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Isolation, identification and toxicity of saponin from different legumes

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Saponin extracts were prepared from peas, beans and soya bean seeds by four different methods. Two biological assays were developed for measuring toxicity of crude saponin extracts based on haemolytic activity and fish mortality. The results indicated that saponin extracts were able to lyse red blood cells with different velocity. The haemolytic activity of bean extracts were significantly higher ($P < 0.05$) than those of soya bean and pea extracts. Sensitivity of blood cells to crude saponin extracts was detected by sheep and rabbit blood cells. The highest haemolytic activities of sheep and rabbit blood cells were 30·0 and 6·24 mg saponin equivalent/g legume sample, respectively. As well as being potent haemolysins, saponin extracts were lethal to guppy fish. The lethal dose (LD_{50}) of saponin to guppy fish was 150 μg/ml. The ethanol/water (1 : 1) extracts showed the highest toxicity as revealed by both assays. Thin-layer chromatography (TLC) of crude saponin extract from beans separated it into six fractions, whereas pea and soya bean were separated into seven and six fractions, respectively. The TLC pattern of standard saponin indicated the presence of two main spots with R_f 0·75–0·85. Further purification of crude saponin extracts from legumes by silica gel column chromatography increased the haemolytic activity of the active principle 5·7, 5·1 and two-fold for bean, soya bean and pea extracts, respectively.

INTRODUCTION

Natural toxicants occurring in human foods and animal feeds present a potential health hazard to man. Saponins are well known as toxic compounds their toxicity being related to their activity in lowering surface tension. Saponins, complex compounds composed of sugar and steroid or triterpenoid moieties, are widely distributed in plants, but are uncommon in animals (Hashimoto, 1979). Isolation and characterisation of saponin from lucerne (*Medicago sativa*) tops and roots have been reported by Shany *et al.* (1970). The structure of saponin from lucerne has been reported by Gestetner (1971) and Massiot *et al.* (1988). Characterisation of saponin from adzuki bean (*Vigna angularis*) seeds has been reported by Kojima *et al.* (1989).

It has been shown that saponins from alfalfa have a variety of pharmacological properties and physiological activities such as haemolysis (Lindahl *et al.*, 1957; Vacek & Sedlak, 1962; Gestetner *et al.*, 1971) and fungal inhibition (Steiner & Watson, 1965). Saponin levels in plants have been determined by many chemical and biological methods as described by Wall *et al.* (1952), Jackson and Shae (1959), Van Atta *et al.* (1961), Coulson and Davis (1962) and Shimoyamada *et al.* (1991). A microbiological bioassay for determining saponin in alfalfa products using the fungus *Trichoderma viride* was described by Zimmer *et al.* (1967). This bioassay was used by Pedersen *et al.* (1967) and Livingston *et al.* (1977) to determine and quantitate the saponin content of several alfalfa varieties. Pedersen (1975) reported that the saponin in germinated alfalfa seeds increased with germination time.

Haemolysis is considered one of the important characteristics of saponin. The haemolytic activity of saponin has been attributed to their interaction with cholesterol in the erythrocyte membrane (Schmidt-Thome & Prediger, 1950, Joos & Ruyssen, 1967). Haemolysis of erythrocytes has been used to demonstrate the presence of saponin in alfalfa plants. Shany *et al.* (1970) showed the alfalfa root extracts have higher surface activity and are more haemolytic than those from alfalfa tops. Haemolytic factors in soyabeans have been reported by Birk *et al.* (1963). Distribution of saponin in soya bean plants has been reported by Shimoyamada *et al.* (1990).

Fish were used as a biological method to evaluate toxicity of certain plant extracts. The toxic effect of crude alfalfa saponin preparations on fish (*Pimephales promelas*) has been reported by Jones and Elliott (1969). Mullet fish (*Mugil cephalus*) were used as a simple and quick method for identifying ichthyotoxic plants (Gates & Wilson, 1960).

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The present study was conducted to isolate and purify saponin from legumes by different methods and to determine toxicity of saponin using biological assays based on haemolytic activity and fish mortality.

MATERIALS AND METHODS

Preparation of saponin extract

Saponin extracts were prepared from the seeds of three types of legumes: *Phaseolus vulgaris* (Giza 6 beans), *Pisum sativum* (progress 9 peas) and *Glycine max* (L.) Merrill (crawford soya bean), which are purchased from Menoufiya market, Egypt. Samples were ground and extracted with petroleum ether in a Soxhlet apparatus to remove pigments and lipids and then submitted to four different methods of saponin extraction.

(1) The saponin extract was prepared according to the method of Livingston *et al.* (1977). Samples (10 g) were extracted by refluxing for 2·5 h with 150 ml ethanol/water (1 : 1) in a water bath at 95°C. Samples were cooled and filtered (Watman No. 1). The solids were washed carefully with water. The filtrates were combined and taken to dryness in a vacuum drier at 45°C.
(2) Legume samples (10 g) were extracted with 150 ml methanol in a Soxhlet apparatus for 50 h. Solvent was removed from extracts by evaporation in rotary evaporator.
(3) The defatted samples (10 g) were mixed with 100 ml distilled water and extracted for 5 h in a boiling water bath. The mixture was centrifuged at 6000 rpm for 10 min, then dried in a vacuum drier at 45°C.
(4) Aliquots (10 g) of the defatted samples were mixed with 100 ml of phosphate buffered saline (PBS) pH 7·3 and shaken for 2 h. The mixture was centrifuged at 6000 rpm for 10 min.

Haemolysin assay

Haemolytic activity of legume crude saponin extracts was determined according to the procedure of Rodriguez *et al.* (1986). Legume crude saponin extracts were prepared as follows. The dried ethanol/water, methanol and water extracts were redissolved in 100 ml PBS (pH 7·3) to avoid lysis of the red blood cells. The PBS extracts were made up to 100 ml total volume. Legume extracts were filtered and refrigerated at 2°C until used. Two types of blood were used for this assay (sheep and rabbit red blood cells). The red blood cells were washed three times with sterile PBS (pH 7·3). A 2·5% suspension of red blood cells was prepared using PBS; 25 μl of a 2·5% suspension of washed red blood cells were placed in separate wells of a microtitre plate. Several concentrations of the legume extracts and pure saponin from saponaria species were prepared to check for haemolytic activity. The volume of each sample tested was 25 μl. The haemolytic activity titre was expressed as minimal haemolysis saponin equivalents, i.e. the lowest concentration of crude saponin extracts causing complete haemolysis when compared to activity exhibited by saponin from saponaria species.

Fish assay

Guppy fish (*Poecilia reticulata*) were purchased from a commercial source and maintained in aerated and dechlorinated tap water for 2 days prior to the test. Legume crude saponin extracts for the guppy assay were prepared as follows. The dried ethanol/water and methanol extracts were redissolved in 100 ml distilled water. The PBS and boiled water extracts were made up to 100 ml total volume. The extracts were refrigerated at 2°C until used. Five fish (average standard length 18–20 mm) were placed in a beaker containing 90 ml distilled water. A 10 ml sample of each extract was added to each beaker. The test was conducted at room temperature (23–25°C). Survival of the fish was monitored for 3–4 h.

Chromatographic analysis

Thin-layer chromatography (TLC)
The saponin extracts were subjected to TLC on silica gel G plates (0·25 mm silica gel) using the solvent system *n*-butanol/ethanol/water (1 : 1 : 1). The developed plates were dried at room temperature. Visualisation of the saponin on developed plates was done by spraying with 50% (v/v) sulphuric acid. The sprayed chromatograms were allowed to dry for 15 min at room temperature, then heated at 110°C until the colours developed reached its maximum. Standard saponin (Sigma Chemical Co., St Louis, MO, USA) from saponaria species was used as a reference on TLC plates.

Column chromatography
A silica gel column (60–120 mesh) was used for saponin purification. Concentrated crude saponin sample (3 ml) was applied to the column. The impurities were washed with *n*-hexane (two bed volume) through a 2·4 × 50 cm bed of silica gel. The column was eluted with *n*-butanol/ethanol/water (1 : 1 : 1). The flow rate was 30 ml/h and the total bed volume was 102 ml. Fractions (5 ml) were collected. Aliquots from fractions were applied as a series of spots to a strip of TLC plate, dried, sprayed with 50% sulphuric acid and heated. Positive fractions were pooled and assayed for quantitative haemolytic activity as previously mentioned.

RESULTS AND DISCUSSION

Haemolysin assay

Results of haemolytic activity on a microtitre well plate for the saponin extract using sheep and rabbit blood cells are shown in Table 1. In this experiment, the

Table 1. Haemolytic activity of crude saponin extracts isolated from different legumes, expressed as mg saponin equivalent/1 g legume samples[a]

Extraction methods	Beans				Peas				Soya beans			
	Sheep blood cells		Rabbit blood cells		Sheep blood cells		Rabbit blood cells		Sheep blood cells		Rabbit blood cells	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Ethanol/water (1 : 1)	30·04	1·55	6·24	0·61	7·51	0·92	3·12	0·48	15·0	1·60	3·12	0·40
Boiling water	20·00	1·44	5·00	0·43	2·50	0·44	1·25	0·16	10·0	1·20	2·50	0·36
Methanol	2·48	0·29	0·64	0·07	0·62	0·11	0·16	0·04	2·48	0·32	0·32	0·40
Phosphate buffer saline	10·00	1·21	1·90	0·36	2·50	0·38	0·95	0·12	5·00	0·81	1·90	0·42

[a] SD, standard deviation; n, 3.

haemolytic activity titre was expressed as minimal haemolysis saponin equivalents (from saponaria species).

The results of this assay indicated that legume extracts varied in their ability to lyse the red blood cells. The bean extracts possessed higher haemolytic activity, expressed as saponin equivalents, than those of soya bean and pea extracts which in turn reflects the different content of these legumes in saponin. The ethanol/water (1 : 1) and the boiled water extracts showed a higher haemolytic activity than those of PBS and methanol extracts as determined by the microtitre plate. The higher haemolytic activity could be explained by the partial hydrolysis of certain amounts of sugars by using boiled water during the preparation of saponin extracts. Thus, it is reasonable to assume that a higher sapogenin to sugars ratio is established, which in turn results in stronger haemolytic activity. Shany *et al.* (1970) and Gestetner *et al.* (1971) showed that the higher the haemolytic activity, the higher the ratio of sapogenin to sugar.

As shown in Table 1, sheep red blood cells appeared to be more sensitive to saponin extracts than rabbit blood cells. Schmidt-Thome and Prediger (1950) showed that the haemolytic activity varies with the source of the erythrocyte, being low for man and high for sheep when tested with saponin.

The erythrocyte technique clearly differentiated between the haemolytic activity of saponin extracts from different legumes and also, evaluated the extraction efficiency by different methods. It was found that beans, peas and soya beans contained saponins with different haemolytic activities. Saponin from peas showed very low haemolytic activity compared to beans and soya beans. These results agree with the findings of Birk and Peri (1980).

Fish assay

Fish assay was used to determine toxicity of crude saponin extracts isolated from different legumes. The guppy (*Poecilia reticulata*) assay developed in the current study appears to be an excellent assay to evaluate the toxicity of saponin. Also, the assay is easy to conduct, rapid, sensitive, adaptable to laboratory conditions and inexpensive.

Table 2 shows the effect of different extraction methods of saponin on guppy fish. The ethanol/water (1 : 1) extract showed the highest toxic effect to guppy fish. The average survival time of the fish by this method were 3·8, 6·6 and 11·4 min for beans, peas and soya beans, respectively. The next most toxic extract was the boiled water, in which the average survival times of the fish were 10·8, 24·4 and 34·8 min. The methanol and PBS extracts showed very low toxic potential to the fish. The average survival time ranged from 112–172·6 minutes.

When guppy fish were exposed to saponin extracts, the fish appeared normal initially followed by reduced activity. The fish then turned upside-down and showed slow movements. Violent activity was observed prior to death associated with severe symptoms of jumping up to the air and down to the water several times; finally the fish appeared calm on the bottom of the container, for some time and died. The possible reasons for toxicity could be explained by the effect of saponin on the permeability of fish gill membranes and disturbance the respiratory system, or the saponin could transfer through the gill membranes and haemolyse the blood causing death. Correlations between foam number, surface tension, haemolytic activity, and toxicity after subcutaneous administration of saponin have been reported by Vacek and Sedlak (1962). The results of the fish assay indicated that the procedure of ethanol/water (1 : 1) extract was the best extraction method for saponin. This agrees well with the previous results of the erythrocyte assay. Also, it was observed

Table 2. Survival time of guppy fish exposed to crude saponin extracts isolated from legumes[a]

Extraction methods	Survival times (min)					
	Beans		Peas		Soya beans	
	Mean	SD	Mean	SD	Mean	SD
Ethanol/water (1 : 1)	3·8	0·84	6·6	0·55	11·4	1·34
Boiling water	10·8	2·78	24·4	5·41	34·8	5·98
Methanol	124	10·58	144·6	21·51	172·6	15·93
Phosphate buffer saline	112	19·24	140·4	17·05	170·6	13·48

[a] SD, standard deviation; n, 5.

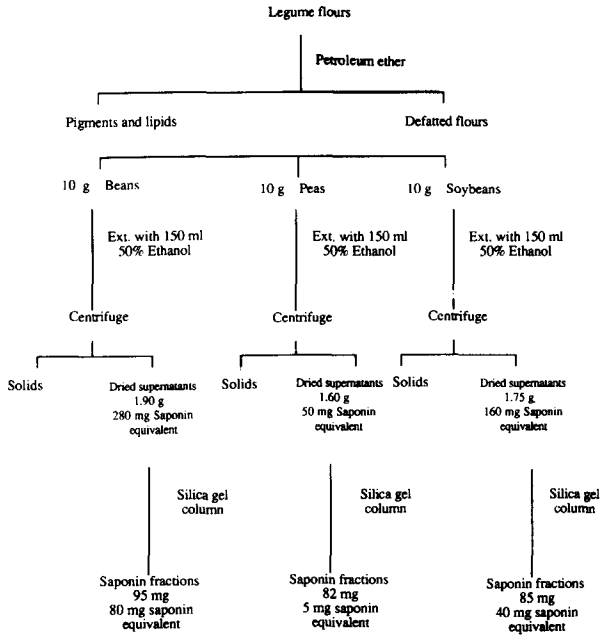

Fig. 1. Flow chart representing extraction, purification and haemolytic activity (sheep red blood cells) of legume flours.

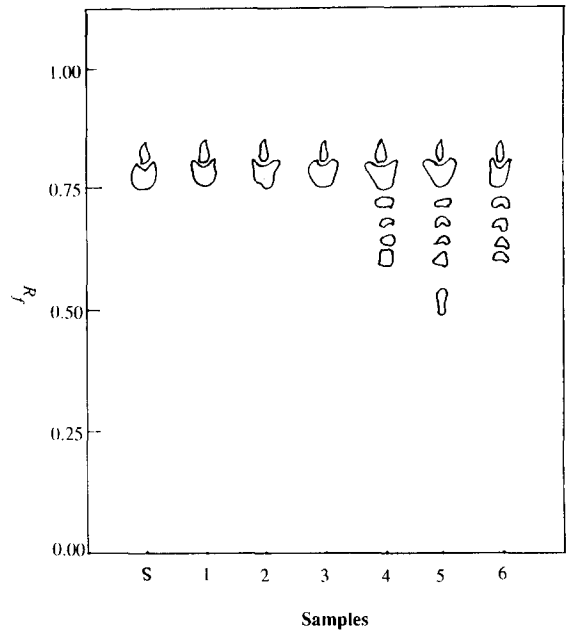

Fig. 2. Thin-layer chromatographic pattern of legume saponin extracts before and after applying to silica gel column. s, standard saponin; 1, beans extract after column; 2, peas extract after column; 3, soya bean extract after column; 4, beans extract before column; 5, peas extract before column; 6, soya bean before column.

that all experimental legume extracts were toxic to fish. In general, the toxicities of saponins isolated from legumes were similar in both assays (fish and erythrocyte) except that peas showed a high toxic potential in the fish assay and very low toxicity in the blood assay, which means that saponins from legumes could behave differently according to chemical composition (Gestetner *et al.*, 1971).

Chromatographic analysis

Figure 1 illustrates the extraction and purification scheme incorporating silica gel chromatography. The positive haemolytic fractions were first observed after elution of 39% of the bed volume and continued until elution of 46% of the bed volume. It should be noted that all toxic fractions were turbid. Fractions exhibiting a positive haemolytic potential were combined and quantitated using the microtitre plate technique. An approximate 5·7-fold increase in haemolytic potency was achieved in bean extract by using column chromatography. Pea and soya bean extracts showed 2- and 5·1-fold increase in haemolytic potency, respectively.

The separation pattern of saponin extracts from legumes on a TLC plate, before and after elution from the silica gel column, is shown in Fig. 2. It can be seen that the saponin extract from beans, before applying to the column, has been separated into six fractions whereas saponin extracts from peas and soya beans have been separated into seven and six fractions each. The distribution pattern of the extracts showed a higher similarity between saponin from beans and soya bean which gave spots at R_f 0·60–0·85. An additional late moving spot could be detected on the chromoatoplate of saponin from pea extract at R_f 0·50. The above results revealed a good relation between fractions exhibiting higher haemolytic activity and the chromatographic pattern on a TLC plate at R_f 0·75–0·85. Examination of the TLC pattern of a saponin standard from saponaria species indicated the presence of two spots with R_f 0·75–0·85. Chromatographically identical spots are obtained after the purification of saponin isolated from legumes using a silica gel column. Similar R_f results of saponin from lucerne were obtained by Gestetner (1971) and Gestetner *et al.* (1971). Thus, it is reasonable to assume that washing the concentrated saponin extract through the column gets rid of the impurities corresponding to the late moving spots in the TLC pattern at R_f 0·50–0·75, which in turn results in stronger haemolytic activity.

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