Chemical characterization and antimicrobial activity of saponins isolated from *Saponaria Cypria,* an endemic species of Cyprus

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ABSTRACT

This study aimed at identifying the main chemical components of *Saponaria cypria* extracts, an endemic species of Cyprus, and compare with the composition of the common *Saponaria officinalis* species. Results on chemical characterisation revealed differences on the major saponin compounds between the two *Saponaria* species. The total amount of saponins was isolated from each extract and was studied for antioxidant activity using the DPPH free radical scavenging assay. Also, the antibacterial activity of the saponin extracts was evaluated by determining the minimum inhibitory concentration and minimum bactericidal concentration against gram positive (*Staphylococcus aureus, Enterococcus faecalis)* and gram negative (*E.coli and Salmonella enteritidis*) bacteria. According to the results, *S. cypria* extracts demonstrated higher antioxidant properties compared to *S. officinalis*. Significant antibacterial activity was identified against all types of bacteria, however *S. aureus* demonstrated increased susceptibility to both *Saponaria* extracts. Specifically, the *S. cypria* acetone extract presented the highest antibacterial activity against *S. aureus.* To the best of our knowledge, this is the first report describing *S. cypria*, its main chemical components and its antimicrobial and antioxidant properties. .

INTRODUCTION

Saponaria plants are rich in active molecules called saponins, glycosylated molecules of an amphiphilic nature which form stable, soap-like foams in aqueous solutions. Saponins are known to inhibit bacteria and may therefore be utilized as an alternative source of active components with antimicrobial activity [1]. *Saponaria* species contain a large amount of saponins [2] and several extraction methods for saponins have been reported in the literature [3,4]. Saponin content seems to be affected by a number of factors, such as environmental conditions and other factors affecting the growth of the plant [5]. Saponins isolated from *S. officinalis* roots, the common species, have been previously characterized in terms of their chemical composition and antibacterial activity [6-9]. However, to this date, *S. cypria*, the endemic species of Cyprus, has not been characterized. The aim of this study was to describe the chemical composition of *S. cypria* in comparison to *S. officinalis.* Moreover, the antioxidant and antimicrobial activity of *S. cypria*, the Cypriot *Saponaria* species, was determined.

MATERIALS AND METHODS

Preparation of extracts: *S. officinalis* and *S. cypria* root extracts were prepared as previously described by Barve *et.al*. [3] utilising different solvents (100% MeOH, 100% EtOH, 100% Acetone, water) in a ratio of 1:15, root to solvent. The crude extracts were finally fully evaporated with a rotary evaporator at 60˚C under vacuum and were all dissolved in methanol at desired concentrations. All extracts were stored at 4˚C for further analysis.

Chemical characterisation of saponin compounds by LC-HRMS and determination of total content: The LC qToF instrument, iFunnel Agilent 6550, was used to perform (or performed) the chromatographic separation and the mass spectrometry detection of Saponaria extracts and operated in negative ionization mode using modified conditions as described by Kanwal *et.al*. [10]. All the chromatographic data were acquired in MS mode and AutoMS/MS mode using collision energies at 10, 20, 40 and 60 volts. The acquired data were manipulated using the Agilent Mass Hunter Qualitative Analysis B.06.60 software and guided towards mass to charge ratio of the compounds and fragmentation patterns in order to formulate their molecular formulas and product ions. Furthermore, they will assist towards chemical structure elucidation of the compounds. The total saponin content of *S. officinalis* and *S. cypria* was measured as described by Medina-Meza et al. [11].The results were expressed as mg of oleanolic acid equivalent per gram of dried crude extract.

Antioxidant activity: Antioxidant activity of *S. officinalis* and *S. cypria* extracts was determined using the DPPH free radical-scavenging assay, as previously described by Naimi *et al*. [12]. The half maximum inhibitory concentration (IC^{50}) of the standard and the several *Saponaria* extracts and the Trolox equivalent antioxidant capacity (TEAC) of the extracts was calculated to determine the antioxidant capacity compared to the standard, Trolox. TEAC was calculated as follows: TEAC = IC^{50} of Trolox (mg⁄ml)⁄IC^{50} of sample (mg⁄ml).

Antimicrobial activity using microbroth dilution and time kill assay: The antibacterial activity of the *Saponaria* extracts was studied by determining the MIC/MBC via the broth micro-dilution method as described by Ballouiri *et.al* [13]. *Saponaria* extracts were also tested for their time kill behaviour against *E. coli* and *S. aureus* bacteria as described by Elisha *et.al.* [14]. All samples were incubated at 37°C and optical density was recorded at a wavelength of 600 nm until the cells reached the stationary phase using a TECAN Sunrise ELISA microplate reader, linked to a computer equipped with Magellan 7.5 software. For the time kill assay a graph of the absorbance (nm) against time (minutes) was plotted for each sample. Ampicillin (starting solution 0.516mg/ml, Sigma Aldirch) or Gentamycin (starting solution 0.064mg/ml, Molekula) were used as positive controls.

RESULTS

Solvent type	*Saponaria Officinalis*	*Saponaria Cypria*
	Extract concentration (mg OA/ g crude extract)	
Methanol	2.38 ± 0.009	0.11 ± 0.510
Ethanol	25.78 ± 0.117	26.55 ± 1.040
Acetone	31.16 ± 1.569	42.25 ± 1.790

Table 1. The total saponin content (TSC) expressed as mg OA equivalent (oleanolic acid) /g crude extract for *Saponaria officinalis* and *Saponaria cypria*.

RESULTS

RT	Formula	$[M-H]^-$ m/z	Main fragment ions from MS/MS data	Identification	References
8.29	$C_{60}H_{96}O_{30}$	1295.580	1133^{h},953^{2h},485.3^{Sh}	Unknown saponin*	----
8.86	$C_{66}H_{104}O_{35}$	1455.616	1275^{h},1231^{h+CO2},$1149^{(2dh)}$,969.4^{ps},501.3^{ag},$439^{H2O-CO2}$,485.1^{t},323^{dh-H2O},179^{mh},113^{f}	Unknown saponin	[1,3]
9.23	$C_{54}H_{86}O_{26}$	1149.526	969.4^{h}, 501.3^{ag}, 483^{ag-H2O} , $439^{(ag-H2O-CO2)}$, 485.1^{t}, 341^{2dh},323^{dh-H2O}, 113^{f}	Unknown saponin	[1,3]
9.57	$C_{60}H_{94}O_{30}$	1293.567	1113^{h}, 969.4^{ps}, 501.3^{ag}, 483^{ag-H2O},$439^{(ag-H2O-CO2)}$, 485.1^{t}, 341^{2dh},323^{dh-H2O},113^{f}	Unknown saponin	[1,]
10.06	$C_{78}H_{122}O_{42}$	1729.733^{2-}	$955^{p+3dh+h+Ac(ps)}$, 113^{f}	QA octosaccharide	[2]
10.10	$C_{60}H_{94}O_{29}$	1277.582	1097^{h},791^{3h}, $485.3^{(ag)}$, 485.1^{t}, 161^{f},125^{f},113^{f},101^{f}	Unknown saponin*	----
10.75	$C_{77}H_{120}O_{43}$	1699.720^{2-}	1681^{H2O},1567^{p},$955^{2p+3dh+Ac(ps)}$,$469.1^{(ag)}$	Saponarioside A	[2,10]
11.57	$C_{71}H_{118}O_{43}$	1657.698^{2-}	$955^{2p+3dh(ps)}$,$485.3^{2p+3dh+h+ua(ag)}$,113^{f}	QA heptasaccharide	[2]
12.62	$C_{69}H_{112}O_{40}$	1579.668	1447^{p},1417^{h}, 1399^{h}, 939,$469.1^{(ag)}$	Unknown saponin*	----
14.43	$C_{73}H_{120}O_{43}$	1683.712^{2-}	1551.7^{p}, $939.4^{2p+3dh+Ac(ps)}$, $469.3^{3p+3dh+h+ua+Ac\ (ag)}$	G octasaccharide	[2]
15.82	$C_{64}H_{104}O_{36}$	1447.622^{2-}	$939.2^{p+2dh+2Ac}$,$469.3^{2p+2dh+h+ua+2Ac(ag)}$	G hexasaccharide	[2]

Table 2. Molecular formulas for the major compounds detected by UHPLC-QTOF-MS analysis in the *S. cypria* extracts. References as follows: [1]Jia *et. al.,*1999; J. Nat. Prod, [2]Budan *et. al*., 2014; Biosci. Biotechnol. Biochem., [3]Koike *et.al.,*1999; J. Nat. Prod.

RT	Formula	$[M-H]^-$ m/z	Main fragment ions from MS/MS data	Identification	References
8.29	$C_{59}H_{94}O_{29}$	1265.578^{2-}	1103^{h}, 1085.5^{h}, 779^{3h}, 617.2^{4h}, 125^{f}, 101^{f}	Saponariosides C,D	[6]
8.47	$C_{72}H_{112}O_{37}$	1567.698^{2-}	1435.9^{p},$939.5^{p+2dh+h+Ac(ps)}$, $469.3^{2p+2dh+2h+ua+Ac(ag)}$	G heptasaccharide	[2]
8.58	$C_{60}H_{96}O_{30}$	1295.589^{2-}	1133^{h}, 1115^{h}, 953^{2h}, 809^{3h}, 485.3^{5h}	Saponarioside E	[6]
10.45	$C_{75}H_{118}O_{39}$	1641.714^{2-}	1509.7^{p}, $939.4^{2p+3dh(ps)}$, $469.3^{3p+3dh+h+ua(ag)}$	G or GA saccharide	[2]
11.35	$C_{77}H_{120}O_{40}$	1683.720	1551.7^{p},$939.5^{2p+3dh+Ac(ps)}$,$469.3^{3p+3dh+h+ua+Ac(ag)}$,$113^{f}$	G octosaccharide	[2,19]
12.44	$C_{68}H_{104}O_{33}$	1447.636	1315^{p}, $939.4^{p+2dh+2Ac}$,$469.3^{2p+2dh+h+ua+2Ac(ag)}$	G hexasaccharide	[2]

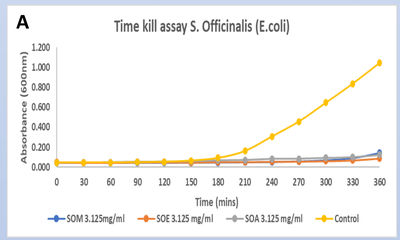
Table 3. Molecular formulas for the major compounds detected by UHPLC-QTOF-MS analysis in the *S. officinalis* extracts. References as follows: [1]Jia *et. al.,*1999; J. Nat. Prod, [2]Budan *et. al*., 2014; Biosci. Biotechnol. Biochem., [3]Koike *et.al.,*1999; J. Nat. Prod., [19]Ekanayaka, E.P.; Celiz, M.D, Plant Physiol. 2015, 167, 1221-1232.

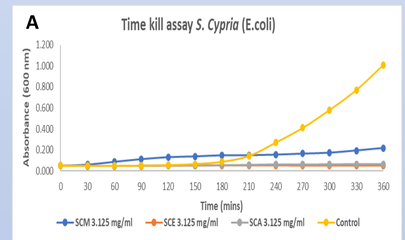
Extract	*E.coli*			*S. aureus*			*E. faecalis*			*S. enteritidis*		
	MIC (mg/ml)	MBC (mg/ml)	MBC/ MIC	MIC (mg/ml)	MBC (mg/ml)	MBC/ MIC	MIC (mg/ml)	MBC (mg/ml)	MBC/ MIC	MIC (mg/ml)	MBC (mg/ml	MBC/ MIC
SOM	3.125	6.250	**2**	3.125	6.250	**2**	6.250	12.50	**2**	3.125	6.250	**2**
SOE	3.125	6.250	**2**	1.563	3.125	**2**	3.125	12.50	**4**	3.125	6.250	**2**
SOA	3.125	6.250	**2**	1.563	3.125	**2**	3.125	6.250	**2**	1.563	6.250	**4**
Amp	0.004	0.004	**1**	-	-	-	0.002	0.002	**1**	0.002	0.002	**1**
Gent	-	-	-	0.004	0.004	**1**	-	-	-	-	-	-
Extract	***E.coli***			***S. aureus***			***E. faecalis***			***S. enteritidis***		
	MIC (mg/ml)	MBC (mg/ml)	MBC/ MIC	MIC (mg/ml)	MBC (mg/ml)	MBC/ MIC	MIC (mg/ml)	MBC (mg/ml)	MBC/ MIC	MIC (mg/ml)	MBC (mg/ml	MBC/ MIC
SCM	3.125	6.250	**2**	1.563	1.563	**1**	3.125	3.125	**1**	3.125	12.5	**4**
SCE	3.125	6.250	**2**	0.391	0.391	**1**	1.563	1.563	**1**	3.125	6.250	**2**
SCA	3.125	6.250	**2**	0.195	0.195	**1**	0.391	0.391	**1**	3.125	6.250	**2**
Amp	0.004	0.004	**1**	-	-	-	0.002	0.002	**1**	0.002	0.002	**1**
Gent	-	-	-	0.004	0.004	**1**	-	-	-	-	-	-

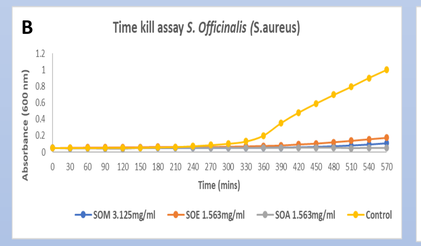
Table 4. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and ratio MBC/MIC of *S. officinalis and S. cypria* extracts against *E. coli, S. aureus, E. faecalis and S. enteritidis* bacteria. Ampicillin and gentamycin were used as control antimicrobial agents. The lower the MIC value, the less extract is needed for inhibiting the growth of the bacteria. Compounds with MIC values of < 0.6 mg/ml are considered strong inhibitors, 0.6-1.6 mg/ml moderate, 1.6-8.0 mg/ml weak and >8.0 mg/ml are considered low bacterial inhibitors [16-17]. MBC is the lowest concentration of the extract that is bactericidal. The lower the MBC value, the less extract is needed to kill the bacteria. Ratio MBC/MIC of < 4 demonstrates a bactericidal effect, ratio MBC/MIC ≥ 4 demonstrates a bacteriostatic effect [18]. SOM: *S. officinalis* methanol; SOE: *S. officinalis* ethanol; SOA: *S. officinalis* acetone; SCM: *S. cypria* methanol; SCE: *S. cypria* ethanol; SCA: *S. cypria* acetone; Amp: Ampicillin; Gen: Gentamycin;

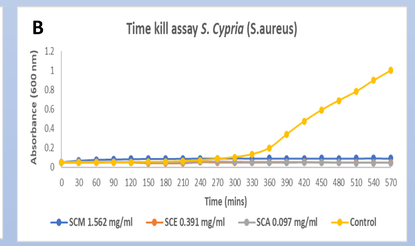
Solvent type	*Saponaria Officinalis*			*Saponaria Cypria*	
	IC_{50} (mg/ml)	TEAC (%)		IC_{50} (mg/ml)	TEAC (%)
Methanol	7.52 ± 0.220	0.11 ± 0.006	***Methanol***	0.19 ± 0.008	4.75 ± 0.115
Ethanol	0.43 ± 0.030	2.05 ± 0.081	***Ethanol***	0.036 ± 0.005	25.22 ± 2.928
Acetone	0.32 ± 0.080	2.74 ± 0.509	***Acetone***	0.053 ± 0.005	16.67 ± 0.818
Trolox	0.008 ± 0.010	-	**Trolox**	0.009 ± 0.010	-

Table 5. The half maximum inhibitory concentrations (IC_{50}) of the standard and the several *Saponaria* extracts were defined as the concentration of the extracts (mg/ml) required to scavenge the DPPH radical by 50%. Antioxidant activity of different extracts of *S. officinalis* and *S. cypria* as expressed as Trolox equivalent antioxidant capacity (TEAC) was calculated as previously described [15]. The higher the TEAC value means the higher the antioxidant activity.









Figures 1: Time kill assay for *Saponaria* extracts: **(A)** Growth curves of *E. coli* with *Saponaria officinalis* and *Saponaria cypria* extracts*;* **(B)** Growth curves of *S. aureus* with *Saponaria officinalis* and *Saponaria cypria* extracts. The graph represents the inhibition of the MIC values for each Saponaria extract.

CONCLUSIONS

The results of the present study revealed a higher saponin yield in *S. cypria* compared to *S. officinalis.* Identification and characterisation of the main saponins in the two species, demonstrated significant differences in the composition of the active saponin molecules. Study of the antioxidant activity demonstrated a higher TEAC activity in *S. cypria*, probably attributed to the higher content of saponins present in this species in comparison to *S. officinalis*. The antimicrobial activity of both species showed a MIC range of 0.19-6.25 mg/ml with a preference to *S. aureus*. *S. cypria* extract exhibited higher antimicrobial activity against all bacteria tested, in comparison to *S. officinalis*. Time kill assay results revealed the inhibition of *E.coli* and *S. aureus* bacterial growth by both species. In conclusion, our data demonstrate differences between the two species, in terms of their saponin content as well as their antioxidant and antimicrobial properties.

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