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MODIFIED SMEAR METHOD FOR SCREENING POTENTIAL INHIBITORS OF PLATELET AGGREGATION FROM PLANT SOURCES[1]

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ABSTRACT.—The smear method developed by Velaskar and Chitre was modified to allow the massive screening of plant extracts and/or fractions for platelet antiaggregating activity. Aspirin and papaverine, known inhibitors of platelet aggregation, were tested by the modified method and showed comparable results with previously reported data. Fifty-four fractions prepared from eighteen plant species were screened for their inhibitory effects on adenosine 5'-diphosphate, arachidonic acid, or collagen-induced rat platelet aggregation. The results suggest that plants could be a potential source of inhibitors of platelet aggregation.

Platelet aggregation is a crucial factor in the pathogenesis of ischemic disease (1,2). Because they readily aggregate in response to a variety of endogenous substances and secrete various substances that cause further aggregation, platelets can initiate thrombus formation and precipitate thromboembolism leading to ischemic diseases (3). In addition, substances secreted from platelets can mediate many other biologic reactions and may also be involved in atherogenesis and other pathologic processes. Hence, drugs that inhibit platelet aggregation or secretion could have wide biological implications. 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). Indeed, a number of valuable drugs are either directly derived from plants or synthesized using plant components as prototypes. In addition, there is a voluminous history of the use of plants in folklore for the treatment of symptoms related to thromboembolic disorders.

Attempts were made to screen plant preparations for their potential antiplatelet aggregating activities with either the turbidimetric method of Born and Cross (5,6) or the smear method of Velaskar and Chitre (7). The method for screening plant materials for their biological activities should be, as Suffness and Douros have pointed out, adaptable to materials which are highly colored, tarry, and poorly soluble in water and chemically complex (8). The turbidimetric method of Born and Cross is a 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 as platelets aggregate.

The smear method developed by Velaskar and Chitre (7) 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. However, only a few tests can be made with a single collection of blood because whole citrated blood, which is stable for only about 15 min, is employed in the original procedure. In

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addition, only those agents that induce platelet aggregation at room temperature can be utilized as aggregating agents 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. Aspirin and papaverine, which are known inhibitors of platelet aggregation, were examined with the modified smear method and demonstrated results comparable with those of the turbidimetric method.

Using the modified smear method, fractions prepared from eighteen plant species were screened for their effects against adenosine 5′-diphosphate (ADP), arachidonic acid (AA), or collagen-induced rat platelet aggregation.

EXPERIMENTAL

PLANT MATERIALS AND REAGENTS.—Specified parts of the crude plant materials were purchased from a local herb drug market and identified taxonomically by Professor Hyung Joon Chi of the Natural Products Research Institute, Seoul National University. The specimens are deposited at the Institute. ADP (adenosine 5′-diphosphate dicyclohexylammonium salt), (AA Carachidonic acid sodium salt) and collagen (acid soluble, from calf skin) were purchased from Sigma Chemical Company.

PREPARATION OF PLANT TEST SAMPLE FRACTIONS.—Plant materials were extracted and fractionated as described in Figure 1. Each specified part of the dried plant was extracted twice with refluxing MeOH for 6 h. 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.

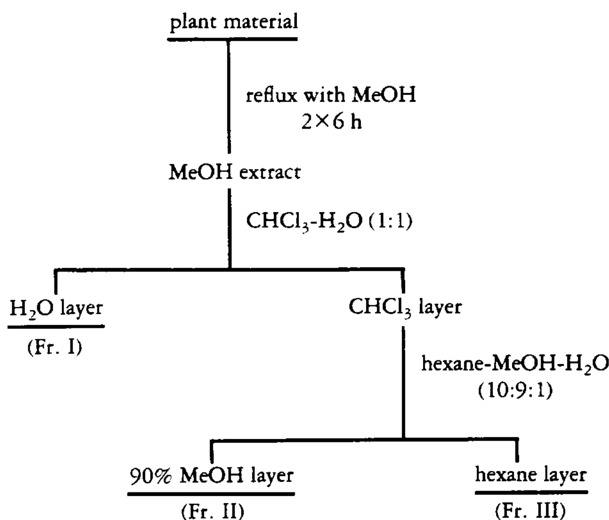


FIGURE 1. Extraction and Solvent Fractionation of Plant Materials

BLOOD COLLECTION AND PLATELET RICH PLASMA (PRP) PREPARATION.—A male Sprague-Dawley rat (220±30 g) was anesthetized with $CHCl_3$ and blood drawn from the heart into a plastic syringe containing 1/10 volume of 2.2% trisodium citrate. The citrated blood was centrifuged at 200×g for 10 min at room temperature, and supernatant platelet rich plasma (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.

SCREENING PROCEDURE (MODIFIED SMEAR METHOD).—H_2O-soluble test samples were dissolved in saline, while H_2O-insoluble samples were first dissolved in EtOH and then homogenized with the addition of saline to give 1% of the final EtOH concentration. 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. The test sample solution (0.02 ml) was added to 0.16 ml of PRP and mixed (saline or EtOH-saline for controls). Two min after 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. 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. The procedure up to this point should be carried out within 3 h of PRP preparation. The glass smears were fixed in EtOH, stained with a Wright-Giemsa stain (7), washed, and dried. 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.

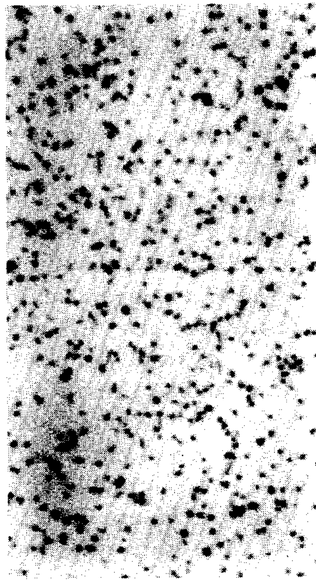
For every batch of PRP prepared, control smears were prepared to insure that PRP alone showed (−), PRP plus an appropriate aggregating agent (+ +) and PRP with aspirin (positive control) at two different concentrations plus aggregating agent (±) or (−). Representative smears of varying degrees of platelet aggregation are shown in Figure 2.

RESULTS AND DISCUSSION

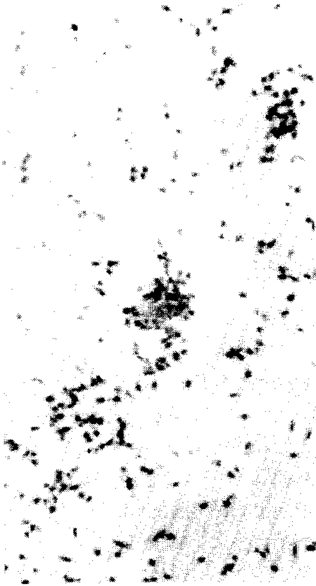
SCREENING METHOD (MODIFIED SMEAR METHOD).—The turbidimetric method of Born and Cross (5,6), which is a widely practiced method for measuring the degree of platelet aggregation in vitro, was determined to be unsuitable for routine screening of plant extracts or fractions. Velaskar and Chitre (7) developed a smear method in which whole citrated blood is smeared on a glass plate to measure circulating platelet aggregates and to measure the response of the total platelet population to aggregating agents. This method was found to be useful inasmuch as neither the color nor solubility of plant-derived 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 place of whole blood, the modified smear method used in this study employs platelet rich plasma (PRP), which is stable for about 3 h, thus providing enough time for preparation 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 nonplatelet cells were removed by centrifugation. Aspirin and papaverine, known inhibitors of platelet aggregation (9,10), were subjected to screening by the present modified method against ADP, AA, and collagen-induced rat platelet aggregation. Although there are species differences between 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. The results summarized in Table 1 are comparable with previously reported data obtained by the turbidimetric method with human blood.

The present modified smear method, therefore, appears to be quite suitable for the primary screening of plant extracts or fractions for antiplatelet aggregating activities.

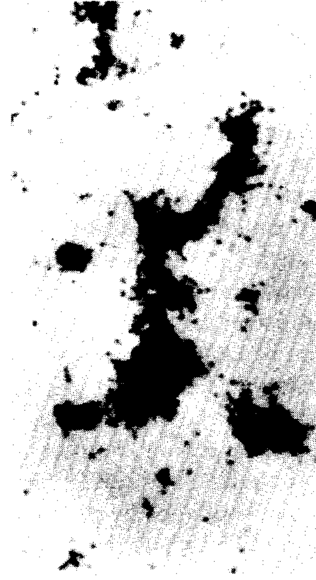
SCREENING OF PLANT PREPARATIONS.—Eighteen plants with a folkloric reputation in Korea were selected for screening. The MeOH extract prepared from each specified part of the plant was partitioned into three fractions following the procedure of Suffness and Douros (4). Fraction I (H_2O fraction) presented no solubility problems;



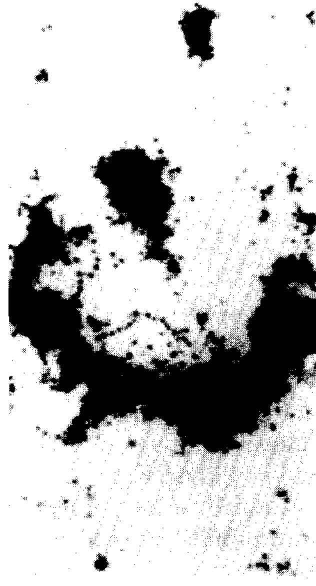
Representative slide of (−): Platelet rich plasma (PRP) plus saline alone.



Representative slide of (+): PRP plus Fr. I of *Conioselinum* sp. and adenosine 5′-diphosphate (ADP).



Representative slide of (++): PRP plus adenosine 5′-diphosphate (ADP).



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

FIGURE 2. Representative Slides of Varying Degrees of Platelet Aggregation

TABLE 1. Inhibitory Activities of Aspirin and Papaverine against Rat Platelet Aggregation

	μg/ml[a]		
	ADP	AA	Collagen
Aspirin	1000	50	250
Papaverine	100	10	25

[a]Minimum concentration of inhibiting agents in which platelet aggregation was fully inhibited. Platelet rich plasma (PRP) was pretreated with either aspirin or papaverine for 2 min before the addition of 1×10^{-6} g/ml ADP, 6×10^{-5} g/ml AA, or 6×10^{-6} g/ml collagen.

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 (15,16); nonetheless, at final concentrations of 1%, EtOH did not significantly affect the aggregation of rat platelets induced by ADP, AA, or collagen.

Platelet activation is a sequence of complicated morphological and functional changes involving a variety of substances and enzymes. Verstraete (17) and Vargaftig *et al.* (18) 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 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 2. 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, three 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 (−) or (±). 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 agents. Fraction I of *Acanthopanax* sp. and Fraction I of *Panax ginseng*, however, showed strong inhibitory effect against ADP-induced platelet aggregation. Fraction 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

TABLE 2. Effects of Plant Preparations against ADP, Collagen, or Arachidonic Acid (AA) Induced Platelet Aggregation

Plant name (Family name)	Parts of Plants used[a]	Fractions[b]	Aggregating Agents[c]		
			ADP	AA	Collagen
Controls					
PRP (without aggregating agents)			−[d]	−	−
PRP (with aggregating agents)			++	++	++
PRP plus aspirin			−(±)[e]	−(±)[f]	−(±)[g]
Acanthopanax sp. (Araliaceae)	ba	I	−(±)	++	++
		II	++	++	++
		III	++	++	++
Achyranthes japonica (Miquel) Nakai (Amaranthaceae)	ra	I	+++	++	++
		II	++	+++	++
		III	+++	++	++
Angelica gigas Nakai (Umbelliferae)	ra	I	++	++	++
		II	++	±(++)	−(+)
		III	++	++	++
Astragalus membranaceus Bunge (Leguminosae)	ra	I	++	++	++
		II	+(++)	++	++
		III	++	++	++
Atractylodes japonica Koidzumi (Compositae)	rh	I	++	++	++
		II	++	+++	++
		III	++	++	++
Carthamus tinctorius Linné (Compositae)	fl	I	++	++	++
		II	++	++	++
		III	+++	++	++
Chrysanthemum indicum Linné (Compositae)	fl	I	++	++	++
		II	+++	+(++)	±(++)
		III	+++	++	++
Conioselinum sp. (Umbelliferae)	rh	I	+(++)	++	±(++)
		II	±(±)	−(±)	++
		III	±(+)	++	++
Crataegus pinnatifida Bunge (Rosaceae)	fr	I	±(+)	++	++
		II	±(+)	++	++
		III	++	++	+++
Fritillaria sp. (Liliaceae)	tu	I	++	++	++
		II	++	++	++
		III	++	++	++
Ledebouriella seseloides Wolff (Umbelliferae)	ra	I	++	++	++
		II	±(++)	++	±(++)
		III	++	++	++
Lycium chinense Miller (Solanaceae)	fr	I	++	++	++
		II	±(+)	−(+)	±(++)
		III	+++	+++	++
Machilus thunbergii Siebold et Zuccarini (Lauraceae)	sb	I	++	++	++
		II	++	++	++
		III	++	+++	++
Panax ginseng C. A. Meyer (Araliaceae)	ra	I	−(±)	++	++
		II	++	++	++
		III	++	++	++
Polygonatum japonicum Morren et Decaisne (Liliaceae)	rh	I	++	++	++
		II	+(++)	±(++)	±(++)
		III	++	++	++
Prunus persica Batsch (Rosaceae)	sm	I	++	++	++
		II	±(+)	++	++
		III	++	++	++

TABLE 2. *Continued*

Plant name (Family name)	Parts of Plants used[a]	Fractions[b]	Aggregating Agents[c]		
			ADP	AA	Collagen
Rehmannia glutinosa Liboschitz . . .	ra	I	++	++	++
(Scrophulariaceae)		II	++	++	++
		III	++	+++	++
Scutellaria baicalensis George . .	ra	I	±(+)	++	±(++)
(Labiatae)		II	±(++)	±(±)	−(±)
		III	+++	++	++

[a]ba, bark; fl, flower; fr, fruit; ra, radix; rh, rhizome; sm, seeds; tu, tuber; sb, stem bark.
[b]Concentration of plant fractions; 5 mg/ml (or 2.5 mg/ml in parenthesis).
[c]ADP, 1×10^{-6} g/ml; arachidonic acid (AA), 6×10^{-5} g/ml; collagen, 6×10^{-6} g/ml.
[d]Degrees of platelet aggregation induced: −, no aggregation; ±, slight aggregation; +, less aggregation; ++, as much aggregation with PRP plus aggregating agent alone; +++, more aggregation.
[e]Aspirin, 1 mg/ml (or 0.5 mg/ml in parenthesis).
[f]Aspirin, 0.05 mg/ml (or 0.025 mg/ml).
[g]Aspirin, 0.25 mg/ml (or 0.1 mg/ml).
The data represents the average of minimum three testings.

species of plants contain both platelet antiaggregating and platelet aggregation inducing components. Incidentally, preliminary work on the isolation of the active components from *Acanthopanax* sp. has been reported by the authors (19). Further, inhibitory activities against various aggregating agents have been reported with ginsenosides, saponins isolated from *Panax ginseng* (20,21).

The screening results from the 54 plant fractions prepared from 18 species, therefore, suggest that plants could be the sources for the identification of new classes of compounds with antiplatelet or antithrombotic potential, and the authors feel further research in these areas is merited.

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