Anti-thrombic Properties of the Oriental Herbal Medicine, Daejowhan

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The anti-thrombic properties of the oriental herbal medicine Daejowhan(DJW, 大造丸) which consists of 11 kinds of herbs (indicated as ratio) of Rehmanniae Radix 24%, Hominis Placenta 5%, Testudinis Carapax 9%, Eucommiae Cortex 9%, Asparagi Radix 9%, Phellodendri Cortex 9%, Achyranthis Radix 7%, Liriopis Tuber 7%, Angelicae Sinensis Radix 7%, Ginseng Radix 5% and Schizandrae Fructus 3% were investigated. The water extracts from DJW inhibited Platelet-activating factor(PAF) induced platelet aggregation. DJW was extracted with methanol and further fractionated by ethylacetate. A 70% methanol extract showed a strong inhibition against PAF-induced aggregation *in vitro* and *in vivo* assays. The ethylacetate soluble fraction was shown to have inhibitory effect on PAF-induced platelet aggregation *in vitro* assay. The ethylacetate soluble fraction specially protected against the lethality of PAF, while verapamil did not afford any protection. These results indicate that the water extracts and alcoholic-fractions inhibit the action of PAF *in vivo* by an antagonistic effect on PAF, so that it may be useful in treating disorders caused by PAF, such as acute allergy, inflammation, asthma, gastrointestinal ulceration, toxic shock and so forth. DJW was investigated regarding its assumed anti-thrombic action on human platelets which was deduced from its ability to suppress Arachidonic acid(AA)-induced aggregation, exocytosis of ATP, and inhibition of Cyclooxygenase(COX) and Thromboxane synthase(TXS) activity. The latter two effects were estimated from the generation of Prostaglandin E_2(PGE_2) and Thromboxane A_2(TXA_2) respectively. Exogenously applied AA (100 μmol / ℓ) provoked a 89% aggregation of platelets, the release of 14 pmol ATP, and the formation of either 225 pg TXA_2 or 45 pg PGE_2, each parameter being related to 106 platelets. An application of DJW 5 min before AA dose-dependently diminished aggregation, ATP-release and the synthesis of TXA_2 and PGE_2 with IC_{50} values of 74, 108, 65, 72 μg/mℓ, respectively. The similarity of the IC_{50} values suggest an inhibition of COX by DJW as primary target, thus suppressing the generation of TXA_2 which induces aggregation of platelets and exocytosis of ATP by its binding on TXA_2-receptors.

Key words : Daejowhan(DJW, 大造丸), inhibition of platelet aggregation, Platelet-activating factor(PAF)

Introduction

According to the traditional oriental medical literature, the formula of DJW first appeared in Fu-Shou-Jing-Fang(扶壽精方, AD 1530) compiled by Wu Min of the Ming Dynasty of China[1]; Li Shi-zhen wrote in his work Compendium of Materia Medica(本草綱目, AD 1578) that Wu Qiu made that formula[2]. In Dong-Eu-Bo-Gam(東醫寶鑑, AD 1613) compiled by Hu Jun, a representative work of oriental medicinal literature[3], Hu Jun quoted DJW from Yi-Fang-Ji-Lue(醫方集略, AD 1541) complied by Guo Jian[4]. Therefore it is not clear who made the formula of DJW.

In oriental medicine DJW has been known to nourish the lung and the kidney, replenish vital essence, nourish blood and invigorate qi. In Dong-Eu-Bo-Gam DJW was used to treat the exhaustion of blood and qi by its action of nourishing *yin* and invigorating *yang*[3].

DJW is a formula of oriental herbal medicines applied in Korea as an effective biological response modifier for augmenting host homeostasis of body circulation[5].

According to the traditional oriental medical literature, the formula of DJW was made from different ingredients. In this study DJW was based on Bang-Yak-Hap-Pyun(方藥合編, AD 1869) compiled by Hwang Do-yoen[6]. DJW consists of crude ingredients from 11 medicinal herbs, *Rehmanniae Radix, Hominis Placenta, Testudinis Carapax, Eucommiae Cortex, Asparagi Radix, Phellodendri Cortex, Achyranthis Radix, Liriopis Tuber, Angelicae Sinensis Radix, Ginseng Radix* and *Schizandrae Fructus. Rehmanniae Radix* has been known to enrich the blood, replesh bone marrow and nourish the kidney[7]. *Hominis Placenta* was used to treat total consumptive disease for males and females[2]

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and had anti-inflammatory and anti-arthritic properties [8]. *Testudinis Carapax* has been known to invigorate *yin*, promote reunion of fractured bones and remove blood stasis[3]. *Eucommiae Cortex* has been known to moisten the liver and invigorate the liver[2]. *Asparagi Radix* has been known to moisten and nourish yin the lung-qi being kept pure and descendent[2]. *Phellodendri Cortex* has been known to purge prime-minister pathogenic fire of the bladder and invigorate deficiency of the kidney[9]. *Achyranthis Radix* has used to treat flaccidity and numbness made up from cold-dampness[10]. *Liriopis Tuber* has been known to cool heat from the lung and tranquilize the mind[7]. *Angelicae Sinensis Radix* was used to treat wind and blood syndrome, consumptive disease and has been known to remove extravasated blood and promote generation of blood[3]. *Ginseng Radix* has been known to highly invigorate primordial-*qi*, invigorate spiritless and promote the production of body fluid and tranquilize the mind[10]. *Schizandrae Fructus* has been known to invigorate consumptive and infirm condition and improve acuity of vision[3].

The pharmacological action of DJW has been studied in regard to ischemic infarction[5]. This herbal medicine has been shown to express diverse activities such as immunomodulating, anti-infarction, anti-allergic and anti-inflammatory effects[11].

Although the effectiveness of DJW for ischemic infarction and inflammatory lung diseases has been widely demonstrated by numerous clinical cases[12], the scientific and acting mechanisms for those effects are not understood and explained. It is generally known that inflammation, hyperlipemia and arteriosclerosis induce disseminated intravascular coagulation[13]. It was shown that the water extracts obtained from DJW was effective in endotoxin-induced experimental disseminated intravascular coagulation in hyperlipemia and normal rats. Also these were inhibitory effects on collagen- and ADP-induced blood platelet aggregation, thrombin-induced conversion of fibrinogen and fibrinolysis *in vitro* experiments[11].

PAF is a fundamental mediator of mammalian cell function which is now thought to play a significant role in a variety of pathophysiological states inducing acute allergy, inflammation, asthma, gastrointestinal ulceration and toxic shock[14-18]. Several PAF antagonists such as ginkgolides, benzofuranoid neolignan and gliotoxin have been reported [14,19-24] and the studies on their biological activities and mechanisms have also been described. During the study for searching PAF antagonists from medicinal plants, it was found that DJW water extracts inhibits the platelet aggregation induced by PAF.

The systematic fractionation of this extract led to a strong inhibitory activity against PAF-induced aggregation. This paper described the inhibitory effect of the water extracts and alcoholic fractions from DJW in PAF-induced aggregation. The anti-thrombic actions of the water-extracted DJW were assumed to result from an inhibition of TXS, an action which may also account for the protective properties against experimental acute and chronic ulcers in rats[11].

For examination of this hypothesis, the water-extracted DJW was investigated for its possible anti-thrombic action on platelets. The anti-thrombic activity of water-extracted DJW was deduced from its ability to suppress platelet aggregation, ATP-exocytosis, and the generation of PGE_2 and TXA_2 by human platelets stimulated with AA.

Materials and Methods

1. Materials

PAF was purchased from Calbiochem (CA, USA) and stored at -70℃ in ethanol. Verapamil, a Ca-antagonist, was obtained from Sigma (MO, USA). OPT-HCl was prepared by addition of 10 volumes of 8 N HCl to an ethanolic solution (0.5% w/v) of recrystallized *ortho*-Phthaldialdehyde (purchased from Wako Pure Chemicals CO., Ltd., Tokyo, Japan). ATP determination was performed with the HS II kit of Boehringer Mannheim Gmbh (Mannheim, Germany). BSA, EDTA, and indomethacin were purchased from Sigma Co. (MO, USA). Microtiter plate based ELISA for the determination of PGE_2 and TXB_2 were provided by Promega (CA, USA). The other chemicals were supplied by Sigma and Merck (Darmstadt, Germany).

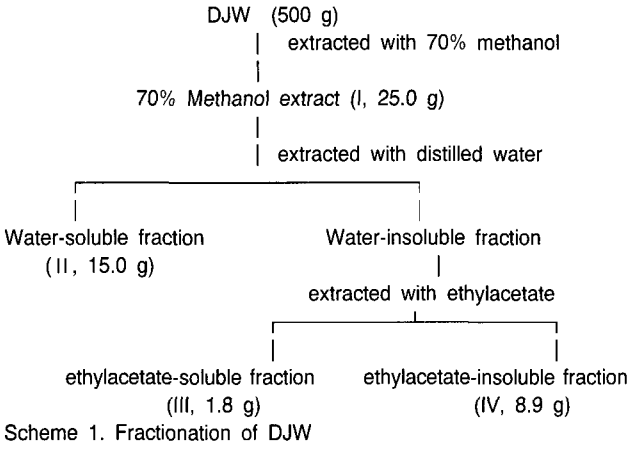
2. Isolation of DJW Extracts

DJW consists of 11 kinds of herbs such as *Rehmanniae Radix, Hominis Placenta, Testudinis Carapax, Eucommiae Cortex, Asparagi Radix, Phellodendri Cortex, Achyranthis Radix, Liriopis Tuber, Angelicae Sinensis Radix, Ginseng Radix* and *Schizandrae Fructus*[5]. The aqueous extracts of DJW and its eleven composed Korean herbs were kindly supplied by the Oriental Medical Hospital of Dongguk University. For methanol extracts from DJW, a 70% methanol extract (I) and various fractions (II, III, IV) derived from fraction I were prepared from crude mixture of DJW as shown in Scheme 1.

3. Animals

Male rabbits (2-2.5 kg) were purchased from DaeHan Experimental Animals and used for the preparation of platelet-rich plasma. Male ICR mice (3 week-old) were supplied by Genetic Resources Center, Korea Research Institute

of Bioscience and Biotechnology, Korea Institute of Science and Technology (KIST) and used in PAF experiments *in vivo*.



Scheme 1. Fractionation of DJW

4. Assay for platelet aggregation for rabbit platelets

PRP was prepared by centrifugation of fresh rabbit blood (190 × g, for 10 min) with 1/10 volume of 3.8% sodium citrate solution. Platelet numbers were adjusted to 6.0 x $10^5/mm^3$. Platelet aggregation was measured by the serotonin release according to the method[22], in which PAF (10^{-7} M) was used as an aggregating agents. A 0.2 ㎖ aliquot of PRP was incubated with 20 ㎕ of a test solution at 37℃ for 7 min, then 20 ㎕ of PAF solution was added to the reaction mixture. Five minutes after the addition of PAF, 1.0 ㎖ of ice-cold EDTA (0.4% w/v solution in isotonic saline) was added to stop the reaction and the platelets were sedimented by centrifugation (1,800 × g for 10 min). The supernatant was aspirated and the platelet pellets were lysed in 0.5 ㎖ of distilled water. Proteins in the lysed pellets were precipitated with 0.1 ㎖ of 6 M trichloroacetic acid. After removing the proteins by centrifugation, 0.5 ㎖ of the solution was added to 3 ㎖ of OPT-HCl. The mixture was then heated at 100℃ for 10 min and cooled in an ice-bath. After washing with chloroform, fluorescence in 3 ㎖ samples of the aqueous phase was measured using a spectrophotometer with wavelengths of 360 and 475 nm. Inhibition rate(%) was calculated by means of the following equation.

$$\text{Inhibition (\%)} = \frac{\text{(sample value - PAF value)}}{\text{(control value - PAF value)}} \times 100$$

5. Preparation of human platelets and aggregation

PRP, prepared from whole blood of apparently healthy volunteers, was anti-coagulated with 1/9 volume citrate-citric acid dextrose (100 ㎜ol/ℓ trisodium citrate, 7 ㎜ol/ℓ citric acid, 140 ㎜ol/ℓ dextrose, pH 6.5) by centrifugation at 100 × g for 15 min at room temperature. Platelets of the supernatant were sedimented by centrifugation at 1000 × g for 10 min. The pellet was washed 2 times with citrate-buffer [0.35% (w/v) BSA, 108 ㎜ol/ℓ NaCl, 2.8 ㎜ol/ℓ KCl, 1.0 ㎜ol/ $CaCl_2$, 1.6 ㎜ol/ℓ $MgCl_2$, 0.3 ㎜ol/ℓ NaH_2PO_4, 9.5 ㎜ol/ℓ $NaHCO_3$, 10.1 ㎜ol/ℓ trisodium citrate, 4.6 ㎜ol/ℓ citric acid] by centrifugation at 555 × g for 10 min. The final pellet was resuspended in citrate-buffer to obtain a cell titer of about 10^9 platelets/㎖. The suspension was shaken in a petri dish until the assays were performed.

For human platelet aggregation assay, an aliquot of the platelet suspension was centrifuged at 7,000 × g for 1 min. The pellet was resuspended in Tyrode's buffer [135 ㎜ol/ℓ NaCl, 3.5 ㎜ol/ℓ KCl, 1.2 ㎜ol/ℓ $CaCl_2$, 2.0 ㎜ol/ℓ $MgCl_2$, 0.3 ㎜ol /ℓ NaH_2PO_4, 0.35% (w/v) BSA, 11.9 ㎜ol/ℓ $NaHCO_3$, pH 7.4] and adjusted to 10^9 platelets/㎖. Platelet-aggregation was carried out on a dual-channel aggregometer (APACT, Ahrensburg, Germany). Aggregation of the stirred suspension was monitored in the final volume of 300 ㎕ at 37℃ turbidimetrically by an increase in light transmission employing cell-free plasma as a reference.

6. ATP determination

ATP released from platelets was detected by the bioluminescence method[25]. An aliquot of the platelet suspension was centrifuged at 7,000 × g for 1 min. The platelet was resuspended in Tyrode's buffer to obtain to 10^9 platelets/㎖ in a final volume of 4.5 ㎖, which was stirred in a plastic tube located in a water bath (37℃). After an equilibration for 5 min, samples of 100 ㎕ were taken as indicated, mixed with 900 ㎕ precooled (0℃) Tyrode's buffer and centrifuged at 7,000 × g for 1 min. For the determination of ATP, 20 ㎕ of supernatant were mixed with 200 ㎕ of 1 mol/ℓ perchloric acid and 50 ㎜ol/ℓ EDTA for 1 min to precipitate soluble proteins. The solution was adjusted to pH 7.5-8.0 by addition of 316 ㎕ of 1 mol/ℓ HEPES (pH 7.75, 25℃). After an incubation for 30 min at 0℃, the solution was centrifuged at 14,000 × g for 10 min to remove potassium perchlorate. 500 ㎕ of supernatant were added to exact same amount of HEPES buffer and the ATP content was detected by the luciferase-bioluminescene method employing the kit HSII (Boehringer Mannheim Gmbh. Mannheim, Germany) and a luminometer (LB 9502, Berthold Gmbh, Bad Wildbad, Germany). The ATP content was calculated according to a calibration curve using different ATP concentrations as standards.

7. Determination of PGE_2 and TXB_2

PGE_2 and TXB_2 were determined by ELISA employing test kits of Progega (CA, UA). An aliquot of platelet

suspension was centrifuged at 7,000 × g for 1 min. The pellet was resuspended in Tyrode's buffer to obtain a cell density of 1.5×10^8 platelets/㎖ on a final volume of 4 ㎖ and transferred into a plastic tube located in a water bath (37℃). After an equilibration for 5 min, 200 ㎕ of stirred suspension were taken as indicated. Considering determination of PGE_2, the TXS-inhibitor E/Z-7-phenyl-7-(pyridyl)-6-heptenoic acid[26] at a concentration of 1 ㎛ol/ℓ was added to the suspension at the onset of equilibration. For the determination of PGE_2 and TXB2, 200 ㎕ suspension were mixed with 1800 ㎕ ice-cooled stop solution (50 ㎛ol/ℓ indomethacin, 25 mmol/ℓ HEPES, pH 7.4). Afterwards platelets were disrupted by sonification at 0℃ for 15 min to allow complete release of both eicosanoids, and the resulting homogenates were centrifuged at 5,000 × g for 10 min. The PGE_2 and TXB_2 contents of the supernatant were determined according to the instructions of the ELISA-kit supplier.

8. Drugs and solvents

AA was dissolved in DMSO to obtain stock solution of 20 mmol/ℓ AA. The final concentration of DMSO in the platelet suspension was amounted to 1% (v/v).

9. PAF-induced mortality in mice

Male ICR mice were intravenously administered 500 ㎎/100 g or 1000 ㎎/100 g of PAF solution. All mice were observed for at least 24 hr after the PAF administration. Each test drug was intraperitoneally administered 15 min before the PAF administration. Results were given as 24 hr survival rates.

10. Statistics

Results are expressed as means±SD. The dose-response curves were deduced from fitting of values according to the logic dose-response function employing the term y = a+b/(1-(x/c)d), where x represents DJW concentration, y is the pharmacologic effect expressed as % of control, and a/b/c/d are fitting constants. The IC_{50} values were calculated from individual fitted dose-response curves and expressed as means±SD.

Results and Discussion

1. Effect of organic solvent(ethanol, methanol, ethylacetate) -extracted DJW on platelet aggregation *in vitro*

As shown in Fig. 1, Fig. 2 and Table 1, the water and methanol extracts inhibited aggregation of platelets. Although various compounds such as swietemahonin, ginkgolides, benzofuranoid neolignans and furanoid lignans have been reported as antagonists of PAF[19-24], these results provided the first examples of water-extracted molecules having an antagonistic effect on PAF. The inhibitory effect of the Methanol extracts was much higher than that of the water extracts. Verapamil[22] as a positive control inhibited dose dependently aggregation of platelets with IC_{50} value of 30.2 ㎍/㎖. The inhibitory activities of I -Ⅳ were weaker than that of verapamil but the case of III was significantly meaningful.

Table 1. Effects of the extracts on PAF-induced aggregation of rabbit platelets in vitro as shown by IC_{50} (㎎/㎖)

	IC_{50} (㎎/㎖)*
Water-Extracts	780.3
I	25.3
II	18.3
III	1.1
Ⅳ	8.3

* IC_{50} (㎎/㎖): Concentration (㎎/㎖) required for 50% inhibition of platelets aggregation Water-Extract : Water extract derived from DJW I : 70% methanol extract derived from DJW II : Water-soluble fraction which extracted I with distilled water III : ethylacetate-soluble fraction derived from water-insoluble fraction which extracted I with distilled water Ⅳ : ethylacetate-insoluble fraction derived from water-insoluble fraction which extracted I with distilled water

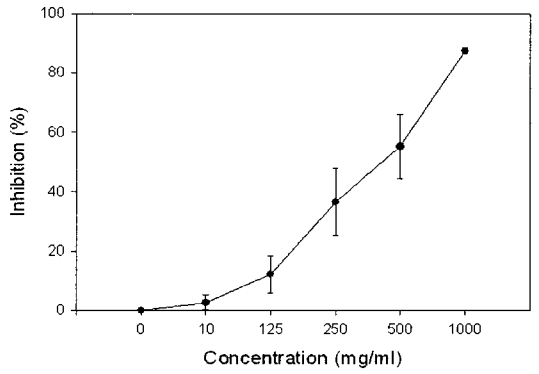


Fig. 1. Effects of the water extracts on PAF-induced aggregation of rabbit platelets *in vitro*

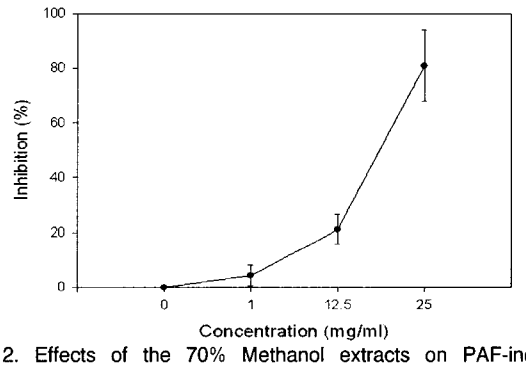


Fig. 2. Effects of the 70% Methanol extracts on PAF-induced aggregation of rabbit platelets *in vitro*

2. Effect of organic solvent(Ethanol, Methanol, ethylacetate) -extracted DJW on PAF-induced mortality in mice

For evaluation of the in vivo anti-PAF effect of the fractions I -Ⅳ, the author have used ICR mice. These mice have been shown by Young et al.[27] and Ekimoto et al.[22] to be

sensitive to PAF-induced mortality. Intravenous administration of PAF into ICR mice caused lethal shock. As shown in Table 2, when administered intraperitoneally, among the fractions I to IV, fraction III specially protected against the lethality of PAF, while verapamil did not afford any protection. It is worth to note that the fraction III have dual activities against PAF aggregation *in vitro* and PAF mortality *in vivo*. Furthermore, it is worthy to note that fraction III had a stronger activity against in vivo PAF aggregation than that of fractions I, II and IV, in spite of its lower potency *in vitro* than that of verapamil. Unfortunately, however, more detailed examinations could not be undertaken because the amount of fraction III available was insufficient to do the anti-PAF effect *in vivo*. These findings indicate that the water extracts and alcoholic-fractions inhibit the action of PAF in vivo, so that it may be useful in treating disorders caused by PAF.

Table 2. Effects of the extracts and fractions on PAF-induced mortality in mice

Fraction	mg/kg	route	survival	survival rate (%)
vehicle (5% DMSO-saline)	0	ip	1/14	7.1
PAF-control	0	ip	3/20	15.0
I	10	ip	6/24	33.3
II	8	ip	11/28	39.5
III	4	ip	14/24	58.3
IV	6	ip	4/20	20.0
Verapamil	3	ip	2/5	40.0

PAF was administrated 200 ㎍/㎏ of solution I : 70% methanol extract derived from DJW II : Water-soluble fraction which extracted I with distilled water III : ethylacetate-soluble fraction derived from water-insoluble fraction which extracted I with distilled water IV : ethylacetate-insoluble fraction derived from water-insoluble fraction which extracted I with distilled water

3. Effect of water-extracted DJW suppressed dose-dependently the aggregation induced by AA in human platelet

As shown in Fig. 3, challenge of platelets with 100 ㎛ol / ℓ AA to human platelets induced an absolute aggregation of almost 90% within about 3 min after its application as deduced turbidimetrically from the increase in light transmission. However, an application of 70 ㎍/㎖ DJW 5 min before AA suppressed aggregation. An application of DJW suppressed dose-dependently the aggregation, but failed to prevent it completely. Even at high DJW concentrations of 500 ㎍/㎖, a transient aggregation of about 15% (peak maximum) could be detected; whereas the COX-inhibitor indomethacin (50 ㎛ol/ ℓ) applied instead of DJW prevented aggregation completely (data not shown). For calculation of the dose-response relation the maximal aggregations, including transient peak-values observed at high DJW concentrations above 300 ㎍/㎖, were expressed as % of control. As demonstrated in Fig. 4 the IC_{50} necessary to diminish aggregation amounted to 74±3.4 ㎍/㎖ DJW.

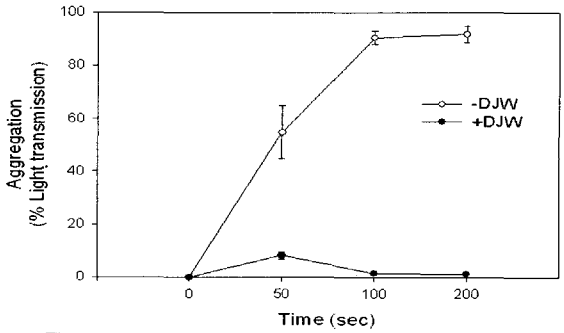


Fig. 3. Time course aggregation of human platelets by 100 ㎛ol/ ℓ AA to human platelets

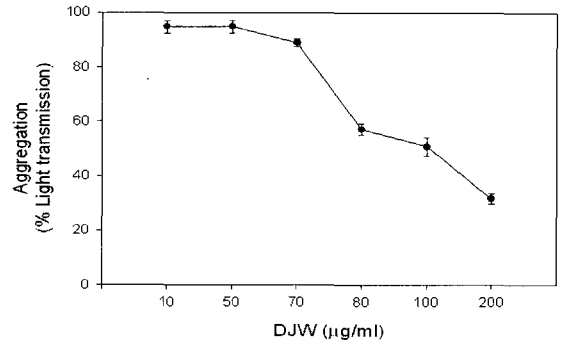


Fig. 4. Dose-dependent inhibition of human platelet aggregation by water-extracted DJW. For the calculation of the dose-response curve DJW-dependent maximal inhibition of aggregation, including peak values of transient aggregation, were expressed as % of control which represents platelets only challenged by AA. Data are depicted as means±SD (n=7).

4. Dose-dependent inhibition of ATP-exocytosis by water-extracted DJW

Similar to the time course of aggregation, AA provoked a release of endogenous ATP which was completed about 10 min after addition of AA (Fig. 5). The ATP content released from platelets, determined 15 min after application of AA, amounted to 12.5±4.2 ㎫ol ATP/10^6 platelets (n=6) which could be suppressed to 2.9±0.8 ㎫ol ATP/10^6 platelets (n=6) if indomethacin (50 ㎛ol/ ℓ) was added instead of DJW (Fig. 5). To calculate the dose-response curve, the amount of released ATP was detected 15 min after addition of AA in the presence of different DJW concentrations. As demonstrated in Fig. 6, the IC_{50} was 108±16.2 ㎍/㎖ (n=5) DJW.

5. Inhibition of TXA_2 generation by water-extracted DJW

The action of DJW on TXS was deduced from the formation of TXA_2, the main metabolite of AA cascade in platelets[28]. TXA_2 spontaneously converts with a half life of 37 sec to TXB_2, which was detected as a representative of TXA_2. As depicted in the Fig. 7, exogenously applied AA provoked immediate formation of TXB_2 from a basal value of 0.5±0.2 to 180.9±28.4 pg $TXB_2/10^6$ platelets (n=5), the latter value determined 12 min after the addition of AA. If 1 ㎛ol/ ℓ of the

TXS-inhibitor E/Z-7-phenyl-7-(pyridyl)-6-heptenoic acid was added to platelets 5 min prior to AA, the generation of TXB_2 diminished to the basal values 0.5±0.2 pg $TXB_2/10^6$ platelets (n=6) observed before the addition of AA, indicating a complete inhibition of TXS. Similar to the action of the TXS-inhibitor, an addition of DJW to platelets suppressed the generation of TXB_2 dose dependently with an the IC_{50} of 65±22 μg/mℓ (n=5) DJW (Fig. 8).

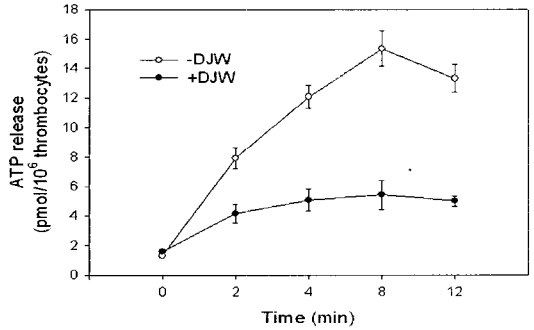


Fig. 5. Time course ATP release (ATP-exocytosis) from platelets (thrombocytes) by 100 μmol / ℓ AA to human platelets

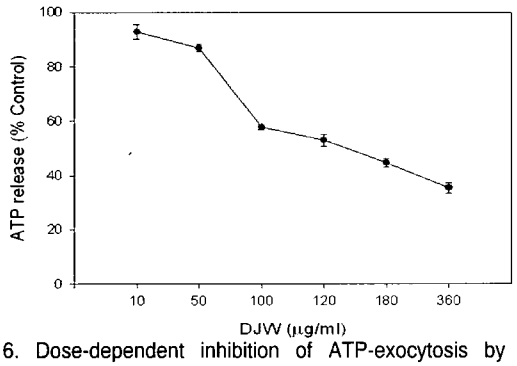


Fig. 6. Dose-dependent inhibition of ATP-exocytosis by water-extracted DJW. The dose-response relation was deduced from the amount of released ATP, detected 5 min after the addition of AA in the presence of different DJW concentrations which were applied 5 min before AA. Results are shown as means±SD (n=5).

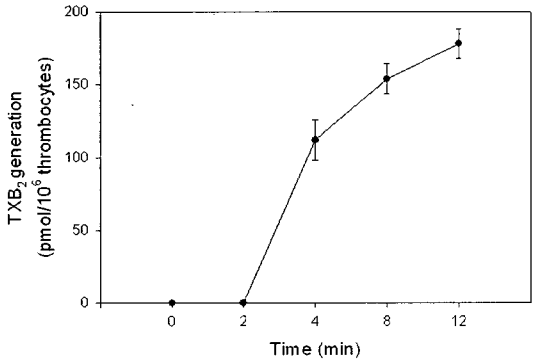


Fig. 7. Time course TXA_2 generation from platelets (thrombocytes) by 100 μmol / ℓ AA to human platelets. Exogenously applied AA is metabolized via COX and TXS to TXA_2, which spontaneously isomerizes to TXB_2; the latter was detected immunologically as a representative of TXA_2. As shown, 100 μmol/ ℓ AA induced an increase of TXB_2 during the first minute after application which remained almost unaffected at least for 15 min of incubation.

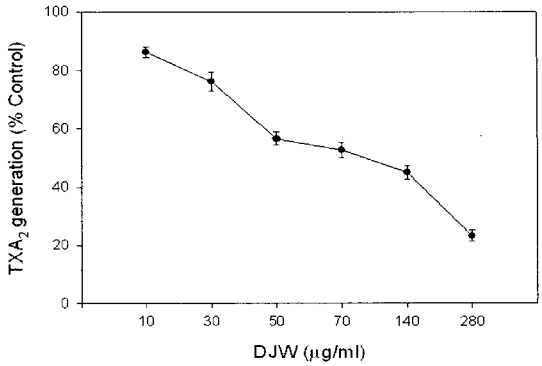


Fig. 8. Dose-dependent inhibition of TXA_2 generation by water-extracted DJW. For the calculation of the dose-response curve the amount of TXB_2, generated 5 min after AA was applied, was determined. DJW, added to platelets 5 min before AA, dose-dependently diminished the generation of TXB_2 expressed as % of control which represents platelets treated with AA only. Data are means±SD (n=5).

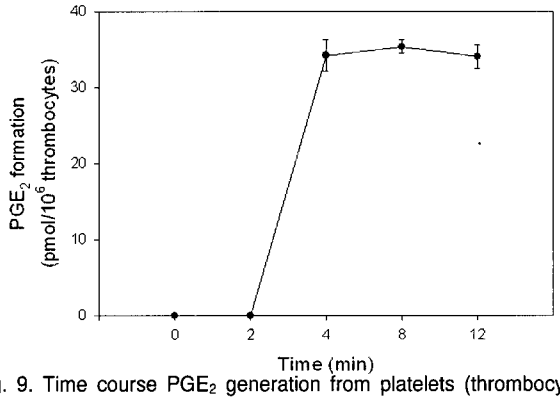


Fig. 9. Time course PGE_2 generation from platelets (thrombocytes) by 100 μmol / ℓ AA to human platelets. Addition of 100 μmol / ℓ AA induced an immediate increase of PGE_2 if platelets were incubated in the presence of TXS inhibitor E/Z-7-phenyl-7-(pyridyl)-6-heptenoic acid (1 μmol / ℓ) which is necessary to shift enzymatic conversion of AA towards PGE_2 generation.

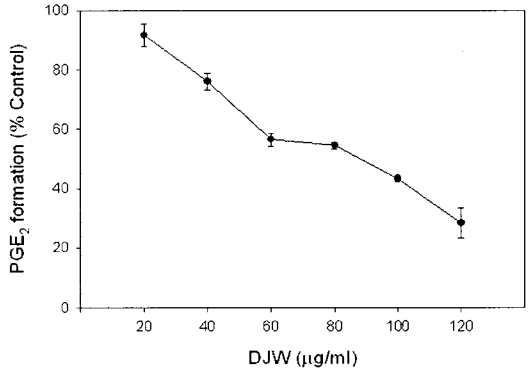


Fig. 10. Dose-dependent inhibition of PGE_2 formation by water-extracted DJW. Since PGE_2 remained stable at least for 15 min during incubation, the amount of PGE_2 was determined 5 min after application of AA. DJW, added 5 min prior to AA, dose-dependently suppressed PGE_2-formation of platelets, expressed as % of control which are platelets challenged by AA only. Data are means±SD (n=5).

6. Inhibition of PGE_2 formation by water-extracted DJW

Exogenously applied AA to platelets was reported to be metabolized by COX and TXS to prostaglandins and

thromboxane A_2[28], the latter released from platelets[29]. Binding TXA2 to its receptors causes an increase of cytosolic Ca^{2+}[30] which triggers exocytosis of inducers like ATP and PAF, thus amplifying aggregation of platelets. Since a synthesis of TXA_2 from exogenously applied AA dependes on both the activity of COX and TXS, DJW-dependent suppression of TXB_2 formation may also be explained by an inhibition of COX due to DJW. Therefore, to estimate the action of DJW on COX, PGE_2 was determined in the presence of different concentrations of DJW and 1 μmol/ℓ of the TXS-inhibitor E/Z-7-phenyl-7-(pyridyl) -6-heptenoic acid, both applied 5 min before AA. The application of the TXS-inhibitor was necessary to shift the formation of AA metabolites from thromboxanes towards prostaglandins, which assures sufficient formation of PGE_2 usually detected as an indicator of COX activity[23]. Challenge of platelets with AA enhanced PGE_2 immediately from a basal value of 1.5±1.0 to 32.5±12.5 pg $PGE_2/10^6$ platelets (n=5), the latter value determined 5 min after the addition of AA (Fig. 9). Since PGE_2 remained stable under the incubation procedure at least for 10 min after addition of AA (Fig. 9), the amount of generated PGE_2 in the presence of DJW was determined 5 min after AA-application to calculate the dose-response curve. As depicted in Fig. 10, DJW dose-dependently diminished the formation of PGE_2 with an IC_{50} of 72±21 μg/mℓ (n=5).

Conclusion

The anti-thrombic properties of the oriental herbal medicine DJW were investigated. Water extracts from DJW inhibited PAF-induced platelet aggregation. DJW was extracted with methanol and further fractionated by ethylacetate. Methanol and ethylacetate extracts showed inhibition against PAF-induced aggregation.

Water-extracted DJW dose-dependently suppressed the aggregation of human platelets, the release of endogenous ATP, and the formation of PGE_2 and TXB_2. The latter two things usually detected to estimate the activity of COX and TXS respectively. Since the IC_{50} values necessary to inhibit COX [72 μg/mℓ DJW] and TXS [65 μg/mℓ DJW] were in the same range, inhibition of COX is suggested to be the primary target of water-extracted DJW thus suppressing the formation of PGE_2 which is metabolized by TXS to TXA_2. An additional substantial inhibition of TXS by water-extracted DJW might be excluded, otherwise IC_{50} inhibition should be significantly smaller than that obtained for COX inhibition because of limited PGE_2 due to reduced COX-activity. Besides the enzyme activities, aggregation of platelets could also be suppressed at an IC_{50} of 70 μg/mℓ DJW, a value closely related to COX-inhibition. This supports the idea that inhibition of COX is the only cause of the toxicating DJW constituents against inflammation of the genito-urinary system, rheumatism, and inflammatory wounds (data not shown). The anti-thrombic properties of water-extracted DJW might be explained by an inhibition of COX comparable to non-steroid analgesics like acetylsalicylic acid.

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References

1. Wu, Min. Fu-Shou-Jing-Fang, Beijing, A book concern of the chinese medicine(中國中醫學出版社). pp 17-18, 1996.
2. Li, Shi-zhen. Compendium of Materia Medica, Beijing, A book concern of the people sanitation(人民衛生出版社). p 1281, 1986, 2956, 2963, 1991.
3. Hu, Jun. Dong-Eu-Bo-Gam, Seoul, Namsandang. pp 445-446, 704, 721, 725, 727, 1991.
4. Hong, Won-Sik. Medical history of China, Seoul, The research of oriental medicine(東洋醫學研究院). p 259, 277, 1984.
5. Cho, Gyu-Sun. The effect of Daejowhan on cerebral and thalamic atrophy following middle cerebral artery occulsion in rats. The Journal of Oriental Chronic Diseases 4(1):163-175, 1998..
6. Hwang, Do-Yeon. Bang-Yak-Hap-Pyun, Seoul, Namsandang. pp 151-153, 1996.
7. Li, Chan. A commentary of medical entrance(編註醫學入門), Seoul, Daesungmunhwasa. p 203, 218, 553, 1994.
8. Gold-alber, P., Chaumontet, M., Capt, M. An experimental study of the anti-inflammatory and anti-arthritic properties of a standardized placental extract. Int. J. Tiss. Reac.Ⅲ(3-4), pp 155-165, 1981.
9. Yang, Dong-Xi. Bencaobeiyaojiexi, Xinzhu, A book concern of guo xing(國興出版社). p 335, 1985.
10. The new medical science in jiangsu(江蘇新醫學院). A great dictionary of chinese medicine(中藥大辭典), Shanghai, A book concern of shanghai science(上海科學技術出版社). p 29, 1982.
11. Min, Sang-Yeon. A Study on Anti-thrombic Action of Korean Herbal Medicine, Daejowhan and Its Herbs. Thesis of master, Dongguk University, Seoul, Korea. 1999.
12. Lai, Tian-Song. clinical concise of oriental herbal prescription(臨床方劑手冊), Beijing, A book concern of people sanitation(人民衛生出版社). pp 315-316, 1992.

13. Hoffman, D.R., Truong, C.T., Johnston, J.M. Metabolism and function of platelet-activating factor in fetal rabbit lung development. Biochim. Biophys. Acta. 879(1):88-96, 1986.
14. Braquet, P., Touqui, I., Shen, T.Y., Vargaftig, B.B. Perspectives in platelet-activating factor research. Pharmacol. Rev. 39, pp 97-109, 1987.
15. Braquet, E., Godfroid, J.J. The relative role of PAF-acether and icosanoids in septic shock. Trends Pharmacol. Sci. 7, pp 397-405, 1986.
16 Sugatani, J., Fujimaru, K., Mizuno, T., Sameshima. The role of platelet activating factor (PAF) in the pathogenesis of gastric ulcer. Prostaglandin Leukot Essent Fatty Acids. Rev. 44(3):134-147, 1991.
17. Imaizumi, T.A., Stafforini, D.M., Yamada, Y., Mclntyre, T.M., Prescott, S.M., Zimmerman, G.A. Platelet-activating factor: a mediator for clinicians. J. Intern. Med. Rev. 238(1):5-20, 1995.
18. Braquet, P., Bourgain, R., Mencia-Huerta, J.M. Effect of platelet-activating factor on platelets and vascular endothelium. Adv. Prostaglandin Thromboxane Leukot Res. Rev. 17B, pp 822-827, 1987.
19. Shen, T.Y., Hwang, S.B., Chang, M.N., Doebber, T.W., Lam, M.H., Wu, M.S., Wang, X.H. and Li, R.J. Conformation and activity of tetrahydrofuran lignans and analogues as specific platelet activating factor antagonists. Proc. Natl. Acad. Sci. USA 82, pp 672-678, 1985.
20. Corey, E.H., Chen, C.P., and Parry, M.J. Tetrahedron Lett. 29, pp 2899-2903, 1988.
21. Hosford, D., Mencia-Hueta, J.M., Page, C. and Braquet, P. Phytotherapy Research 2, pp 1-7, 1988.
22. Ekimoto, H., Irie, Y., Araki, Y., Han, G.Q., Kadota, S. and Kikuchi, T. Platelet aggregation inhibitors from the seeds of Swietenia mahagoni: inhibition of in vitro and in vivo platlet-activating factor-induced effects of tetranortriperpenoids related to swietenine and swietenolide. Planta Med. 57, pp 1609-1611, 1991.
23. Heuer, H.O. Current status of PAF antagonists. Clin. Exp. Allergy. Rev. 22(11):980-983, 1992.
24. Desquand, S. Effect of PAF antagonists in experimental model. Therapeutical. Therapie. Rev. 48(6):585-597, 1993.
25. O'Brien J.R. J. Clin. Pathol. 15, pp 452-455, 1962.
26. Kato, K., Ohkawa, S., Terao, S., Terashita, Z., and Nishikawa, K.J. Med. Chem. 28, pp 287-294, 1985.
27. Young, J.M., Maloney, P.J., Jubb, S.N. and Clark, J.S. Prostaglandins. 30, pp 545-549, 1985.
28. Siess, W. Physiol. Rev. 69, pp 58-178, 1989.
29. Parise, L.V., Venton, D.L., LeBreton, G.C. Pharmacol. Exp. Ther. 228, pp 240-244, 1984.
30. Brace, L.D., Venton, D.L., LeBreton, G.C. Am. J. Physiol. 249, H1-H7, 1985.