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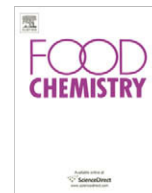

Haemolytic and antimicrobial activities of saponin-rich extracts from guar meal

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ABSTRACT

Saponin-rich GM extract was prepared by refluxing 25 g of GM with 250 ml of EtOH/H_2O (1:1, v/v) for 3 h then filtering and distilling EtOH at 50 °C. The refluxed extract was partitioned with equal volume of BuOH obtaining crude saponin-rich GM extract with 4.8 ± 0.6% DM of GM that was purified by RP-HPLC eluting 20%, 60% and 100% MeOH fractions with 2.04 ± 0.32%, 0.91 ± 0.16% and 1.55 ± 0.15% DM of crude saponin-rich GM extract, respectively. Further purification of 100% MeOH fraction using NP-HPLC eluted four peak sub-fractions at 16, 39, 44 and 46 min. All saponin-rich GM fractions eluted were evaluated for both haemolytic and antimicrobial activities using 96-well plates in eight concentrations. Results indicated that only 100% MeOH fraction and its 16 min peak sub-fraction exhibited both haemolytic and antibacterial activities against *Staphylococcus aureus*, *Salmonella Typhimurium* and *Escherichia coli*, but 20% and 60% MeOH fractions stimulated *Lactobacillus* spp. growth.

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1. Introduction

Antibiotics used are no longer desirable because of concerns about bacterial resistance (Wallace, 2004). Increased awareness of the potential problems associated with the use of antibiotics stimulates research efforts to identify alternatives to their use. Novel approaches in the development of new antimicrobial have been carried out, such as compounds to treat diseases or to improve animal growth include dietary use of probiotics, prebiotics (Higgins et al., 2007; Murry, Hinton, & Morrison, 2004; Patterson & Burkholder, 2003), organic acids (Van Immerseel et al., 2006), medicinal herbs (Arab, Rahbari, Rassouli, Moslemi, & Khosravirad, 2006; Du & Hu, 2004).

Original candidate chemical structures for many pharmaceutical compounds that promote human health originated from chemicals found in plant extracts (Patterson & Burkholder, 2003). Among these plant compounds are saponins from *Quillaja*, *Yucca* and alfalfa which have beneficial effects on animal health (Avato et al., 2006; Sen, Makkar, & Becker, 1998a). Saponins are glycoside compounds whose chemical structures are composed of a fat-soluble nucleus (aglycone) that is either a triterpenoid (C-30) or neutral or alkaloid steroid (C-27) attached to one or more side chains of water-soluble sugars (glycone) through ester linkages to the aglycone nucleus at different carbon sites. Triterpenoid saponins predominate in soybean, alfalfa and *Quillaja* (Haralampidis, Trojanowska, & Osbourn, 2002) while steroid saponins predominant in *Yucca*, tomato and oats (Kaneda, Nakanishi, & Staba, 1987).

Among the various biological effects of saponins are haemolytic activity (Khalil & El-Adawy, 1994; Woldemichael & Wink, 2001) and antibacterial activity (Sen, Makkar, Muetzel, & Becker, 1998b). Some saponins are beneficial while others are considered hazardous to animals (Cheeke, 1998). Biological activities of saponins are affected by factors such as the saponin nucleus type, number of sugar side chains and type of functional groups (Osbourn, 2003).

Guar, *Cyamopsis tetragonoloba* L. (syn. *C. psoraloides*) or cluster bean is a drought tolerant annual legume grown primary for the guar gum (galactomannan polysaccharide) that has many industrial and food processing applications. Guar meal (GM) is a combination of hull and germ fractions that is produced as a by-product of guar gum manufacture. Guar is used in some parts of the world, primarily India and Pakistan, as a human and animal food. Guar also contains 13% dry matter (DM) crude saponin (Curl, Price, & Fenwick, 1986). No research has evaluated the biological activities of guar saponin. Therefore, this study reports isolation of saponin-rich GM extract and evaluates its fractions for haemolytic and antibacterial activities against two gram-positive bacteria (*Lactobacillus* spp. and *Staphylococcus aureus*) and two gram-negative bacteria (*Escherichia coli* and *Salmonella Typhimurium*) in a dose dependent manner.

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2. Materials and methods

2.1. Isolation of saponin-rich GM extract

Ground GM (Rhodia Inc., Vernon, TX) was extracted by refluxing 25 g with 250 ml of EtOH/H_2O (1:1, v/v) for 3 h. The refluxed extract was cooled and filtered through 150 then 125-μm pore size filter papers. EtOH was removed from filtered extract by evaporation under reduced pressure in a rotary evaporator (Buchi, Rinco Instrument Co., Inc., Greenville, IL, Switzerland, model 310391) until two-thirds of the initial volume was removed. Remaining aqueous extract was partitioned with BuOH (1:1, v/v) overnight at room temperature (RT) using a separatory funnel. Upper BuOH extract was collected in a glass flask and lower aqueous extract was collected and further partitioned with BuOH two more times to increase the yield of crude saponin-rich GM extract. BuOH extracts were pooled and evaporated to dryness as described above. A minimum volume of H_2O was added to dry BuOH extract and resulting material was freeze-dried, weighed and stored at RT.

2.2. Preparative chromatography of saponin-rich GM extract

A 40 mm × 150 mm inner diameter flash chromatography column was packed using 30 g of 230–400 mesh C-18 media (EMD Chemicals Inc.) in 100% isopropanol. Column was incorporated into a Bio-Rad Biologic chromatography workstation with a model 2128 fraction collector and equilibrated with isopropanol for 5 min at a flow rate of 30 ml/min. Freeze-dried BuOH extract was dissolved into H_2O and injected onto reverse-phase high pressure liquid chromatography (RP-HPLC) and developed with a step gradient of MeOH/H_2O (20:80, 60:40 and 100:0, v/v), respectively over a 96 min run time at a flow rate of 4 ml/min. Peaks eluting with 20%, 60% and 100% MeOH fractions were detected at 254 nm (Waters 994, programmable photodiode array detector), collected and pooled separately into three fractions and evaporated to dryness using the rotary evaporator (Fig. 1). A minimum volume of H_2O was added to each dried MeOH fraction and resulting material was freeze-dried, separately weighed and stored at RT until used. The procedure was repeated to obtain sufficient pooled material.

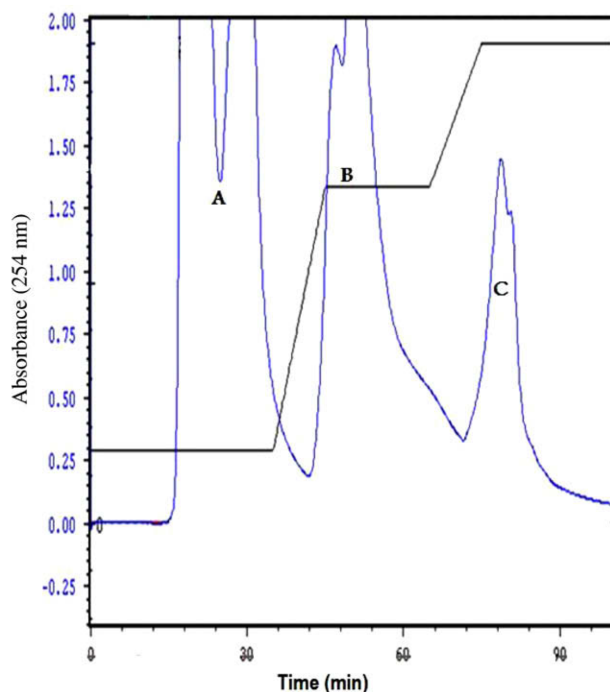

Fig. 1. An example of an elution profile of reverse-phase high pressure liquid chromatography (RP-HPLC) of saponin-rich guar meal *n*-BuOH extract. Fractions were eluted at 20% (A), 60% (B) and 100% (C) MeOH fractions, respectively.

2.3. Isolation of 100% MeOH sub-fractions

The 100% MeOH fraction was further separated into sub-fractions using preparative normal-phase high pressure liquid chromatography (NP-HPLC) column (Waters, model 510) packed with silica gel. In a small test tube 15 mg of freeze-dried 100% MeOH fraction was dissolved in 1 ml of acetonitrile and 50–250 μl of the resultant solution was injected onto a NP-HPLC (Waters, RCM 25 × 10). Silica gel preparative NP-HPLC column was preequilibrated and developed with isocratic acetonitrile: H_2O + 0.025% trichloroacetic acid (50:50, v/v) at a 4 ml/min flow rate using an isocratic mobile-phase over a 50 min run time. Four major sub-fraction peaks were detected at 254 nm (Waters 994, programmable photodiode array detector) and collected with a fraction collector (Gilson model 203) at 16, 39, 44 and 46 min (Fig. 2). This procedure was repeated to obtain sufficient pooled material. Pooled material was evaporated under reduced pressure to remove the last traces of acetonitrile. A minimum volume of H_2O was added to the dry fractions and the resulting material was freeze-dried, weighed and stored at RT until used.

2.4. Haemolytic activity assay

Chicken blood was collected from three mature roosters using 20 ml syringes with 18 gauge needles. Syringes were filled first with 10 ml of stock solution containing sodium citrate as anticoagulant dissolved in phosphate buffered saline (PBS) (15.1 mg/ml PBS (1:1, v/v). Blood samples were decanted into test tubes and placed in an ice bath. Chicken red blood cells (cRBCs) were separated by centrifugation (1000*g* for 5 min) and resuspended with PBS. This procedure was repeated three additional times until the supernatant was colourless. A final 2% cRBCs suspension was prepared by suspending 200 μl of cRBCs with 800 μl of PBS. Fresh cell suspensions were prepared daily.

Stock solutions of 2 mg of 20%, 60% and 100% MeOH fractions and four sub-fractions of 100% MeOH fraction (16, 39, 43 and 46 min retention time) were dissolved in 1 ml of PBS and filtered

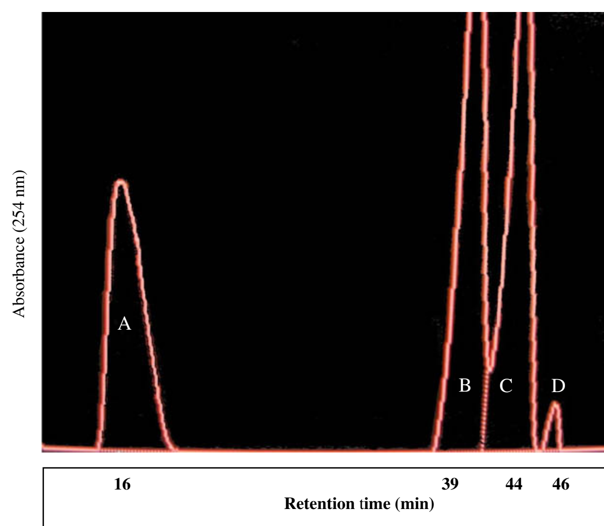

Fig. 2. An example of an elution profile of normal-phase high pressure liquid chromatography (NP-HPLC) of four sub-fractions of 100% MeOH fraction of GM extract at 16 (A), 39 (B), 44 (C) and 46 min (D) retention time.

using 0.2-μm filters. Haemolytic assay was conducted in 96-well flat bottom microtitre plates with low evaporation lids (Microtest™, Becton Dickinson, USA). One hundred microlitres of PBS were added to all wells of the plate except the wells in the first two columns in which 100 μl of H_2O were added. Then, 100 μl of each 20%, 60% and 100% MeOH fraction stock solutions were pipetted into the first row of columns 3–11 with three columns for each fraction. The contents of the first row of wells in columns 3–11 were serially diluted through row eight by aspirating and redispensing three times then transferring 100 μl to the next row. This procedure was repeated until 100 μl were discarded from each column after the last dilution. One hundred microlitres of 2% cRBCs suspension were added to all wells of the plate. This procedure resulted in eight dilutions ranging from 0.01–1.0 mg MeOH fraction/ml of PBS. The first two columns containing H_2O with cRBCs were 100% haemolysis positive control wells, while column 12 containing PBS and cRBCs alone served as the 0% haemolysis negative control wells. Each plate included three wells for each concentration of 20%, 60% and 100% MeOH fraction with two plates/replicate with three replicate plates/experiment.

Same procedure was used to assay the haemolytic activity of four sub-fractions of 100% MeOH fraction (16, 39, 43 and 46 min), but with two microtiter columns for each sub-fraction and two columns for negative and positive controls. Each plate included two wells for each concentration of each fraction with two plates/replicate with three replicate plates/experiment.

Each plate was sealed with Parafilm (Pechiney Plastic Package, Neenah, WI) and covered with a polystyrene plate lid. Plates were kept for 3 h at RT. Preliminary scanning of haemolysis in culture plate wells at wavelengths 405, 455, 520 and 650 nm showed that 650 nm was most efficient. The 650 nm wavelength allowed for optimum readings of cRBCs lysis with little interference. Turbidity was measured by reading well optical density at 650 nm using a multi-well plate Bio-Rad® ELISA reader and observing a decrease in optical density associated with cRBCs lysis.

2.5. Antibacterial activity assay

Minimum inhibitory concentration (MIC) values were used as a measure of antibacterial activity of 20%, 60% and 100% MeOH fractions against *S. aureus* (ATCC 49525), *Lactobacillus* spp., *S. Typhimurium* (NCIM 2719) and *E. coli* (ATCC 933). Average concentration of each bacterium was 1.90×10^6, 7.20×10^7, 5.74×10^5 and 3.88×10^6 colony forming units/ml (cfu/ml), respectively. Antibacterial activity of the four sub-fractions (16, 39, 43 and 46 min) of 100% MeOH fraction was evaluated against *S. aureus* (ATCC 49525), *S. Typhimurium* (NCIM 2719) and *E. coli* (ATCC 933). Average concentration of each bacterium was 1.90×10^6, 7.20×10^7 and 5.74×10^5 cfu/ml, respectively. Cfu/ml density of cultures was confirmed after distribution into assay plates by the following procedure. All bacterial stock solutions were serially diluted 10-fold and plated by inoculating 100 μl onto tryptic soy agar (TSA) (Difco™, Becton Dickinson, USA) plates. Plates were incubated for 24 h at 37 °C. Colonies were counted to calculate cfu/ml of the stock bacteria solutions.

Stock solutions of growing bacteria and standard stock concentrations of each fraction were prepared. A stock solution of each bacterium was prepared by inoculating 100 μl of overnight cultures of each bacterium into 10 ml of sterile tryptic soy broth (TSB) (Bacto™, Becton Dickinson, USA) and incubating overnight at 37 °C. Prior to use, all bacterial stock solutions were passed to new TSB every 8 h three times over a 24 h period to reach log phase growth for use in the study. A stock solution of each extract was prepared by dissolving 25 mg of each fraction/ml of TSB and filtering solutions through 0.2-μm filters before use in 96-well plates. A stock solution of ampicillin was prepared at a concentration of 2 mg/ml of TSB as a positive control.

MIC assays were conducted in 96-well flat bottom microtiter plates with low evaporation lids. One hundred microlitres of TSB were added to all wells except the wells in the first column where 200-μl aliquots of TSB were added. The first column contained only TBS to assure absence of TSB contamination. Column 2 contained ampicillin as a positive control to show minimal growth (0% bacterial growth) while columns 3–11 contained GM fractions with three columns for each fraction. TSB with bacteria alone in column 12 served as a negative control for maximum growth (100% bacterial growth). This procedure resulted in eight serially 2-fold concentration dilutions ranging from 0.10–12.5 mg of each fraction/ml of TSB, and 0.01–1.0 mg of ampicillin/ml of TSB as described previously. Each plate included three replicates of each fraction at each concentration with two plates/replicate and three replicate plates/experiment.

Same procedure was used to assay the antibacterial activity of the four sub-fractions of 100% MeOH fraction (16, 39, 43 and 46 min), by adding 50 μl of each bacterium to each well. MeOH fraction concentrations ranged from 0.005–0.666 mg/ml of TSB. Each plate included two wells for each concentration of 100% MeOH sub-fraction with two plates/replicate with three replicate plates/experiment. Preliminary scanning of bacterial growth in culture plate wells at wavelengths 405, 455, 520 and 650 nm showed that 650 nm was most efficient. The 650 nm wavelength allowed for optimum readings of bacterial growth with little interference in absorption by saponin-rich fractions. Turbidity (growth or inhibition of the bacterial growth) was examined at 0 h and after 24 h of incubation using a Spectra Max 190 plate reader and Soft Max Pro. 3.0 Software (Molecular Devices Corp., Sunnyvale, CA). Bacterial growth was determined by comparison with negative controls in which 100% bacterial growth was recorded.

2.6. Statistical analysis

Each treatment group resulted from 18 individual values ($n = 3$ replicates, two plates/replicate with three wells/plate) of 20%, 60% and 100% MeOH fractions and 12 individual values ($n = 3$ replicates, two plates/replicate with three wells/plate) of four sub-fractions of 100% MeOH fraction for the haemolytic and antibacterial activities, respectively. Means of fractions at each concentration were compared to the negative control group in order to establish significant differences among treatment groups in haemolytic and antibacterial activities. MIC for each treatment was recorded at the lowest concentration which inhibited growth compared to the negative control group. Data obtained were subjected to one-way ANOVA and were expressed as mean ± standard error of mean (SEM) using the GLM programme of statistical software (SPSS 14.0, SPSS Inc., Chicago, IL). Treatment means were separated ($P \leqslant 0.05$) using Duncan's multiple range test (Duncan, 1955).

3. Results and discussion

Initial EtOH of GM extracts were subsequently partitioned by BuOH and subjected to RP-HPLC. This extract separated into three peaks, eluting with 20%, 60% and 100% MeOH fractions. Yields averaged 2.04 ± 0.32%, 0.91 ± 0.16% and 1.55 ± 0.15% DM of BuOH extract, respectively. Further purification of 100% MeOH fraction using preparative NP-HPLC resulted in eluting four sub-fraction peaks at 16, 39, 44 and 46 min.

Saponins frequently are isolated by boiling in MeOH (Oleszek et al., 1992), EtOH (Levy, Zehavi, Naim, & Polacheck, 1989) and BuOH (Massiot, Lavaud, Benkaled, & Le Men-Olivier, 1992). In this experiment, GM was extracted by EtOH:H_2O (1:1, v/v) and the

Table 1
Haemolytic activity of saponin-rich GM extract separated by RP-HPLC into 20%, 60% and 100% MeOH fractions.

Saponin extract	Positive control[A]	Negative control[B]	Methanol extracts		
			20%	60%	100%
mg/ml	*Optical density (OD[C])*				
1.00	0.16 ± 0.01[c]	0.86 ± 0.05[a]	0.74 ± 0.01[a]	0.72 ± 0.01[a]	0.39 ± 0.04[b]
0.50	0.17 ± 0.01[c]	0.81 ± 0.03[a]	0.78 ± 0.01[a]	0.77 ± 0.01[a]	0.44 ± 0.04[b]
0.25	0.15 ± 0.01[c]	0.87 ± 0.04[a]	0.78 ± 0.02[a]	0.78 ± 0.02[a]	0.54 ± 0.04[b]
0.13	0.16 ± 0.02[b]	0.84 ± 0.03[a]	0.78 ± 0.01[a]	0.79 ± 0.01[a]	0.88 ± 0.02[a]
0.06	0.18 ± 0.02[b]	083 ± 0.08[a]	0.79 ± 0.01[a]	0.81 ± 0.02[a]	0.86 ± 0.02[a]
0.03	0.16 ± 0.01[b]	0.85 ± 0.05[a]	0.76 ± 0.01[a]	0.80 ± 0.02[a]	0.81 ± 0.01[a]
0.02	0.16 ± 0.01[b]	0.82 ± 0.01[a]	0.77 ± 0.01[a]	0.79 ± 0.01[a]	0.81 ± 0.01[a]
0.01	0.16 ± 0.01[b]	0.85 ± 0.04[a]	0.77 ± 0.01[a]	0.76 ± 0.01[a]	0.76 ± 0.01[a]

[a–c]Means (±SEM, $n = 3$) within a row that do not share a common superscript are significantly different ($P \leqslant 0.05$).
[A] Water replaced saponin solutions as a positive control since this treatment results in 100% haemolysis.
[B] Phosphate buffered saline replaced saponin solutions as a negative control since this treatment results in 0% haemolysis.
[C] Lower values are associated with increased cell lysis.

resultant extract was partionated with BuOH (1:1, v/v) to elute crude saponin-rich GM extract. Khalil and EI-Adawy (1994) extracted saponins from peas, beans and soybean seeds by refluxing seed samples using four different methods. It was extracted with EtOH/H_2O (1:1, v/v) for 2.5 h in a H_2O bath at 95 °C, pure MeOH in a Soxhlet apparatus for 50 h, H_2O for 5 h in a boiling H_2O bath, and PBS at pH 7.3 while shaking for 2 h. It was found that the EtOH/H_2O (1:1, v/v) extract showed the highest toxicity as assayed by haemolytic activity and fish mortality. Extraction with BuOH efficiently isolates monodesmosidic and short-sugar-chain bisdesmosidic saponins, however, the results in partial or total loss of long-chain bisdesmosidic and trisdesmoside saponins (Oleszek, 1996).

Oleszek (1988) noted that saponin-rich plant extract purified using graded MeOH concentrations showed that eluants up to 40% MeOH remove carbohydrates and some phenolics. Eluants containing 50–60% MeOH remove only the bisdesmoside saponins, while 70–80% MeOH removes monodesmoside saponins. This phenomenon may explain the antibacterial and haemolytic properties of the presumed saponin structures isolated by 100% MeOH fraction rather than 20% and 60% MeOH fractions reported later in this study. Curl et al. (1986) mentioned that the predominant GM saponins have two sugar side chains, one attached at C-3 and another at C-29. It is not known whether the saponins isolated in 100% MeOH and 16-min sub-fraction are bisdesmoside or monodesmoside saponins.

Further purification was performed using a three-step MeOH gradient in combination with a RP-HPLC and three MeOH fractions were eluted. Pure saponins can not usually be obtained by RP-HPLC alone (Oleszek et al., 1992). Individual saponins can be selectively isolated from BuOH extract subjected to RP-HPLC followed by NP-HPLC with H_2O:acetonitrile as solvent systems (Kesselmeier & Strack, 1981). In this experiment, further saponin purification was performed by subjecting 100% MeOH fraction to NP-HPLC, where four sub-fraction peaks were eluted at different (16, 39, 43 and 46 min) retention times.

The 20% and 60% MeOH fractions were not haemolytic at any concentration tested. H_2O positive control (100% haemolysis) and PBS negative control (0% haemolysis) were used as standard references. The 100% MeOH fraction was haemolytic until diluted to less than 0.25 mg fraction/ml (Table 1). Further purification of 100% MeOH fraction by HPLC yielded four sub-fractions. The 16-min sub-fraction was the only sub-fraction of 100% MeOH fraction with haemolytic activity. This fraction was haemolytic until diluted to less than 0.06 mg fraction/ml of PBS concentration (Table 2). Lindahl, Davis, and Tertell (1957) reported that haemolysis can be used to identify the presence of saponins in plant extracts. However, haemolytic activity is not an attribute of all saponins. While *Quillaja* saponins exhibit haemolytic activity (Jenkins & Atwal, 1994), other saponins from soybeans, which are a mixture of soyasapogenol glycosides, are not haemolytic (Gestetner, Birk, & Tencer, 1968).

Authors have reported that aglycone:glycone ratios, number of polar groups and carbohydrate side chain length are the determinant features in haemolytic activity (Segal, Shatkovsky, &

Table 3
The MIC[a] of antibacterial activity for 20%, 60% and 100% MeOH fraction separated by RP-HPLC from saponin-rich GM extract against *S. aureus*, *Lactobacillus* spp., *E. coli* and *S. Typhimurium*.

Bacterium	Methanol extract		
	100%	60%	20%
	MIC (mg/ml)		
S. aureus	3.13	12.5	12.5
Lactobacillus spp.	0.78	PB[b]	PB
E. coli	1.56	12.5	ND[c]
S. Typhimurium	0.78	12.5	12.5

The means of MIC values of each saponin source against different bacteria are shown in the same column.
[a] Minimal inhibitory concentration is the lowest concentration of antibiotic or saponin that inhibits growth of a microbe.
[b] PB, prebiotic effect detected as indicated by microbial growth stimulation at ⩾0.39 mg/ml saponin extract.
[c] ND, MIC assay did not show antibacterial activity at the concentrations tested.

Table 2
Haemolytic activity of four sub-fractions from the 100% MeOH fraction from GM eluted by NP-HPLC at at 16, 39, 44 and 46 min.

Saponin extract	Positive control[A]	Negative control[B]	Peak retention times (min)			
			16	39	44	46
mg/ml	*Optical density (OD[C])*					
1.00	0.30 ± 0.01[b]	0.77 ± 0.05[a]	0.31 ± 0.01[b]	0.87 ± 0.01[a]	0.81 ± 0.01[a]	0.84 ± 0.04[a]
0.50	0.30 ± 0.01[b]	0.77 ± 0.05[a]	0.30 ± 0.01[b]	0.82 ± 0.01[a]	0.80 ± 0.01[a]	0.81 ± 0.04[a]
0.25	0.30 ± 0.01[b]	0.77 ± 0.05[a]	0.33 ± 0.01[b]	0.80 ± 0.02[a]	0.80 ± 0.02[a]	0.79 ± 0.04[a]
0.13	0.30 ± 0.01[b]	0.77 ± 0.05[a]	0.36 ± 0.01[b]	0.80 ± 0.01[a]	0.80 ± 0.01[a]	0.82 ± 0.02[a]
0.06	0.30 ± 0.01[b]	0.77 ± 0.05[a]	0.41 ± 0.01[b]	0.79 ± 0.01[a]	0.81 ± 0.02[a]	0.83 ± 0.02[a]
0.03	0.30 ± 0.01[b]	0.77 ± 0.05[a]	0.67 ± 0.01[a]	0.77 ± 0.01[a]	0.78 ± 0.02[a]	0.81 ± 0.01[a]
0.02	0.30 ± 0.01[b]	0.77 ± 0.05[a]	0.75 ± 0.01[a]	0.78 ± 0.01[a]	0.77 ± 0.01[a]	0.80 ± 0.01[a]
0.01	0.30 ± 0.01[b]	0.77 ± 0.05[a]	0.75 ± 0.01[a]	0.78 ± 0.01[a]	0.77 ± 0.01[a]	0.80 ± 0.01[a]

[a–b]Means (±SEM, $n = 3$) within a row that do not share a common superscript are significantly different ($P \leqslant 0.05$).
[A] Water replaced saponin solutions as a positive control since this treatment results in 100% haemolysis.
[B] Phosphate buffered saline replaced saponin solutions as a negative control since this treatment results in 0% haemolysis.
[C] Lower values are associated with increased cell lysis.

Table 4
Antimicrobial activity of four sub-fractions from the 100% MeOH fraction from GM eluted by NP-HPLC at 16, 39, 44 and 46 min against *S. aureus*.

Saponin extract	Positive control[A]	Negative control[B]	Peak retention times (min)			
			16	39	44	46
mg/ml	*Optical density (OD[C])*					
0.666	0.05 ± 0.01[c]	0.51 ± 0.05[a]	0.23 ± 0.01[b]	0.54 ± 0.01[a]	0.55 ± 0.01[a]	0.55 ± 0.04[a]
0.333	0.05 ± 0.01[c]	0.51 ± 0.05[a]	0.33 ± 0.01[b]	0.46 ± 0.01[a]	0.41 ± 0.01[a]	0.42 ± 0.04[a]
0.167	0.05 ± 0.01[b]	0.51 ± 0.05[a]	0.43 ± 0.01[a]	0.43 ± 0.02[a]	0.38 ± 0.02[a]	0.38 ± 0.04[a]
0.083	0.47 ± 0.01[a]	0.51 ± 0.05[a]	0.49 ± 0.01[a]	0.42 ± 0.01[a]	0.40 ± 0.01[a]	0.40 ± 0.02[a]
0.042	0.48 ± 0.01[a]	0.51 ± 0.05[a]	0.55 ± 0.01[a]	0.38 ± 0.01[a]	0.39 ± 0.02[a]	0.40 ± 0.02[a]
0.021	0.47 ± 0.01[a]	0.51 ± 0.05[a]	0.51 ± 0.01[a]	0.40 ± 0.01[a]	0.39 ± 0.02[a]	0.39 ± 0.01[a]
0.011	0.47 ± 0.01[a]	0.51 ± 0.05[a]	0.49 ± 0.01[a]	0.40 ± 0.01[a]	0.40 ± 0.01[a]	0.41 ± 0.01[a]
0.005	0.52 ± 0.01[a]	0.51 ± 0.05[a]	0.55 ± 0.01[a]	0.46 ± 0.01[a]	0.45 ± 0.01[a]	0.45 ± 0.01[a]

[a–c]Means (±SEM, $n = 3$) within a row that do not share a common superscript are significantly different ($P \leqslant 0.05$).
[A] Contained only ampicillin at 0.005–0.666 mg/ml.
[B] Contained neither ampicillin nor saponin.
[C] Lower values of optical density were indicative of decreased bacterial growth and increased antibacterial activity.

Milo-Goldzweig, 1974). Haemolytic activity is thought to be due to effects on cell membrane permeability by forming pores in membranes, altering the sodium–potassium and calcium–magnesium ATPase activities, or insertion of the hydrophobic saponin nucleus into the lipid bilayer (Hu, Konoki, & Tachibana, 1996).

MIC is the lowest concentration of antibiotic or, in this case, saponin-rich GM extract that can inhibit growth of a bacterium. The 100% MeOH fraction was more effective than either 20% or 60% MeOH fractions against *S. aureus, Lactobacillus* spp., *E. coli* and *S. Typhimurium* (Table 3).

It is of interest that 20% and 60% MeOH fractions showed a mild prebiotic effect with *Lactobacillus* spp. at concentrations ⩾0.39 mg fraction/ml. MICs were 3.13 and 0.78 mg fraction/ml for 100% MeOH fraction against *S. aureus* and *Lactobacillus* spp., respectively. A similar MIC was observed for 100% MeOH fraction exposed to *E. coli* and *S. Typhimurium* at 1.56 and 0.78 mg fraction/ml, respectively (Table 3).

The 16 min sub-fraction of 100% MeOH fraction was the only effective fraction against *S. aureus* at concentrations equal to or above than 0.333 mg fraction/ml (Table 4). None of the sub-fractions of 100% MeOH fraction exhibited any antibacterial activity against *E. coli* and *S. Typhimurium* at the concentrations tested. None of the sub-fractions of 100% MeOH fraction were not tested against *Lactobacillus* spp. Results suggested some antibacterial activity was lost during the sub-fractioning of the RP-HPLC 100% MeOH extraction. In general, many saponins are considered natural antimicrobial compounds making up a plant's defence systems (Morrissey & Osbourn, 1999). Some studies reported antibacterial activity of dietary saponins in ruminant animals. For example, adding *Yucca* extracts to the ruminant animal diet negatively affected cellulolytic bacteria without any effect on amylolytic bacteria (Wang, McAllister, Yanke, & Cheeke, 2000). In another study adding saponin-rich *Yucca* powder or extract to ruminant animal diets inhibited gram-positive bacteria (Cheeke, 1998). Also, some studies noted that commercially available *Quillaja* saponin (*Quillaja saponaria*) and *Yucca* saponin (*Yucca schidigera*) showed different antibacterial activity against *E. coli*, suggesting that saponins from various sources, extracted using different procedures, differ in their biological activities, most likely due to different chemical structures (Sen et al., 1998b).

Kuete, Tangmouo, Penlap Beng, Ngounou, and Lontsi (2006) reported significant antibacterial effects of saponin-rich MeOH fractions from the stem bark of *Tridesmostemon omphalocarpoides* (Sapotaceae). These observations agree with our results where 100% MeOH fraction exhibited antibacterial activity against all bacteria tested. Many saponins show a dose response effect on bacterial activation such as saponins isolated from *Bauhinia variegata* L. bark that exhibited more sensitivity for gram-negative bacteria than gram-positive bacteria at concentrations ranging from 2.5 to 10 mg/ml (Morrissey & Osbourn, 1999).

In contrast, our results showed that saponin-rich GM fraction was more active against gram-positive bacteria than gram-negative bacteria. These findings agree with results obtained by several other studies (Avato et al., 2006). These differences may be due to the degradation of these saponins by some glucosidase enzymes produced by gram-negative bacteria. Variability of saponin glycone side chains in terms of number, chemical composition specific point of attachment to the steroid or triterpenoid nucleus is critical to the saponins biological effects (Osbourn, 2003).

Mode of action of antibacterial activity of saponins against both gram-negative and gram-positive bacteria is not yet clear. Avato et al. (2006) noted that the aglycone part of the saponin is the antibacterial determinant suggesting that the sugar moiety is not important for the antimicrobial efficacy while another study reported that saponins hydrolysed by bacterial enzymes to its corresponding aglycone resulted in decreased antibacterial activity (Mandal, Sinha Babu, & Mandal, 2005).

In conclusions, BuOH extracts from GM yielded 4.8 ± 0.6% DM crude saponin-rich GM extract. BuOH extract purified by RP-HPLC resulted in three peaks eluting with 20%, 60% and 100% MeOH fraction to yield averages of 2.04 ± 0.32%, 0.91 ± 0.16% and 1.55 ± 0.15% DM of BuOH extract, respectively. Further NP-HPLC purification of 100% MeOH fraction eluted four peaks with 16, 39, 44 and 46 min. Haemolytic activity was observed only in 100% MeOH fraction and its 16 min sub-fraction. Antimicrobial activity of 100% MeOH fraction was most effective against *S. aureus* and *E. coli* bacteria. Only 100% MeOH fraction exhibited both haemolytic and antibacterial activities against *S. aureus*, *Lactobacillus* spp., *S. Typhimurium* and *E. coli*. The 20% and 60% MeOH fractions were neither haemolytic nor antibacterially active, however, *Lactobacillus* spp. showed growth at concentrations ⩾0.39 mg/ml. Saponins may be interacting in some way with the bacterial cell walls, however, the specific mode of action is not yet clear. Therefore, further research is required to describe the mode of action of saponins against both gram-positive and gram-negative bacteria.

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