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Saponins

Key Words: Steroidal saponins; triterpenoid saponins; sapogenins; hemolytic activity; foaming; hypocholesterolemic effects; medicagenic acid; sapogenin; diosgenin; vanillin-perchloric acid reagent; TLC plate; fenugreek; Quillaja; Yucca.

1. Introduction

1.1. Nature, Mechanism of Action, and Biological Effects

Saponins comprise a large family of structurally related compounds containing a steroid or triterpenoid aglycone (sapogenin) linked to one or more oligosaccharide moieties by glycosidic linkage **(Fig. 1)**. The carbohydrate moiety consists of pentoses, hexoses, or uronic acids. The presence of both polar (sugar) and nonpolar (steroid or triterpene) groups provides saponins with strong surface-active properties that then are responsible for many of its adverse and beneficial effects. The primary biological effect of saponins is the interactions with cellular and membrane components. For example, saponins hemolyze red blood cells by nonspecific interactions with membrane proteins, phospholipids, and cholesterol of erythrocytes.

Saponins are characterized by their hemolytic activity and foaming properties and are responsible for imparting a bitter taste and astringency to plant materials containing high concentrations of saponins. Nonetheless, saponins are reported to affect the permeability of the small intestinal mucosal cells and thus have effect on active nutrient transport. Saponins have also been shown to inhibit various digestive enzymes, including trypsin and chymotrypsin, and are also known to inhibit protein degradation by forming saponin-protein complexes. On the other hand, positive nutritional effects of specific saponins such as hypocholesterolemic effects and improvement of growth in various

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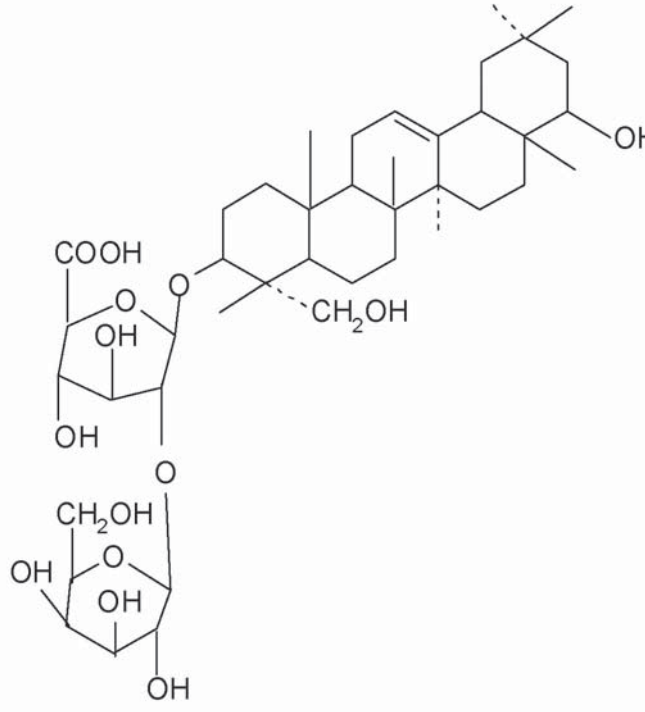
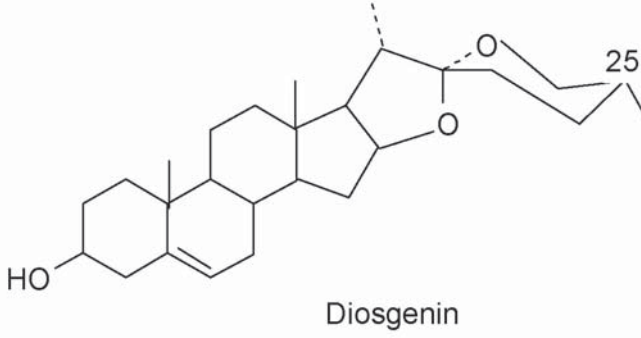
I. Triterpene saponins



Soyasaponin III

2. Steroid saponins



Diosgenin

Fig. 1. Structure of triterpenoid and steroid saponins.

animal species have also been reported. *Medicago sativa* (Alfalfa, Lucerne) contains many saponins. Medicagenic acid is unique to alfalfa. Alfalfa saponins may lower growth rate in chicks and egg production of hens when included in poultry diets above 5%.

The cardiac glycosides are also saponins. Digitalin is the cardiac glycoside in *Digitalis* species and other *Serophulariaceae*. The closely related cardiac glycoside, strophanthin, is found in the Apocynaceae. Livestock poisoning caused by strophanthin has occurred in Kenya when sheep and cattle consumed leaves of *Acokanthera longiflora* and *Acokanthera schimperi*. Death is caused by heart failure. Extracts of these plants are used as arrow poisons.

1.2. Present in

Glycine max, Medicago sativa, M. lupulina, M. media, Chenopodium quinoa, Glycyrrhiza glabra, Phaseolus vulgaris, Vigna angularis, Asparagus officinalis, dried pea, mung bean, runner bean, butter bean, kidney bean, haricot bean, field bean, broad pea, lentil, yellow split pea, chickpea, sunflower, sugar beat, spinach, oats, yam, fenugreek, gilla bean, moth bean, *Amaranthus caudatus, Thea sinensis, Quillaja saponaria, Yucca mohavensis, Y. schidigera, Aloe barbadensis, Lathyrus hirsutus, Smilax aristolochiifolia, Saponaria officinalis, S. sapindus, Aesculus hippcastum* (horse chestnut), *Chenapodium ambrosioides, C. quinoa, Sesbania sesban, S. bispinosa, Trigonella monspeliaca, Zizyphus jujuba, Treculia africana* (African breadfruit), *Artocarpus altilis* (Polynesian breadfruit), *Lotus corniculatus.*

1.3. Principle of Assays

This chapter presents four methods: determination of total steroidal saponins, based on Baccou et al. ***(1)***; determination of total saponins, based on Hiai et al. ***(2)***; determination of saponins based on hemolytic activity, based on Francis et al. ***(3)***; and qualitative evaluation of saponins, based on Burbano et al. ***(4)***.

The method for quantification of total steroidal saponins is based on the reaction of steroidal saponins with anisaldehyde and ethyl acetate in acidic medium to give a colored complex, the absorbance of which is measured at 430 nm ***(1)***.

In the method for determination of total saponins, steroidal sapogenins with or without double bond at C-5, triterpenoid sapogenins, and sterol and bile acids that have an OH group at their C-3 position react with vanillin in acidic medium to give chromogens with the absorbance maxima at 455 to 460 nm or 460 to 480 nm or at 544 nm, depending on the nature of the saponins ***(2)***. The chromogen formed is not dependent on the nature of sugar moieties.

In the third method based on hemolytic properties, saponins in a hemolytic unit are determined visually by twofold serial dilution of the plant extract ***(3–5)***. The qualitative evaluation of saponins is based on separation of saponins on the thin-layer chromatography (TLC) plate and location of saponin spots by their violet blue color produced by spraying the vanillin–perchloric acid or sulfuric acid reagent. The principle that saponins have hemolytic activity is also used for detecting hemolytic saponins. The developed TLC plates are sprayed with 6% cattle erythrocyte solution. The clear zones against the red blood erythrocyte area indicate the presence of saponins.

2. Materials

2.1. Determination of Total Steroidal Saponins

1. *Reagent A:* Add 0.5 mL of anisaldehyde to 99.5 mL of ethyl acetate and mix thoroughly.
2. *Reagent B:* 50 mL of concentrated sulfuric acid (95–98% w/w) plus 50 mL of ethyl acetate.
3. *Standard saponin solution:* Weigh 10 mg of diosgenin and dissolve it in 100 mL of ethyl acetate (0.1 mg/mL).

2.2. Determination of Total Saponins

1. *Vanillin reagent (8%):* Dissolve 800 mg of vanillin in 10 mL of 99.5% ethanol (analytical grade).
2. *72% (v/v) sulfuric acid:* To 28 mL of distilled water, add 72 mL of sulfuric acid (analytical grade, 95%, w/w).
3. *Standard saponin solution:* Weigh 10 mg of diosgenin, dissolve in 16 mL of methanol, and add 4 mL of distilled water. The final concentration of diosgenin in the solution is 0.5 mg/mL of 80% aqueous methanol. Mix thoroughly and start pipetting immediately.

2.3. Determination of Saponins Based on Hemolytic Property

1. *Phosphate buffer saline, PBS (pH 7.2):* Dissolve 8.2 g NaCl, 0.136 g KH_2PO_4, 0.224 g KCl, and 1.14 g Na_2HPO_4 in 1 L of distilled water (composition is 140 mM NaCl, 3 mM KH_2PO_4, 8 mM KCl, and 1 mM Na_2HPO_4).
2. *Preparation of 3% suspension of red blood cells in phosphate-buffered saline (PBS):* Take blood from cattle into heparinized tubes containing beads. The beads can be removed soon after taking the blood. Centrifuge the blood at 1500 *g* for 5 min and wash the packed erythrocyte cells three times with the PBS (pH 7.0) by centrifugation and subsequent removal of supernatants. The remaining layer of packed erythrocyte cells is diluted to 3% with the PBS.

2.4. Qualitative Evaluation of Saponins

1. *Vanillin-perchloric acid reagent:* Prepare 1% vanillin in ethanol (w/v) and 2% perchloric acid in ethanol (v/v) in a separate bottle. Combine equal volumes of vanilline and perchloric acid reagents.
2. *Sulfuric acid reagent:* Prepare freshly a mixture of ethyl acetate/ethanol/concentrated H_2SO_4 (10 : 10 : 18; v/v/v). Acid should be added drop by drop to the mixture kept on an ice-bath.
3. *Phosphate buffer saline, PBS (pH 7.2):* Dissolve 8.2 g NaCl, 0.136 g KH_2PO_4, 0.224 g KCl, and 1.14 g Na_2HPO_4 in 1 L of distilled water (composition is 140 mM NaCl, 3 mM KH_2PO_4, 8 mM KCl, and 1 mM Na_2HPO_4).
4. *Preparation of 6% suspension of red blood cells in PBS:* Take blood from cattle into heparinized tubes containing beads. The beads can be removed soon after taking the blood. Centrifuge the blood at 1500 *g* for 5 min and wash the packed erythrocyte cells three times with the PBS (pH 7.0) by centrifugation and subsequent removal of supernatants. The remaining layer of packed erythrocyte cells is diluted to 6% with the PBS. This erythrocyte solution for spraying on the plates should be prepared freshly.

3. Methods

3.1. Determination of Total Steroidal Saponins

3.1.1. Preparation of Extract

Take 10 g of defatted sample (finely ground, preferably using a ball mill) in a 250-mL flask and add 100 mL of 50% aqueous methanol. Keep it on a magnetic stirrer overnight at room temperature. Centrifuge the contents at 3000 *g* for 10 min and collect the supernatant. Repeat extraction with the same solvent by stirring on a magnetic stirrer for overnight. After centrifugation combine the first supernatant with the second one. If any particles are floating on the surface of the solvent, the filtration through Whatman filter paper is necessary. Evaporate methanol from the solution under vacuum at approximately 42°C by using a rotary-evaporator. Then centrifuge the aqueous phase at 3000 *g* for 10 min to remove the water insoluble materials. Transfer the aqueous phase into a separating funnel and extract with equal volume of chloroform (three times) to remove pigments. Finally extract concentrated saponins in the aqueous solution with equal volume of n-butanol (two times). Evaporate the solvent n-butanol under vacuum at a temperature not higher than 45°C or by nitrogen flushing. Dissolve the dried fraction containing saponins in 5 to 10 mL of distilled water and transfer the solution into a separate preweighed container. Freeze-dry the fraction and calculate the percent recovery of saponins (*see* **Notes 1** to **3**).

3.1.2. Preparation of Calibration Curve

1. Place 0, 20, 40, 60, 80, and 100 µl of the diosgenin standard solution (0, 2, 4, 6, 8, and 10 µg) in test tubes and make up the volume to 2 mL with ethyl acetate.
2. Add 1 mL of reagent A and 1 mL of reagent B.
3. After stirring, place the test tubes at room temperature for 30 min.
4. Measure absorbance at 430 nm against the reagent blank (0 µl of the diosgenin standard solution).

3.1.3. Determination of Saponins

1. Dissolve a known amount of extracted (freeze-dried) crude saponins in 80% aqueous methanol.
2. Take its suitable aliquots (corresponding to a sapogenin content of between 0 and 40 µg) in test tubes. Place the tubes in a boiling water bath or in a hot air bath at 100°C in order to remove alcohol (flushing with nitrogen gas could also be done).
3. After cooling, add 2 mL of ethyl acetate and carry out the determination as for diosgenin described in **Section 3.1.2**.

3.2. Determination of Total Saponins

3.2.1. Preparation of Calibration Curve

1. Place 0, 50, 100, 150, 200, and 250 µl of the diosgenin standard solution (0, 25, 50, 75, 100, and 125 µg) in test tubes and make the volume up to 0.25 mL with 80% aqueous methanol.
2. Add 0.25 mL of the vanillin reagent, and then 2.5 mL of 72% (v/v) sulfuric acid slowly on the inner side of the wall.
3. Mix the solution well and transfer the tubes to a water bath adjusted at 60°C.
4. After 10 min, cool the tubes in ice-cold water for 3 to 4 min, and measure absorbance at 544 nm against the reagent blank (0 µl of the diosgenin standard solution).

3.2.2. Quantitation of Total Saponins

Dissolve a known amount of extracted freeze-dried saponin residue (*see* **Section 3.1.1.**) in 80% aqueous methanol. From this, take an aliquot of 0.25 mL and carry out the determination as for the standard saponin.

3.3. Determination of Saponins Based on Hemolytic Property

1. Place 50 µL of the red blood cells suspension (3%) in separate wells of a microtiter plate.
2. Dissolve 10 to 20 mg of the freeze-dried saponin-enriched fraction (*see* **Section 3.1.1.**) in 1 mL of the PBS. Prepare a series of twofold diluted solution with the PBS.

3. Add a 50-µL aliquot of these twofold diluted solutions of saponins with the PBS to each well and incubate the mixture at room temperature for 2 h.
4. At the end of the incubation, visually determine which hemolyzed erythrocyte well is just before the well completely containing the nonhemolytic erythrocytes. A clear concentric circle around the red blood cells is indicative of a nonhemolytic well, and the spread of red color in the well and absence of a clear zone around red blood cells shows hemolysis.
5. The hemolytic activity is expressed as the inverse of the minimum amount of saponin extract/mL assay medium in the highest dilution that started producing the hemolysis. The results can also be expressed as compared to the hemolytic activity of a commercial standard triterpenoid saponin mixture from Quillaja bark, run simultaneously with the test sample. Any other commercially available hemolytic saponin can also be used as a reference.

3.4. Qualitative Evaluation of Saponins

1. Prepare developing solvent mixture of chloroform/methanol/water (65 : 35 : 10, v/v/v). Pour about 120 mL of this solvent mixture into a chromatographic tank and saturate the tank for overnight.
2. Dissolve 5 mg of the freeze-dried crude saponin residue (*see* **Section 3.1.1.**) in 1 mL of 50% aqueous methanol. From this stock, take 5 µl and load on each lane, 2 to 2.5 cm above from the bottom of plate, on a TLC plate (20 cm × 20 cm, Silica gel 60; Merck catalogue No. 1.05721).
3. After completely drying the spots, insert the TLC plate in the chromatographic tank containing the developing solvent mixture and place in the proper position.
4. When the developing solvent reaches approximately 1 cm below the top of the TLC plate (could take 3 to 4 h), remove the plate carefully and allow it to air-dry in room temperature.
5. Then spray the spraying reagent (the vanillin-perchloric acid reagent or sulfuric acid reagent) and heat at 100°C for 5 min. Saponins as violet or blue spots are located visually on the plates.
6. For evaluating the hemolytic nature of saponins, develop another set of the TLC plates in the same manner. Soon after air-drying the developed plates, spray uniformly the 6% blood erythrocytes on the surface of the plate. After 2 to 3 min, the location of white spot on a red background indicates a hemolytic activity of the separated saponins.
7. The results can be interpreted in terms of how many and which saponin spots out of the total spots located visually using the vanillin-perchloric acid reagent or sulfuric acid reagent produce hemolytic activities.

4. Notes

1. For leaf samples, after extraction of saponins in 80% aqueous methanol, the pigment can be removal by using chloroform. Pigment removal is not necessary for the grain samples.

2. Without the purification step, 80% aqueous methanol extract of sample may be directly used for the estimation of total saponins. However, many moieties present in the extract could interfere in the assay, giving an overestimate of the saponin values.
3. To get more purified and specific targeted saponins, the acetone precipitation of saponins from the *n*-butanol fraction may be carried out.

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