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Platelet Anti-Aggregating Plant Materials

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Abstract—The smear method developed by Velaskar and Chitre was modified to allow the screening of plant extracts and/or fractions for platelet anti-aggregating activity. The modified smear method was also found suitable for massive screening of pure compounds. Sample fractions prepared from various plant extracts were examined for their effects against ADP, arachidonic acid (AA) or collagen induced platelet aggregations. Several solvent fractions of plant extracts including water fraction prepared from the methanol extract of *Acanthopanax* sp. was inhibitory against rat platelet aggregations. The activity guided treatments and fractionations of the water fraction from *A. senticosus* Max yielded two anti-platelet aggregatory substances, 3, 4-dihydroxybenzoic acid (I) and its artefact ethyl 3, 4-dihydroxybenzoate (II). The inhibitory activities of I and II against rat platelet aggregation were compared with that of aspirin, a known inhibitor of platelet aggregation. Discussions also included the results of the investigations on the structural activity relationships among the various dihydroxybenzoic acid derivatives against platelet aggregations induced by either one of ADP, AA or collagen.

Keywords—Platelet anti-aggregating activity · modified smear method · adenosine 5′-diphosphate (ADP) · arachidonic acid (AA) · collagen · *Acanthopanax senticosus* Max · 3, 4-dihydroxybenzoic acid · ethyl 3, 4-dihydroxybenzoate

Platelets play an important role in the process of haemostasis and in its pathological counterpart, arterial thrombosis. Since platelets readily aggregate in response to a variety of endogenous substances and secrete various substances that cause further aggregation, platelets can initiate thrombosis formation and precipitate thromboembolism leading to ischemic diseases.[1~3] In addition, substances secreted from platelets can mediate many other biologic reactions and may also be involved in artherogenesis and other pathologic processes. Hence, the possibility that antiplatelet drugs may be useful in the prophylaxis or therapy of platelet-induced thromboembolism and other platelet-mediated pathologic processes has arisen considerable interests in recent years. However, the present state of knowledge has not approached the point where one can rationally design new classes of molecules which could be expected to be effective. Plants have been considered especially valuable in the empirical search for new drugs in such situations because plants contain broad classes of compounds as their constituents.[4]

1) Modified Smear Method for Screening Potential Inhibitors of Platelet Aggregation from Plant Sources

There are, as Suffness and Douros[5] pointed out, special problems to be considered for the selection of the method for screening plant materials for their biological activities. The method is required

to be highly selective to limit the number of leads for following-up evaluations, highly sensitive in order to detect low concentrations of active compounds and specific to be insensitive to a wide variety of inactive compounds. Furthermore, the methodology must be adaptable to materials which are highly colored, tarry, poorly soluble in water and chemically complex.

The existing methods of measuring the degree of platelet aggregation were examined in an attempt to screen plant extracts or crude fractions prepared from the extracts for their potential antiplatelet aggregating activities. The turbidimetric method (optical density method) developed by Born and Cross[6,7] is the most widely practiced procedure to measure the degree of aggregation of platelets induced by various stimuli *in vitro*. However, this turbidimetric method was unsuitable for measuring the effects of highly colored, tarry and poorly soluble plant extracts or fractions because it measures the changes in turbidity of platelet-rich plasma (PRP) as platelets aggregate. Chandler's tube technique[8] measures the thrombin-induced platelet aggregation from the recalcified citrated-PRP. This technique measures the time required to give "snowstorm" effect as the platelet aggregates become visible. This method also was unsuitable to screen plant extracts or fractions. The smear method developed by Velaskar and Chitre[9] seemed more suitable for testing plant-derived samples since the blood is smeared on a glass slide, stained and the degree of aggregation of platelets directly examined under a microscope. This method was found to be useful inasmuch as neither the color nor solubility of plant-dirived test samples adversely affects the determinations as platelets are selectively stained and the degree of aggregation is directly examined under a microscope. In addition, this method requires a small volume of blood per test and does not involve the use of special instruments. However, because the smears must be prepared within 15 min of the collection of the blood, only a few tests can be completed with a single blood collection, thus rendering the smear method inadequate for massive screening. In addition, only those agents that induce platelet aggregation at room temperature can be utilized as aggregating agent due to the use of whole blood. Because of these limitations, modifications were made in the smear method to make it more suitable for the screening of plant extracts and/or fractions.[10] In place of whole blood which is employed in the original procedure, the modified smear method employs PRP which is stable for about 3 hr thus providing enough time for preparations of smears with various test samples, in addition to positive and negative controls. Employment of PRP also made possible experimentation with aggregating agents, such as arachidonic acid (AA) and collagen, which need incubation to induce platelet aggregation. The original procedure of Velaskar and Chitre could only employ those aggregating agents that induce platelet aggregation at room temperature. Furthermore, obtaining readings became much easier by using PRP smears compared to those prepared with whole blood, since most of the non-platelet cells were removed by centrifugation. The stepwise procedure of the modified smear method is described below.

(1) A male Sprague-Dawley rat (220±30 g) is anesthetized with chloroform and blood drawn from the heart into a plastic syringe containing 1/10 volume of 2.2% trisodium citrate.

(2) The citrated blood was centrifuged at 200 x g for 10 min at room temperature and supernatant PRP was obtained. PRP prepared from centrifugation was directly employed for the following tests without further adjustment of platelet counts since consistent aggregations were obtained from different batches of PRP preparations.

(3) The solutions of the aggregating agents were prepared in saline to give the following final concentrations: adenosine 5′-diphosphate (ADP), 1×10^{-6} g/ml; arachidonic acid (AA), 6×10^{-5} g/ml; collagen, 6×10^{-6} g/ml.

(4) The test sample solution (0.02 ml) was added to 0.16 ml of PRP and mixed.

(5) Two min after the incubation at 37° (for AA or collagen as aggregating agents) or at room temperature (for ADP), 0.02 ml of the appropriate aggregating agent (or saline as a control) was added and the tube was vigorously agitated for 10 sec.

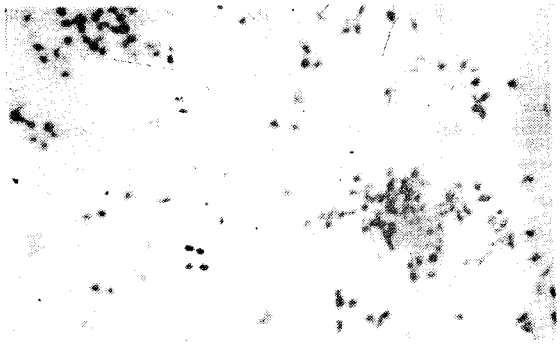
(6) Thin smears were prepared on glass slides after 4 min (for AA) or 6 min (for collagen) incubation at 37°or after standing at room temperature for 4 min (for ADP) and dried quickly in the air.

(7) The glass smears were fixed in EtOH, stained with a Wright-Giemsa stain[7], washed and dried.

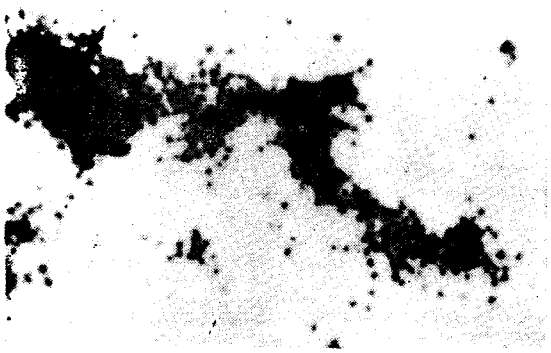
(8) The smears were subjected to examination under an ordinary light microscope using an oil immersion objective lens (1000 times). The degree of aggregation was graded as described: (−), no aggregation as shown with PRP plus saline alone; (±), slight aggregation of platelets; (+), less aggregation than with PRP plus saline and an appropriate aggregating stimuli; (++), as much aggregation as with PRP plus saline and an appropriate aggregating stimuli; and (+++), more aggregation than with PRP plus saline and an appropriate aggregating agent. Representative smears of varying degrees of platelet aggregation are shown in Figure 1.

Although there are species differences between

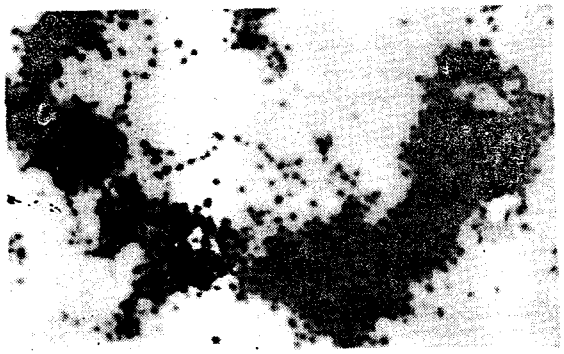
Representative slide of "−": PRP plus saline alone.



Representative slide of "+": PRP plus Fr. I of *Conioselinum* sp. and ADP.



Representative slide of "++": PRP plus ADP



Representative slide of "+++": PRP plus Fr. I of *Achyranthes japonica* and ADP

Fig. 1. Representative slides of varying degrees of platelet aggregation.

platelets from different laboratory animals in their functions and responses to various aggregating and also antiaggregating agents,[11~14] the rat was chosen as a source of blood since its platelets respond to most of the aggregating agents which induce aggregation in human platelets. Aspirin and papaverine, known inhibitors of platelet aggregation[15,16] were subjected to screening by the present modified method against ADP, AA, and collagen-induced rat platelet aggregation and the results were comparable with previously reported data obtained by the turbidimetric method with human blood.[10]

The present described method seemed also suitable for preliminary massive screening of pure organic compounds since the technique is simple and inexpensive requiring no sophisticated instruments.

2) Screening of Plant Preparations

Plants with a folkloric reputation in Korea were selected for screening and each specified parts of the crude plant material was purchased from a local herb drug market. Plant materials were extracted and fractionated as described in Figure 2 following the procedure of Suffness and Douros[4]. The dried plant was extracted twice with refluxing MeOH for 6 hr. The extract was then filtered and the filtrate concentrated under reduced pressure. The resulting MeOH extract was then partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer was evaporated and the residue partitioned between 9 : 1 MeOH—H_2O and hexane. The residues obtained from the H_2O layer (Fr. I), 90% MeOH layer (Fr. II) and hexane layer (Fr. III) were used as test sample fractions.

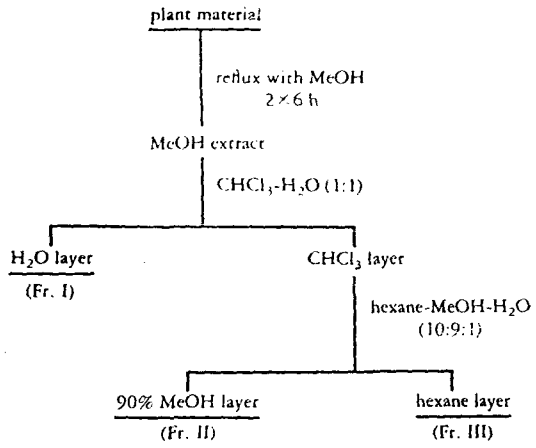


Fig. 2. Extraction and solvent fractionation of plant materials

Using the modified smear method, fractions prepared from eighteen plant species were screened for their effects against ADP, AA or collagen-induced rat platelet aggregation. Fraction I (H_2O fraction) presented no solubility problems. However, most of Fraction II (90% MeOH fraction) and Fraction III (hexane fraction) were not very soluble in H_2O and raised a problem in preparing test solutions in saline. The addition of DMSO, tween or EtOH aided the solubilization of each of these fractions. DMSO and tween also impaired platelet aggregations. EtOH has been reported to either inhibit or potentiate human platelet aggregation induced by various aggregating agents[17,18]; nontheless, at final concentrations of 1%, EtOH did not significantly affect the aggregation of rat platelets induced by ADP, AA or collagen. Therefore, H_2O-insoluble samples were first dissolved in EtOH and then homogenized with the addition of saline to give 1% of final EtOH concentrations.

Platelet activation is a sequence of complicated morphological and functional changes involving a variety of substances and enzymes. Verstraete[19] and Vargaftig *et al.*[20] have reviewed the various aspects of induction of platelet aggregation with different aggregating stimuli and examined the inhibitors of platelet aggregation acting at different aggregating steps. Collagen is one of the primary agonists which induces platelet aggregation through interactions with the specific receptors of the platelet membrane. ADP, which has a role as the mediator of aggregation due to other agents such as collagen or thrombin, is released from the first stimulated platelets and

Table I. Effects of plant preparations against ADP, collagen, or arachidonic acid(AA) induced platelet aggregation

Plant name (Family name)	Part of plants[a]	Aggregating agents[b] and plant fractions[c]								
		ADP			AA			Collagen		
		I	II	III	I	II	III	I	II	III
Acanthopanax sp. (Araliaceae)	ba	−(±)	++	++	++	++	++	++	++	++
Achyranthes japonica (Miquel) Nakai (Amaranthaceae)	ra	+++	++	+++	++	+++	++	++	++	++
Angelica gigas Nakai (Umbelliferae)	ra	++	++	++	++	±(++)	++	++	−(+)	++
Astragalus membranaceus Bunge (Leguminosae)	ra	++	+(++)	++	++	++	++	++	++	++
Atractylodes japonica Koidzumi (Compositae)	rh	++	++	++	++	+++	++	++	++	++
Carthamus tinctorius Linne (Compositae)	fl	++	++	+++	++	++	++	++	++	++
Chrysanthemum indicum Linne (Compositae)	fl	++	+++	+++	++	+(++)	++	++	±(++)	++
Conioselinum sp. (Umbelliferae)	rh	+(++)	±(±)	±(+)	++	−(±)	++	±(++)	++	++
Crataegus pinnatifida Bunge (Rosaceae)	fr	±(+)	±(+)	++	++	++	++	++	++	+++
Fritillaria sp. (Liliaceae)	tu	++	++	++	++	++	++	++	++	++
Ledebouriella seseloides Wolff (Umbelliferae)	ra	++	±(++)	++	++	++	++	++	±(++)	++
Lycium chinense Miller (Solanaceae)	fr	++	±(+)	+++	++	−(+)	+++	++	±(++)	++
Machilus thunbergii Siebold et Zuccarini (Lauraceae)	sb	++	++	++	++	++	+++	++	++	++
Panax ginseng C. A. Meyer (Araliaceae)	ra	−(±)	++	++	++	++	++	++	++	++
Polygonatum japonicum Morren et Decaisne (Lilia ceae)	rh	++	+(++)	++	++	±(++)	++	++	±(++)	++
Prunus persica Batsch (Rosaceae)	sm	++	±(+)	++	++	++	++	++	++	++
Rehmannia glutinosa Liboschitz (Scrophulariaceae)	ra	++	++	++	++	++	+++	++	++	++
Scutellaria baicalensis George (Labiatae)	ra	±(+)	±(++)	+++	++	±(±)	++	±(++)	−(±)	++

[a]ba, bark; fl, flower; fr, fruit; ra, radix; rh, rhizome; sm, seed; tu, tuber; sb, stem bark.

[b]ADP, 1×10^{-6} g/ml; arachidonic acid (AA), 6×10^{-5} g/ml; collagen, 6×10^{-6} g/ml.

[c]I, H_2O fraction; II, 90% MeOH fraction; III, hexane fraction; Concentrations of plant fractions, 5 mg/ml (or 2.5 mg/ml in parenthesis)

[d]Degrees of platelet aggregation induced; −, no aggregation; ±, slight aggregation; +, less aggregation; ++, as much aggregation with PRP plus aggregating agent alone; +++, more aggregation.

[e]The data represent the average of minimum three testings.

is responsible for the further recruitment of remaining platelets and thus for the formation of the aggregates. The arachidonate pathway of aggregation is thought to be mediated by thromboxane A_2 formed from the prostaglandin endoperoxides derived from AA. An antagonist or an inhibitor of platelet aggregation is expected to block at least one of the above mentioned aggregating stimuli (ADP, AA and collagen) induced aggregation.

A total of 54 plant test fractions, prepared from 18 species, were tested for their effects against ADP, AA or collagen-induced platelet aggregations and the results are summarized in Table 1. PRP aggregated with the addition of an aggregating stimuli (ADP, AA or collagen), while PRP itself did not aggregate. Aspirin was employed as a positive control agent. For every batch of PRP prepared, control smears were prepared to insure that PRP alone did not aggregate (−), PRP plus an aggregating agent gave appropriate aggregation (+), and PRP with aspirin plus an aggregating agent reduced aggregation. Those samples with inhibitory activities against platelet aggregation should result in reduced aggregation (−) or (±), as was the case with aspirin. Most of the plant samples tested showed no effects upon platelet aggregation induced by any of the three aggregating agnts. Fraction I of *Acanthopanax* sp. and Fraction I of *Panax ginseng*, however, showed strong inhibitory effect against ADP-induced platelet aggregation. Fr. II of *Conioselinum* sp. and Fraction II of *Lycium chinense* showed strong inhibitory effects against AA-induced aggregation, and Fraction II of *Angelica gigas* and Fraction II of *Scutellaria baicalensis* against collagen-induced aggregation. Many other plant samples showed rather mild inhibitory activities. Several plant sample fractions, such as fractions of *Achyranthes japonica*, induced more aggregations than the controls (PRP plus aggregating agent). The above results are indicative that various species of plants contain both platelet antiaggregating and platelet aggregation inducing components and therefore, suggestive that plants could be the sources for the identification of new classes of compounds with antiplatelet or antithrombotic potential.

3) Identification of Antiplatelet Aggregating Substances from *Acanthopanax senticosus* Max.

The water fraction (Fr. I) of *Acanthopanax* sp. is one of the two fractions showed strong inhibitory activities against ADP-induced platelet aggregation during our screenings. *A. senticosus* Max collected from Chunnam, Korea was also inhibitory. Thus, the work was proceeded to the activity guided treatments and fractionations of the water fraction prepared from *A. senticosus* and yielded two anti-platelet aggregatory substances, 3, 4-dihydroxybenzoic acid (comp. 1) and its artefact ethyl 3, 4-dihydroxybenzoate (comp. 2). Comp. 1 and comp. 2 showed comparable inhibitory activities against collagen induced platelet aggregations to those of aspirin. Against ADP induced platelet aggregation, comp. 1 was as potent as aspirin while comp. 2 was significantly more potent than aspirin. Both comp. 1 and comp. 2 were less inhibitory than aspirin against AA induced platelet aggregations, Comp. 2 was one-half active as aspirin however, was five times more active than comp. 1.[21]

Twenty-four different dihydroxy-benzoic acid derivatives were purchased to investigate the structural-activity relationships (SAR) for inhibitory activities against platelet aggregations induced by ADP, AA or collagen. These include various positional isomers of dihydroxybenzoic acids, their esters, mono- or di-methylated analogs at phenolic functions, etc. Esterification of 3, 4-dihydroxy- or 3, 5-dihydroxybenzoic acids was favorable for the inhibitory activities, however methylation of the phenolic hydroxyl groups was

unfavorable among the compounds tested. Although extensive conclusions could not be obtained with the screening results with only the limitted number of compounds, the observations are valuable for the designing of the follow-up researches toward the investigation of more potent anti-platelet aggregating agents.

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