5.

Assay for Hemolytic Activity

Hemolysis is the breaking open of red blood cells and the release of hemoglobin into the surrounding fluid (plasma, *in vivo*). *In vivo* (inside the body) hemolysis, which can be caused by a large number of conditions, can lead to anemia. A number of organic compounds (natural and synthetic) have the ability to hemolyse red blood cells. This property is referred to as hemolytic activity. A large majority of organic compounds having this activity have been found to be organic compounds belonging to the class of steroidal or terpenoidal glycosides generally referred to as saponins. This activity disappears when the carbohydrate components of the saponins are removed by chemical or enzymatic cleavages. It is therefore possible to test for saponins in plant/animal extracts by performing this bioassay.

The test is usually carried out using agar plates that are prepared coated with 5-10% mammalian blood. Test organic compounds are inserted into small wells dug in the blood coated agar plates and when left for about 6-12 hrs, hemolytic activity can be seen around the top of the wells, where complete digestion of the red blood cell contents had taken place, if the test extract contained saponins.

Screening for Saponins

Purpose: To screen parts of plants or animals for saponins.

Rationale: Saponins are an important class of natural products. They have very interesting biological activities such as anti-inflammatory, molluscicidal, fungistatic, toxic and spermicidal activities. A number of medicinal/ayurvedic plants contain saponins. The medicinal properties of these plants have been attributed (in part) to the presence of saponins in these plants.

Procedure: There are two experiments which could be used to detect the presence/absence of saponins. If both the experiments give positive results, then the presence of saponins in the materials being tested can be confirmed. In the first test called the FROTH TEST, the material can be directly used or a methanol extract of the material can be used instead. In the second test which is called the HEMOLYSIS TEST, only the organic solvent extract is tested.

Froth Test

1 Weigh about 100 mg of the test sample and place it into a clean, dry test tube. For the control, use 2 ml of the "Saponin Control" in a separate test tube. In the absence of a saponin control, use powdered soyabean seeds, which are known to contain saponins.

2 Add 10 ml of distilled water to each tube, stopper, and shake vigorously for about 30 secs. Allow the tubes to stand in a vertical position and observe over a 30 minute period of time.

3 If a "honeycomb" froth greater than 3 cm above the surface of the liquid persists after 30 minutes, then the test sample is presumed to contain saponins.

Hemolysis Test

1 Obtain a blood coated agar plate and, using a small test tube, scoop a cup of blood agar from three areas of the plate - each equidistant apart.

2 Using a 1-2 mm diameter glass rod, heat the rod in a Bunsen burner flame, and immediately seal off the agar at the bottom of each cup so that when the liquid samples are introduced into the cups, they do not spread beneath the surface of the blood agar. The heating may have to be repeated several times.

3 Using a dropper or a small pipette, add enough of the test extract (80% MeOH) to fill two-thirds of the cup. Fill the second cup with the saponin control and the third cup should be filled with the solvent control (MeOH).

4 Allow the plate to stand undisturbed for 24 hrs. At the end of this period of time, observe the blood agar plate for any clear zone of hemolysis surrounding any of the three cups. If present, measure (in mm) the hemolytic zone from its end to the edge of the cup and record the results.

You may wish to read more about this test, its usefulness and its limitations in the following publications:

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Hemolysis Test for Saponins—A Caution

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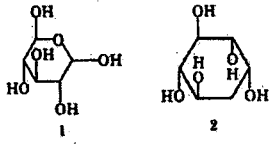
Saponins are an important class of natural products. Saponins have biological properties such as anti-inflammatory (*1–3*), molluscicidal (*4–6*), fungistatic (*7*), toxic, (*8*), and spermicidal (*9*) activities. Search for new saponins is, therefore, a worthwhile exercise. Two phytochemical screening tests are employed (*10*) in the search for saponins in the extracts from plants and marine organisms. The formation of a honeycomb froth (froth test) when an extract is shaken with water and the hemolysis of red blood cells (hemolysis test) by an extract when left in contact with red blood cells are taken to indicate the presence of saponins in the test extract. These tests are simple enough to allow a large number of extracts to be assayed for the presence of saponins in medicinal plants and marine organisms (*10*). A negative hemolysis result is interpreted as an absence of saponins. In this paper the efficacy of the hemolysis test has been examined using several pure natural products whose structures are known. An attempt has been made to explain the effect of the structure of the compound on the hemolytic activity. Hemolytic activity has been discovered for compounds that are not saponins.

Results and Discussion

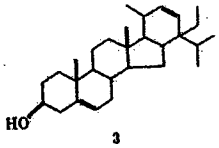
Eight compounds (1–8) belonging to different classes of natural products and possessing one or more hydroxyl groups were subjected to the hemolysis test. The compounds tested included a carbohydrate (glucose, 1), a hydroxylated cyclic alkane (*vibo*-quercitol 2), a sterol (sitosterol, 3), saponins (4–6), a triterpene (phytolaccagenic acid, 7), and an ecdysone (8).



1

2

5 Saponin standard



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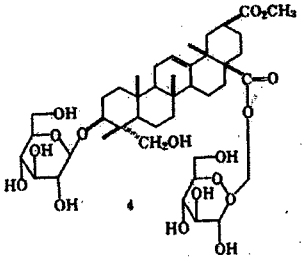

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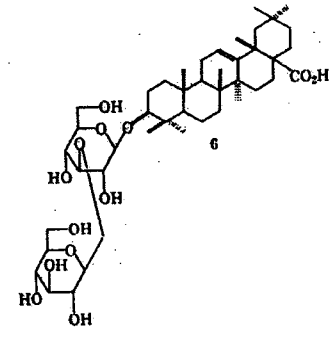
Two blood-coated agar plates were used in the test. The test was performed as described previously (*10*) and the results are summarized in the table. All compounds were tested at concentrations of 0.1 M. At this concentration glucose (1) did not hemolyze red blood cells. But the pentahydroxy cycloalkane (2) gave a weakly positive test. β-Sitosterol (3) gave a negative test. But the triterpenoid, 7, gave a weakly positive test. Surprisingly the diglucoside of 7 (4) produced only a very weak hemolysis. At the same concentration 7 hemolyzed red blood cells better than 4. At lower concentrations 4 showed no hemolysis. The saponin, randianin (6), gave a positive hemolysis test. The hemolytic activity of 20-hydroxyecdyson (8), a nonsaponin, compared well with that of saponin 6.

Hemolytic Activity of Some Natural Products

Compound	Hemolysis[a] Zone (diameter, cm)
1	0.0
2	0.30
3	0.00
4	0.10[b]
	0.00[c]
5	2.00
6	0.70
7	0.40
8	0.80

[a] Measured from the center of cup to edge of zone.
[b] Unless specified otherwise the concentration of each compound used was 0.01 M.
[c] At 0.001 M concentration.



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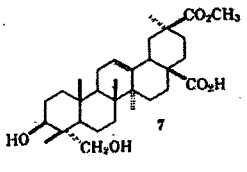

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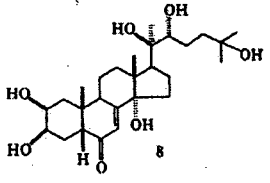

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The results indicate that for hemolytic activity the necessary structural requirements are the presence of a hydrocarbon backbone and a number of hydroxyl groups. The hemolytic activity in 2 and 8, which are not saponins, confirms this view. Thus a positive hemolytic activity observed during a

phytochemical screening test need not necessarily indicate the presence of saponins in the test extract. On the other hand, a negative hemolysis test may not necessarily mean the absence of saponins in the test extract as evidenced in the test results for 4 at different concentrations (see table). Caution should be exercised in using the hemolysis test results.

Experimental

Glucose (1), sitosterol (3), and the authentic saponin (5) were purchased from Aldrich Chemical Company Ltd. Compounds 2, 4, 6, 7, and 8 were available from previous work (*11–14*). 0.1 M concentrations of the compounds 1–8 were prepared in methanol: water (9:1). Preparation of the blood-coated agar plates and sample applications have been described earlier (*10*). Hemolysis zones were measured from the center of the cup and are reported in the table.

Acknowledgment

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Screening for Saponins Using the Blood Hemolysis Test

An Undergraduate Laboratory Experiment

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Saponins are steroid/triterpenoid glycosides. Many saponins are found in plants as well as in some marine organisms such as sea cucumbers and starfish. Saponins are poisonous to fish and lower animals. They are produced by marine organisms for their defense against predators. Plant saponins have been shown to have interesting biological activities such as spermicidal activity (*1*) and molluscicidal activity (*2*). Saponins from some sea cucumbers show (*3*) a high order of activity against pathogenic fungi such as *Trichophyton mentagrophytes, Microsporum gypseum, Candida albicans,* and *Candida utilis.* The interesting pharmacological properties associated with the Chinese drug ginseng, which is considered a panacea and a drug for longevity, are attributed to the various saponins present (*4*) in ginseng. Plant saponins such as dioscin are commercially sought after as starting materials for the synthesis of steroid hormones. Saponins are therefore, chemically and pharmacologically, an interesting group of natural products.

Saponins, when shaken with water, reduce the surface tension of water and produce a honeycomb froth. Saponins behave like soap, thus, their name is derived from the Latin word for soap. All saponins hemolyze blood, that is, they break down the red blood cells. This property is used in the screening test for saponins (*5*). Screening of plant materials or marine organisms has been hitherto confined to phytochemical and marine research groups. Screening tests for saponins can be incorporated into undergraduate laboratory courses. This would enable a large number of biological specimens to be examined for saponins in a short time and also would enable undergraduates to learn about such screening techniques. Besides, positive results would mean the discovery of new sources of saponins.

Collection of Material and Identification

The collection and identification of materials has to be done by the instructor. About 10 g of each of the biological materials would be necessary for the hemolysis test. The help of biologists could be sought for the identification of the material collected for examination.

In one laboratory course at the University of the South Pacific, 21 biological materials were examined. Each student was assigned one material. The biological materials included medicinal plants and marine organisms. The material (10 g) was extracted with aqueous methanol (75%) using a Soxhlet extractor. The concentrated extract was used in the hemolysis test as described below.

Hemolysis Test (*5*)

Seven blood-coated agar plates were used in the screening test. Three students were given one plate. Each agar plate contained five samples: three students' samples, one positive control (a saponin), and one solvent control (75% MeOH–H_2O).

Preparation of Blood-Coated Agar Plates

Commercially available blood-agar base (8 g) and distilled water (200/mL) were sterilized, cooled to 45 °C. Five percent fresh defibrinated blood was added and was mixed thoroughly. The blood-coated agar medium was poured into sterilized Petri dishes. The plates can be stored in the refrigerator prior to use.

Application of Samples

Using a small test tube, cups of blood agar were removed from the agar plate from five areas of the plate, equidistant from one another (see diagram). Using a 1-mm-diameter glass rod, which had been heated previously in a Bunsen burner flame, the agar at the bottom of each cup was sealed off so that the liquid samples when introduced into the cups would not spread beneath the surface of the blood agar. Saturated solutions of the extracts from three biological

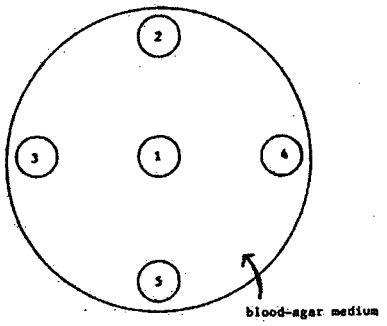

Diagram: Blood-agar plate with five cups for introduction of samples.
1. Cup to introduce solvent control.
2. Cup to introduce saponin control.
3–5. Cups to introduce extracts from three materials.

Yield of Extracts and Hemolysis Zones

Material	Yield of Methanol Extract[a]	Hemolysis Zone[b]
Sea cucumber		
Holothuria edulis[c] (body wall)	0.6	1.3
Bohadschia argus (body wall)	0.7	1.4
Psolus fabricii (body wall)	1.5	3.2
Plant material		
Dioscorea esculenta[c] (yam)	1.0	1.3
Derris trifoliatus (root)	0.9	1.3

[a] Yield is expressed as a weight percent with respect to the material.
[b] Hemolysis zone is indicated by the distance from the edge of cup to the farthest end of the zone (in mm). The hemolysis zones for the controls were 75% MeOH–H_2O (nil) and saponin control (0.9 mm).
[c] *H. edulis* (local name: *dairo*) and *D. esculenta* (local name: *sarandra*) are both foods eaten by the indigenous people of the South Pacific Islands.

materials were introduced into three of the cups using separate droppers. The other two cups, in each of the blood-agar plates, were filled with 75% MeOH–H_2O and a solution of a known saponin, respectively.

The Petri dishes containing the blood agar were covered and were left for a 24-h period, and then the agar plates were observed for any clear zones of hemolysis surrounding the cups. If a hemolytic zone was present, then the distance (in mm) from the farthest point of hemolysis to the edge of the cup was measured.

Out of the 21 materials examined by the students, five gave positive results for the hemolysis test. The materials that gave the positive tests and their hemolysis results are given in the table.

Acknowledgment

I am grateful to W. Kenchington for identification of sea cucumbers and to S. Vodonaivalu for identification of medicinal plant materials.

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