SUPPLEMENTARY MATERIAL

Title: Direct detection of saponins in crude extracts of soapnuts by FTIR

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Direct detection of saponins in crude extracts of soapnuts by FTIR

Abstract: Direct detection of saponins in soapnuts (*Sapindusmukorossi)* using Fourier Transform Infrared (FTIR) spectroscopy is investigated in this project. Potassium bromide (KBr) powder was mixed with extracted powder of soapnuts and compressed to a thin pellet for examination process. The outcome of the FTIR spectra of saponin demonstrated characteristic triterpenoidsaponin absorptions of OH, C=O, C-H, and C=C, while the glycoside linkages to the sapogenins were indicated by the absorptions of C-O. The significance of this study is that saponin absorption peaks are directly detectable in crude aqueous and 95% ethanol extracts of soapnuts powder using FTIR spectroscopy thereby eliminating the need of further expensive and exhaustive purification steps. The extracts of soapnuts were screened for saponins along with controls by phytochemical tests and advanced spectroscopic techniques like Ultra Fast Liquid Chromatography and UPLC QTOF MS/MS were also implemented to validate the saponins.

Keywords: saponin; soapnuts; FTIR; extraction; phytochemical; UFLC; UPLC QTOF MS/MS

1. Experimental

1.1 Materials

Dry fruits of *Sapindusmukorossi*(Lot# HAUS57/30185-1) commonly known as soapnuts were purchased from ISKA GmbH, Germany. Laboratory reagent grade standard saponin (from *Quillajasaponaria*) was purchased from Fisher Scientific, UK. Absolute ethanol and HPLC grade acetonitrile, obtained from Sigma, Germanyand Milli-Q water from Milllipore filtration unit was used for UPLC and UFLC. Tryptone soya agar (CM0131) obtained from Oxoid, UK and defibrinatedblood from sheep obtained from Saudi Prepared Media Laboratory (Ltd), KSA was used for the preparation of blood agar plate.

1.2 Sample preparation and extraction of saponins

The dark brown colouredsoapnuts were sorted and outer pericarps were separated from the seeds manually. The soap nuts (500g) were placed in a vacuum oven (Isotemp Vacuum Oven, Model 281A, Fisher Scientific, UK) at 60^{o}C for 48hours and were ground into a fine powder by using a dry grinder (Stardust), and stored in airtight plastic containers. Dry

soapnuts(*Sapindusmukorossi*) powder (50g) was used for extraction with 95% ethanol at a ratio of 1:10 with 500 ml of extractant in erlenmeyer flasks covered with aluminum foil to reduce evaporation. The suspension was agitated on a hotplate with magnetic stirrer (Thermo Nuova) for 3 hours at 45ºC and centrifuged using Sorvall Lynx model 4000, at 12000 rpm for 30min at 25ºC. The supernatant obtained was carefully decanted into freeze dryer bottles (Labconco) and placed on a tray layered with aluminum foil. The pellet was collected and added to 500ml of 95% ethanol to extract and centrifuge at same conditions as described previously. The extracts were frozen at -80ºC for 16-24 hours and loaded onto a freeze dryer (LabconcoFreezone 18 Freeze dry/Shell Freeze system). The freeze dried sample was then stored in airtight bottles at room temperature.The phytochemical tests on the extracts were carried out according to standard methods as described by Tewari et al. (2011) and Sotheeswaran (1988). While, the qualitative analyses of saponins was conducted with the help of modern spectroscopic methods like UPLC and UFLC QTOF MS/MS.

1.3 Phytochemical tests for saponins

***1.3.1 Foam test*:** Froth forming capacity of saponin was demonstrated by a foam test. The samples containing saponin when shaken with water for 10 minutes until a honeycomb froth was formed. Then a 200mg of the extracts, standard and water were thoroughly shaken with 2 ml of distilled water and left undisturbed for a further ten minutes. The extent of foam produced was observed after 10min and the final samples were screened for saponins

1.3.2 Hemolysis test:All saponins can hemolyse blood, that is, they break down the red blood cells (Fong et al. 1977). This technique was used in the screening test for saponin by application of the saponin containing sample on an agar base containing blood.

1.3.2.1 Preparation of Blood Agar Plates

Standard conical flask of 250 ml was used for preparation of solid media. The flask was filled with 100 ml distilled water and 4 g of Tryptone Soya agar powder. The conical flask was placed on the hotplate stirrer (CB302), to boil the solution to dissolve completely. Then, the mixture was sterilised using Autoclave (TOMY SX-500) at 121^{o} C for 15 minutes. The solution was

cooled to 45°C, and then 7ml of the sheep blood was pipetted carefully into the petri dishes to avoid bubbles. Subsequently, the solution was allowedto solidify at room temperature for 20 minutes.

1.3.2.2 Application of Samples

Four wells, 4mm deep were cut on the solidified agar equidistant from each other using a sterile well cutter. 120µl each of the aqueous extract, 95% ethanolic extract, standard Quillajasaponin (positive control) at a concentration of 125mg/ml dissolved in sterile distilled water and sterile water (negative control) were carefully dispensed into the wells and kept covered for 24 hours at ambient temperature. The blood agar plate was observed for zone of clearance. Also, the distance from the farthest point of hemolysis to the edge of the well was measured and recorded.

1.4 Fourier TransformInfra-Red Spectroscopy (FTIR)

Dry powder of the extract obtained was mixed with KBr salt, using a mortar and pestle, and compressed into a thin pellet. Infrared spectra were recorded as KBr pellets on a Jasco Vacuum FT/IR 6300 between 4000–500 cm^{-1}. Infrared absorptions were recorded for the direct detection of triterpenoidsaponins as described byKareru et al. (2008).

1.5 Ultra Performance Liquid Chromatography Quadrupole-Time Of Flight-Mass Spectrometry (UPLC QTOF MS/MS)

All mass spectra of the soapnutsextracts and standardQuillajasaponinwereconducted on aXevo G2-S QTOF LC-MS/MSmicromass spectrometer from Waters, UK. An Acquity UPLC class I system from Waters was operated at room temperature with a BEH C_{18}column (50 x 2.1mm id, 1.7 µm). The mobile phase consisted of water(A) and acetonitrile (B),and was used at a flow rate of 0.6 ml/min under gradient program 40–60% B in 30min. The mass spectral identification of saponins was established by monitoring the ESI spectrum in positive mode.

1.6 Ultra-Fast Liquid Chromatography (UFLC)

Theseparation of saponinswas achieved using a UFLC from Shimadzu (Japan) system equipped with PDA detector on aShimpack ODS C_{18} column(50 x 3mmid, 2.2µm). The mobile phase consisted of water (A)and acetonitrile (B). Gradient elution was performed as follows:5-100% B

in 8min, at a flow rate of 2 ml/min by monitoring the eluentat 206 nm. The injection volume and column temperature was 40µl and 25ºC respectively.

References

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Table S1. Hemolytic zones observed in hemolysis test

Sample	Haemolytic zone* (mm)
Aqueous extract	9
95% Ethanol extract	9
Standard saponin (Fisher)	10
Sterile water (control)	0

*Hemolysis zone is indicated by the distance from the edge of the cup to the farthest end of the zone (in mm)

Table S2. Comparison of characteristic molecular mass fragments in the mass spectrum of the soapnut extracts and standard Quillajasaponin

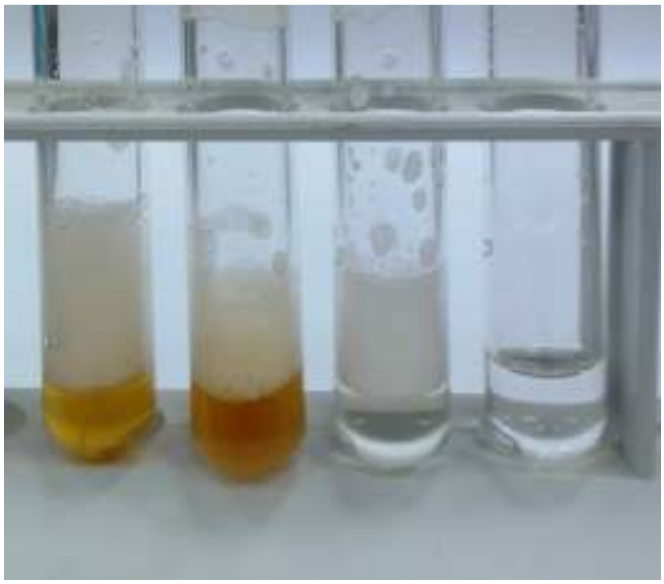
	Charcteristic Molecular mass of triterpenoid saponins							
	Aqueous extract of soapnuts		95% Ethanol extract of soapnuts		Standard Quillaja saponin		Reference*	
	Mass (m/z)	RT (min)	Mass (m/z)	RT (min)	Mass (m/z)	RT (min)	Mass (m/z)	Type of saponin from reference*
1	ND	ND	ND	ND	1076	0.47	1074	Mukurozi-saponin X
2	881	6.63	881	6.60	895	6.41	882	Sapinoside B
3	757	17.63	757	17.59	751	21.28	750	Sapinoside A
4	1024	5.10	1024	5.10	ND	ND	1024	Mukuroziosside1b
5	1148	5.90	1148	5.90	ND	ND	1148	MukuroziosideIIa
6	1206	0.64	1207	0.64	1206	15.93	1206	Mukurozi-saponin Y
7	ND	ND	947	30.3	ND	ND	947	Mukurozi-saponin E1/ Sapinoside L

*Heng et al. (2014); RT=Retention time; ND=Not detected

Table S3. Comparison of retention time of peaks obtained by UFLC

	UFLC Retention time (min)		
	Aqueous extract (a)	95% ethanol extract(b)	Standard Quillajasaponin(c)
1	ND	ND	0.61
2	ND	ND	1.072
3	ND	ND	1.654
4	ND	ND	2.142
5	ND	ND	2.437
6	2.827	2.82	2.702
7	3.025	3.024	ND
8	3.36	3.361	ND
9	3.535	3.532	3.494
10	3.785	3.787	ND
11	4.112	4.112	4.141
12	ND	ND	4.633
13	4.87	4.85	4.803
14	5.042	5.023	4.981
15	5.341	5.115	ND
16	ND	5.334	ND

ND=Not detected



a b c d

Figure S1. Froth test with a) aqueous extract b) 95% ethanol extract c) standard saponin as positive control and d) water as negative control.

Figure S2. Hemolysis test with a) aqueous extract b) 95% ethanol extract c) standard saponin as positive control and d) water as negative control.

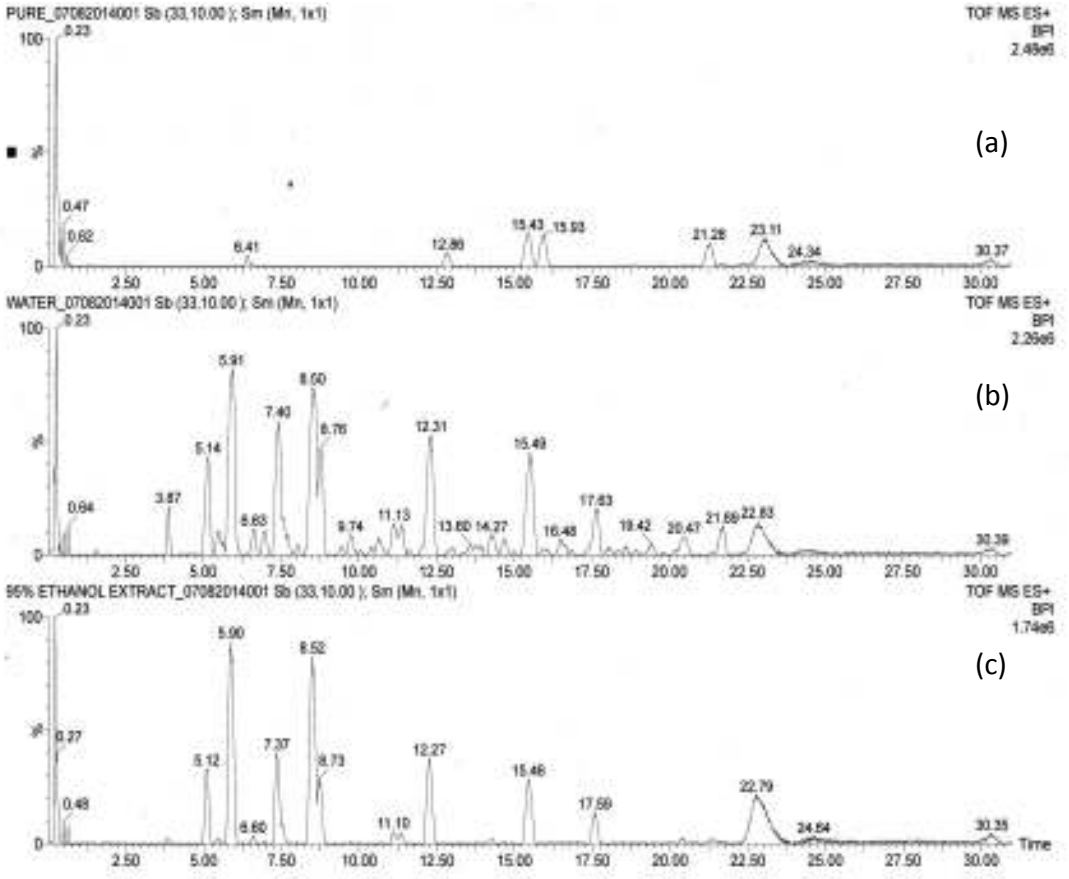


Figure S3.UPLC-MS chromatogram a) Quillaja saponin (Fisher) b) aqueous extracted soapnut saponin, c) 95% ethanol extracted soapnut saponin

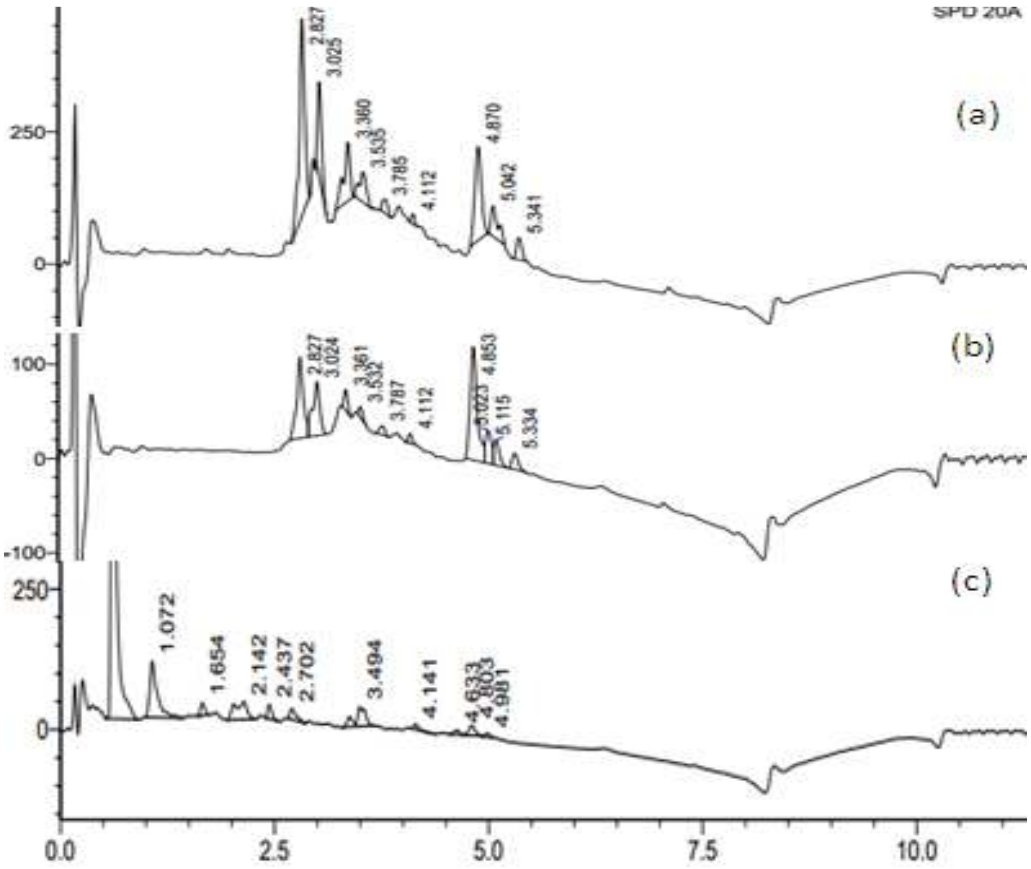


Figure S4. UFLC chromatograms of a) aqueous extracted soapnut saponin, b) 95% ethanol extracted soapnut saponin, c) Quillaja saponin (Fisher) on a Shimpack XR ODS (3mm id × 50mm, 2.2μm) column; gradient elution with solvents water (A)and acetonitrile(B) at 5-100%B in 8min at flow rate 2ml/min; detection at 206nm.