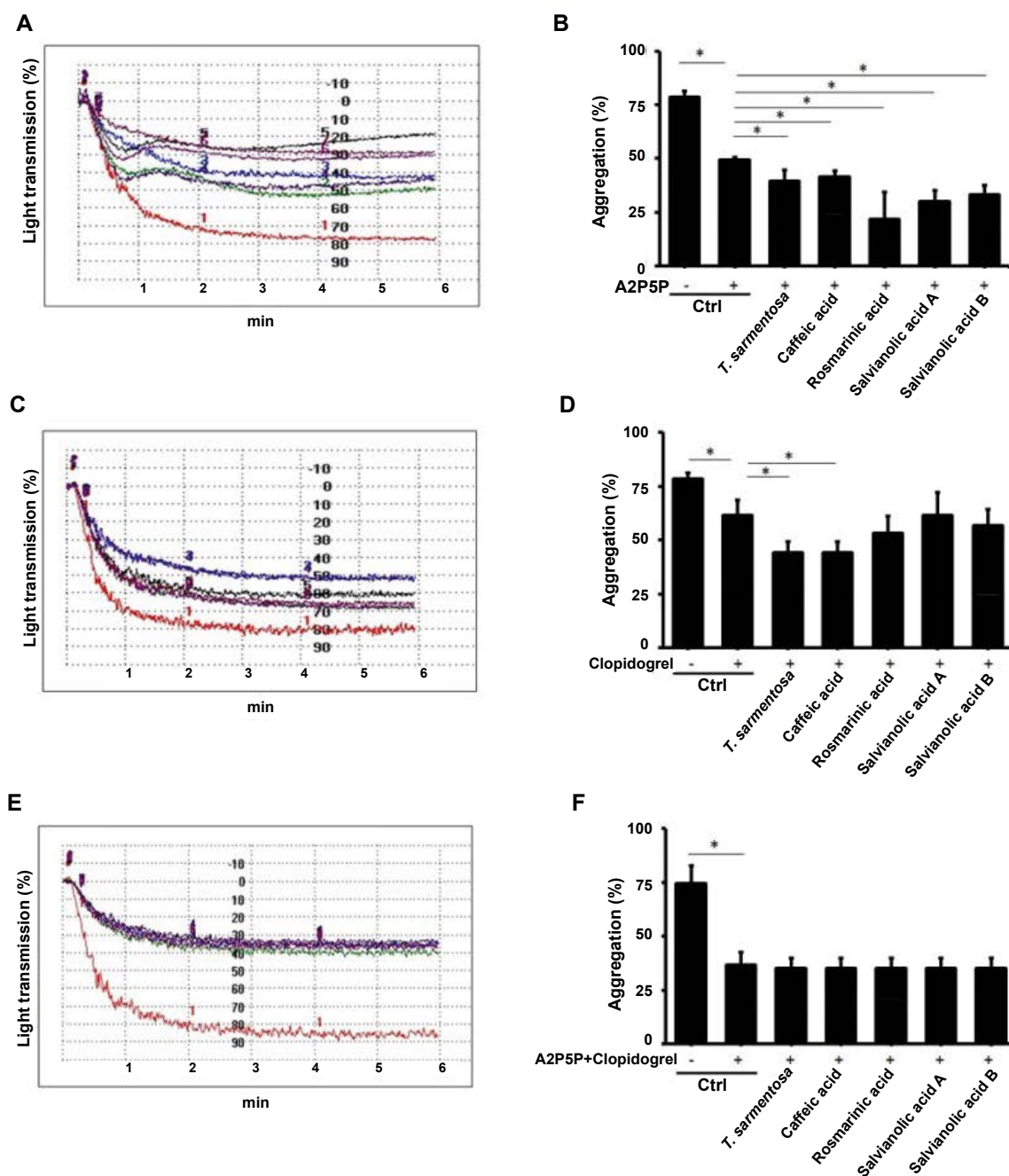


Fig. 3: The effect of P2 receptor antagonists on *T. sarmentosa*-mediated platelet aggregative suppression

A. — Rosmarinic acid+A2P5P; — salvianolic acid B+A2P5P; — salvianolic acid A+A2P5P; — *T. sarmentosa*+A2P5P; — caffeic acid+A2P5P; — control+A2P5P; — control: C. — *T. sarmentosa*+clopidogrel; — caffeic acid+clopidogrel; — rosmarinic acid+clopidogrel; — salvianolic acid B+clopidogrel; — salvianolic acid A+clopidogrel; — control+clopidogrel; — control: E. — rosmarinic acid+A2P5P+clopidogrel; — salvianolic acid B+A2P5P+clopidogrel; — salvianolic acid A+A2P5P+clopidogrel; — *T. sarmentosa*+A2P5P+clopidogrel; — caffeic acid+A2P5P+clopidogrel; — control+A2P5P+clopidogrel; — control

that *T. sarmentosa* stem extract and caffeic acid inhibited platelet aggregation via P2Y₁ and P2Y₁₂ receptors. And, rosmarinic acid, salvianolic A, and salvianolic B blocked platelet aggregation through P2Y₁₂ receptor.

To further understand the mechanism involved in *T. sarmentosa*-mediated platelet aggregation suppression, we examined the effect of *T. sarmentosa*

stem extract on ADP-induced calcium influx and cAMP suppression in PRP. Our data show reduced calcium responsiveness ranging from 23.1 % to 52 % in PRP pretreated with *T. sarmentosa* stem extract and caffeic acid (fig. 4A). Rosmarinic acid, salvianolic A, and salvianolic B had no effect on the ADP-induced calcium influx. In addition, a reduction of cAMP production was observed in forskolin-stimulated treated with ADP.

Preincubation with *T. sarmentosa* reverted the cAMP production in a dose-dependent manner in forskolin-induced PRP treated with ADP (fig. 4B). Similarly, an increased in cAMP was observed in forskolin-and ADP-treated PRP pre-incubated with the components studied of *T. sarmentosa* stem extract.

The TXA₂ released during platelet aggregation stimulates activation of new platelets. To explore the effects of *T. sarmentosa* on ADP-enhanced platelet aggregation, we measured the expression of TXB₂, the stable metabolite of TXA₂. To induce TXB₂ production, PRP were stimulated with fibrinogen (30 µM) and ADP. As expected, *T. sarmentosa* stem extract inhibited TXB₂ production in a dose-dependent manner. Similar inhibitory activity was observed in PRP pre-treated with the components studied of *T. sarmentosa* stem extract (fig. 5A). TXA₂ is generated by activated platelets, which convert arachidonic acids through the cyclooxygenase pathway. We determined the effect of *T. sarmentosa* stem extract on ADP-induced arachidonic acid production. As expected, decreased arachidonic acid production was observed in ADP-induced PRP treated with *T. sarmentosa* stem extract or the components studied of *T. sarmentosa* stem extract (fig. 5B).

Glycoproteins found on platelets play crucial roles in platelet-mediated aggregation and interactions with the extracellular matrix. The glycoprotein complex IIb/IIIa changes its conformation leading to binding of adhesive proteins that contain RGD motif during platelet activation. Whole blood flow cytometry can analyse the exposed glycoproteins on the surface of platelets and measure platelet activation *in vivo*.

Therefore, in order to elucidate the antiaggregative ability of *T. sarmentosa* stem extract on platelets *in vivo*, we detected the expression of surface CD61 (glycoprotein IIIa) and PAC-1 (glycoprotein IIb/IIIa). As shown in fig. 6A and 6B, the surface CD61 expression of platelets was slightly enhanced by 1.1-fold; PAC-1 was significantly increased by 11.4-fold upon ADP addition. We observed dose-dependent decrease of surface PAC-1 expression in *T. sarmentosa* stem extract-treated whole blood. Results show it did not affect the surface CD61 expression of platelets (fig. 6A and B). We examined the effect of the components studied of *T. sarmentosa* stem extract on the surface CD61 and PAC-1 expression of platelets. Treatment of whole blood with the components studied of *T. sarmentosa* stem extract significantly decreased surface PAC-1 expression by 65 % to 82 %. Similarly, the components studied of *T. sarmentosa* stem extract had no effect on the surface CD61 expression of platelets (fig. 6C and D).

The results here demonstrate that aqueous stem extracts of *T. sarmentosa* significantly decreased ADP-induced platelet aggregation. Also, aqueous stem extract of *T. sarmentosa* alleviated ADP-mediated calcium influx and production of cAMP, TXA₂, and arachidonic acid in platelets and the activation surface marker PAC-1 expression of platelets. These findings suggest that aqueous stem extract of *T. sarmentosa* affects platelet activity.

The constituents of the aqueous extract of *T. sarmentosa* stems were further investigated in *T. sarmentosa*-mediated suppression platelet aggregation. Caffeic acid decreased Ca²⁺ mobilization and elevated cAMP

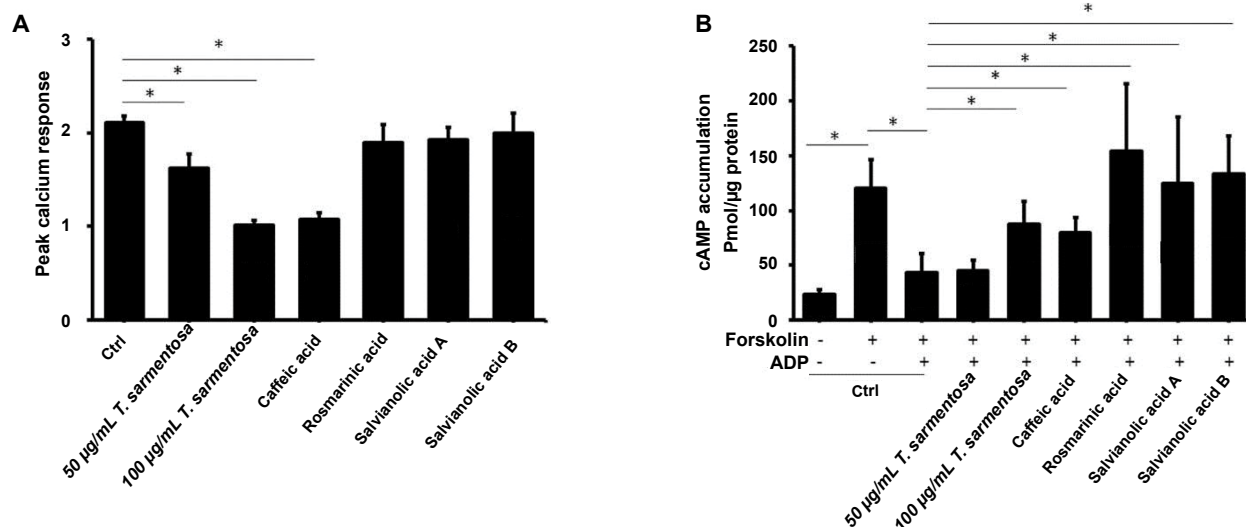


Fig. 4: The effect of *T. sarmentosa* stem extract on P2Y₁ and P2Y₁₂ receptors

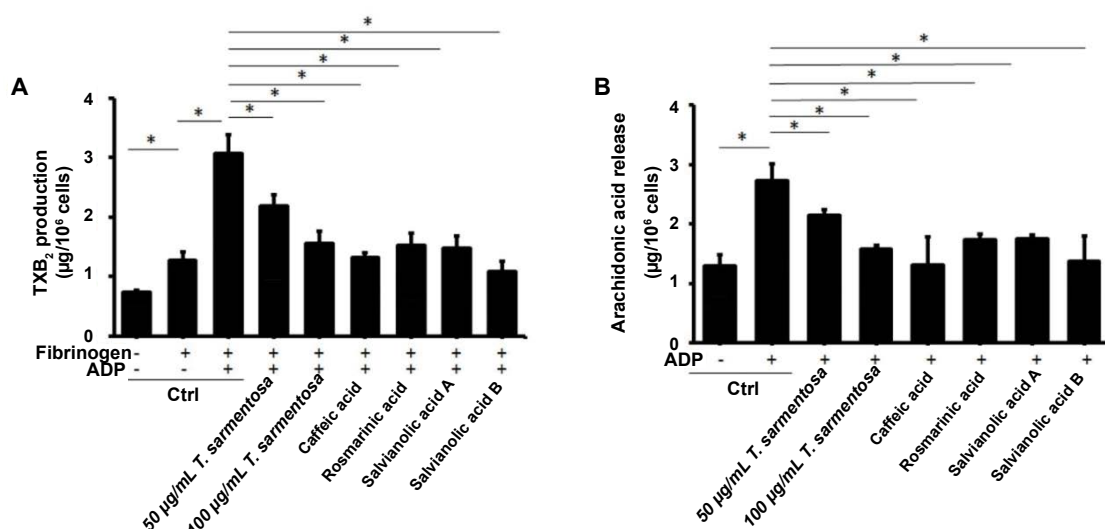


Fig. 5: The effect of *T. sarmentosa* stem extract on ADP-induced TXA₂ and arachidonic acid production in human platelets

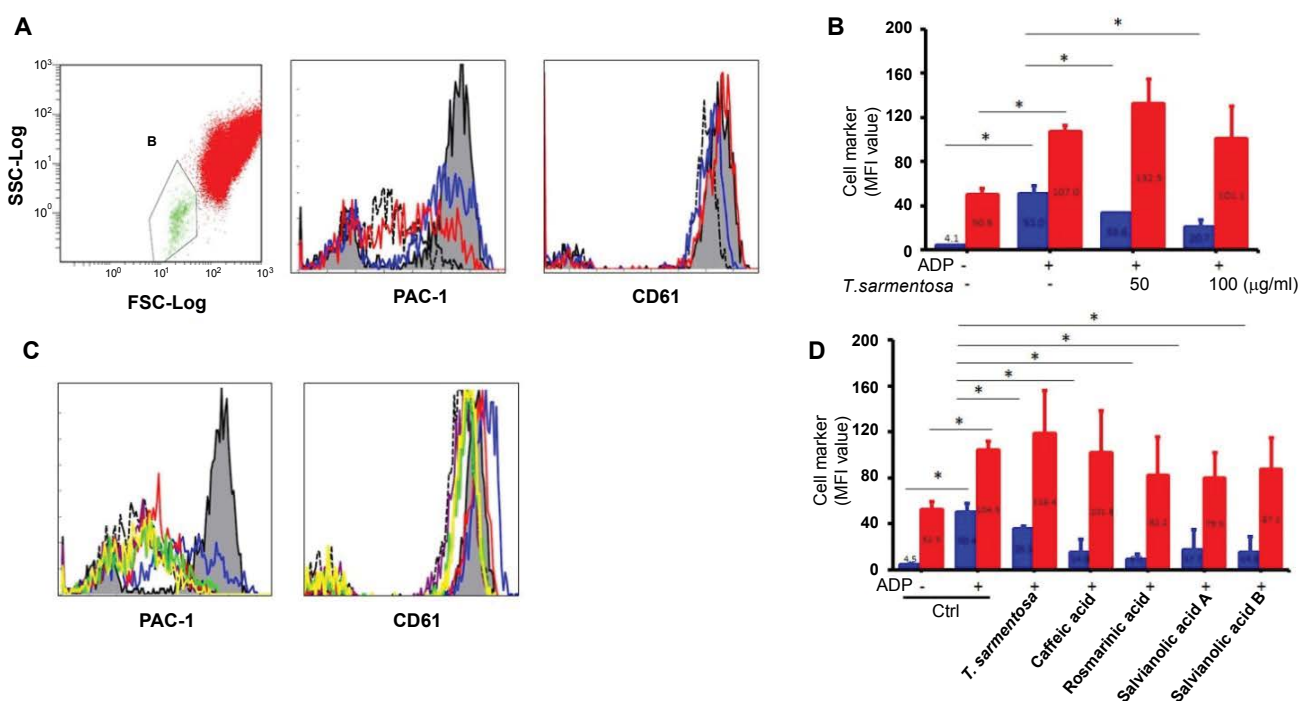


Fig. 6: The effect of *T. sarmentosa* stem extract on ADP-induced activation markers expression of platelet

A. Control; — ADP; — 50 µg/ml *T. sarmentosa*+ADP; — 100 µg/ml *T. sarmentosa*+ADP. B: ■ PAC-1; ■ CD61. C: Control; — ADP; — *T. sarmentosa*+ADP; — caffeic acid+ADP; — rosmarinic acid+ADP; — salvianolic acid A+ADP; — salvianolic acid B+ADP. D: ■ PAC-1; ■ CD61

levels in ADP-stimulated platelets^[20]. Rosmarinic acid has been reported to possess antithrombotic and antiplatelet effects^[21,22]. Both salvianolic A and B have been shown to inhibit platelet activation^[23,24]. Taken together, these findings demonstrate *T. sarmentosa* stem extract suppressed platelet aggregation.

Although the individual components showed similar antiaggregative abilities, the molecular mechanisms for ADP-mediated platelet activity were somewhat

different for each component studied of *T. sarmentosa* stem extract. The cAMP production is affected by all components studied of *T. sarmentosa* stem extract. Calcium influx was inhibited by caffeic acid and aqueous stem extract of *T. sarmentosa*. However, rosmarinic acid, salvianolic A, and salvianolic B did not. All components studied of *T. sarmentosa* stem extract significantly inhibited the ADP-induced production of TXA₂, arachidonic acid and the surface marker PAC-1 on platelets.

Caffeic acid plays an important role in *T. sarmentosa*-mediated regulation of phagocytic uptake of neutrophils and macrophages through Akt/MEK pathway^[4-5]. Fewer reactive oxygen species were observed in *T. sarmentosa* treated macrophages, particularly with salvianolic acid A and salvianolic B^[4]. The present study shows that all components studied in *T. sarmentosa* suppressed platelet aggregation, indicating each component studied mediated the effects of *T. sarmentosa* on different cells in various ways.

Antiplatelet drugs are widely used in primary and secondary prevention of thrombotic cerebrovascular and cardiovascular disease. Antiplatelet drugs are classified as reversible or irreversible, according to the inhibition process involved in platelet activation. Aspirin is an irreversible inhibitor of enzyme cyclooxygenase, resulting in reduced platelet production of TXA₂^[25]. Dipyridamole is a phosphodiesterase inhibitor that increases the cyclic AMP by preventing conversion to AMP in platelets^[26]. Clopidogrel affects the ADP-dependent activation of IIb/IIIa complex via ADP receptor P2Y₁₂^[27]. Glycoprotein IIb/IIIa receptor antagonists, including abciximab and eptifibatide, block a receptor on platelets for fibrinogen and von Willebrand factor^[28]. A daily low dose of aspirin is considered safe at reducing the risk of heart attacks and strokes^[29]. Given that *T. sarmentosa* enhances the immune response^[4,5], prevents chemical-induced hepatotoxicity^[2], and suppresses platelet aggregation, it is considered a traditional Chinese herb promoting health.

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Conflicts of interest:

There are no conflicts of interest.

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