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Extraction and Surface Activity of Australian Native Plant Extracts: *Alphitonia excelsa*

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Abstract: Saponin surfactants extracted from plants have significant potential applications in many industries. The interfacial properties of extracts of *Alphitonia excelsa*, a native Australian plant rich in saponins, have been characterised to assess their suitability as dual-purpose foaming and antibacterial additives. Two sources of the plant (Adelaide Botanic Gardens and homelands of Chuulangun Aboriginal Corporation) were investigated to look for alteration of properties as a result of differences in cultivation and geographic location. Two methods of saponin extraction (water and water/ethanol mixtures) were investigated to determine differences in extraction efficiency and performance. Distinct differences were observed between the traditional analytical analysis (for saponin content) of the extracts based on source and extraction method; however, these differences were not as stark when considering the effect of the extracts on air–water interfacial tension and dilatational rheology, with extraction method proving to be the single biggest factor in extract efficacy. The data obtained point toward the presence of an altered array of surface-active species (different relative amounts of particular saponins in the water/ethanol extracted material) as a function of the extraction method. All extracts presented some antibacterial effect, albeit modest. This work highlights that the extraction method needs to be carefully considered and tailored for a given application.

Keywords: saponin; *Alphitonia excelsa*; LC-MS; interfacial tension; interfacial rheology; antibacterial

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1. Introduction

Structured colloidal systems such as food, cosmetic, and pharmaceutical formulations require the addition of surfactants for long-term stability. Colloidal systems are subject to an array of destabilisation mechanisms, including coalescence; flocculation; sedimentation; creaming; and Ostwald ripening [1]. Consumer demand for environmentally friendly products without the use of synthetic additives has resulted in increased interest in naturally derived stabilising compounds [1,2]. Natural extracts are often added to colloidal formulations for their medicinal benefits, such as antioxidant and anti-inflammatory effects. However, it has been noted that the effect of these extracts on the physical structure and functions of the colloidal systems has been largely ignored, when in fact there exists the possibility that natural extracts could prevent the need for other stabilisers altogether [3].

Alphitonia excelsa (Fenzl) Benth., commonly called the 'soap tree' or 'red ash', has been used by Aboriginal Australians for both its medicinal and foaming properties [4–7]. The Kuuku I'yu, traditional owners on their homelands located in Cape York Peninsula, Queensland, use the leaves of this plant species to make a soap/lather to wash the skin. Addition-

ally, *A. excelsa* has been used for its antimicrobial properties [5,7] and anti-inflammatory activity [4,6,8]. The foamability of *A. excelsa* is a result of saponins present within the leaves of the plant [4,5,7]. Extracts from plants such as *A. excelsa* may provide not only structural stability to colloidal systems but may also impart secondary benefits through their antimicrobial and anti-inflammatory properties.

Saponins are a class of naturally occurring phytochemicals present in a large number of plant species [9]. They have found use in food [1,2,10,11], cosmetic [2,3,12,13], and pharmaceutical [2,10,11,13–15] formulations due to their excellent foaming and emulsifying properties. The structure of saponin molecules consists of a hydrophobic backbone (or aglycone) connected to one or more hydrophilic sugar (glycone) residues via glycoside bonds [16,17]. Saponins are then classified based on their aglycone skeleton, either triterpenoid or steroid, and the number of sugar residues bound to it. Triterpenoid saponins are more prevalent in nature than steroid saponins and the majority of saponins are bidesmodisic (i.e., containing two sugar residues) [16]. In addition to their excellent interfacial stabilisation ability, saponins are known to exhibit a range of potential health benefits, such as hypercholesterolemic, antitumour, anti-inflammatory, antiallergy, and antimicrobial activity [18–20].

A significant amount of literature exists regarding the interfacial properties of *Quillaja saponaria* Molina extracts, while only a few studies investigate saponins from other botanical sources [9]. To the best of our knowledge, the interfacial properties of *A. excelsa* extracts have not yet been studied in detail; most existing research focuses on the medicinal properties of the extracts, based on the ethnomedicinal knowledge of Australian Aboriginal communities. A study by Blyth et al. used crude extracts from *A. excelsa* for the removal of polycyclic aromatic hydrocarbons from soil [4]; however, no further studies using *A. excelsa* extracts for their physical properties can be found. Saponin-rich extracts, such as those of *Saponaria officinalis* L. [21], *Aesculus hippocastanum* L. [22], and *Verbascum nigrum* L. [1], have been studied as stabilisers in food and personal care applications,

The present study aims to probe the viability of simple *A. excelsa* extracts as dual-purpose stabilising additives for colloidal systems, with a focus on foams. *A. excelsa* leaf material obtained from two geographical locations is extracted with water and water/ethanol. The extracts are analysed in terms of their total saponin content. LC-MS analysis allows for putative identifications of the compounds present within each extract resulting from comparisons with MS database information. The extracts are also analysed in terms of their interfacial activity and interfacial dilatational rheology, whereby the surface tension is monitored until equilibrium conditions are reached, prior to the application of sinusoidal perturbations to the bubble volume. Finally, the antimicrobial activity of the extracts against Gram-negative and -positive bacteria is tested. The results highlight the importance of extraction methodology in determining the amount and variety of saponins that can be obtained from *A. excelsa* in terms of the effectiveness of the extract in altering interfacial properties, and all applications of the extract as a source of surfactants will be influenced by this.

2. Materials and Methods

2.1. Chemicals and Plant Materials

Wild-harvested leaf samples of *A. excelsa* were provided by the Chuulangun Aboriginal Corporation and were collected in July 2019 near Chuula homestead, Kuuku I'yu homelands, Cape York Peninsula, Queensland, Australia, and a voucher specimen was deposited at the South Australian State Herbarium (voucher number AD191480). Cultivated leaf samples were collected from the Adelaide Botanic Gardens (Adelaide, South Australia, Australia) in September 2019 (voucher number AD191481). Ninety-eight percent sulphuric acid, ethanol (AR grade), methanol (LC grade), and acetonitrile (LC grade) were obtained from ChemSupply (Adelaide, Australia). Dimethyl sulfoxide (DMSO), vanillin (ReagentPlus® 99%), and oleanolic acid (>97%) were purchased from Sigma Aldrich (Sydney, Australia) and used without further purification. Aqueous solutions were prepared using Milli-Q

water (Millipore, Burlington, MA, USA) with a resistivity of 18.2 MΩ cm, surface tension of 72.4 mN/m at 22.0 °C, and maximum total organic carbon component of 3 ppb.

2.2. Methods

2.2.1. Extract Preparation

A. excelsa leaf samples were dried at 40 °C in a laboratory oven for 72 h before being packaged and stored in a freezer at −18 °C. Crude *A. excelsa* extracts were prepared by crushing 2 g of dried leaf material in a mortar and pestle before immersing in either 50 mL Milli-Q water or 50:50 Milli-Q/ethanol in a sealed conical flask. The suspended leaf material was stirred at room temperature for 24 h before being filtered under vacuum through a filter paper (Whatman, Marlborough, MA, USA), followed by a 0.45 µm PES syringe filter (Sartorius, Germany) to remove any particulates. The ethanol was removed by a rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) before samples were split into clean, glass vials and frozen at −18 °C. The frozen samples were then dried using a MudulyoD FreezeDryer (Thermo Electron Corporation, Emeryville, CA, USA). The powdered samples were combined and stored in a dark cupboard under a dry nitrogen atmosphere. There were four samples in all; two samples from Cape York Peninsula (Milli-Q and 50:50 Milli-Q/ethanol) and two samples from the Botanic Gardens (Milli-Q and 50:50 Milli-Q/ethanol). Samples will be referred to as BG-H2O and BG-Et for the Botanic Gardens water and water/ethanol extracts, and CYP-H2O and CYP-Et for the Cape York Peninsula water and water/ethanol extracts.

2.2.2. Saponin Quantification

The total saponin content (TSC) of the *A. excelsa* extracts was determined using the vanillin–sulphuric method [23]. Briefly, the dried extracts were dissolved in 50:50 Milli-Q/ethanol to a concentration of 0.5 mg/mL and filtered through a 0.45 µm PTFE syringe filter (Millipore). A 0.25 mL aliquot of each sample was transferred to clean glass vials, along with 0.25 mL of 8% w/v vanillin in ethanol and 2.5 mL of 72% v/v sulphuric acid in water. A blank was also prepared using 0.25 mL of the ethanol/water mixture in place of a sample. The samples were vortexed to ensure complete mixing, then heated in a water bath at 60 °C for 15 min. The vials were removed and allowed to cool at room temperature prior to UV-Vis analysis. The absorbance of each sample at 560 nm was measured using a Cary 50 UV-VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The TSC was determined from a standard curve of oleanolic acid, ranging from 0 to 1500 mg/L, and prepared under the same previously described conditions as the samples. The results are expressed as milligrams of oleanolic acid equivalents per gram of extract (g OE/100g). The analysis of samples was performed in triplicate using independent solutions.

2.2.3. LC-MS Analysis

Metabolites from plant extracts (BG-H2O, CY-H2O, BG-EtOH, CY-EtOH) were diluted 1/2 from 1 mg/mL stock in 5% methanol. Following dilution, 10 µL of each extract (as well as a pooled control) was injected for analysis using a 1290 UPLC coupled online to a 6545XT Qq-TOF MS (Agilent Technologies) equipped with a JetStream^{TM} ESI ionisation source. Metabolites were separated using a Zorbax Eclipse Plus C18 (5 cm × 2.1 mm ID, Agilent technologies). The column was thermostatically controlled at 50 °C and the flow rate was 0.4 mL/min. The mobile phases consisted of two solvents: Buffer A was water with 0.1% acetic acid and Buffer B was methanol with 0.1% acetic acid, based on [24]. The linear gradient solvent system was as follows: 0–2 min, 5% B; 2–32 min, 5–65% B; 32–33 min, 65–95% B; 33–35 min, 95% B; 35–36 min, 95–5% B; 36–42 min, 5% B. The sample was directed to the MS after 2 min loading. The ESI source was operated in negative ions mode, and full scan mass spectral data were acquired from m/z 100 to 1700 at 4 spectra/s. The optimum source parameters were as follows: capillary voltage (3.5 kV); nozzle voltage (500 V); drying gas flow (12.0 L/min); drying gas temperature (290 °C); nebulizing gas pressure (45 psi); sheath gas temperature (275 °C); sheath gas flow

(12.0 L/min); Fragmentor voltage (175 V); octopole RF voltage (750 Vpp); collision energy (5V). Extracts were collected in MS mode in triplicate runs, as well as one MS/MS analysis. Pseudomolecular ions $[M\text{-}H]^-$ were selected as precursor ions and subjected to MS/MS analysis using the following settings: For collisional induced dissociation fragmentation energy, the formula "(slope)*(m/z)/100 + Offset" was used to define voltage applied. For singly charged ions, the slope was 3.6 and offset +9, with nitrogen used as a collision gas. A medium (~4 m/z) width was used, and MS/MS spectra were acquired at 3 spectra/s. Active exclusion was enabled, with exclusion after 1 spectrum and release after 0.1 min. LC-MS analyses were exported to mzData format using Qualitative Workflow software (B.08.00, Agilent technologies). The exported data were analysed using MZmine 2 v2.53 [25] according to the following procedure; centroided mass lists generated with a noise cut-off of 4.0E2, chromatogram generation using ADAP Chromatogram builder function [26] with tolerance 0.01 *m/z* or 10 ppm, group intensity threshold 5.0E2, minimum group size in # of scans 10. Deconvolution of chromatograms (ADAP) used an S/N threshold of 10 and a coefficient area/threshold of 110, isotope grouping, mass tolerance of 0.01 *m/z* or 10 ppm, and retention time tolerance of 0.5 min. Features were aligned using a Ransac aligner, with a tolerance of 0.04 *m/z* or 5 ppm, retention time tolerance of 0.2 min, 10,000 iterations, minimum data points 30%, and filtered using 'Feature list rows filter' with minimum peaks in a row as 2. Missing values were gap-filled using the 'Same RT and m/z range gap filler with 0.015 *m/z* or 15 ppm, retention time tolerance of 1 min. Compounds generated from LC-MS/MS data from each extract were searched against the METLIN accurate mass library (v8.0, 80058 compounds) using Qualitative Workflow software (B.08.00, Agilent Technologies). Agilent Profinder was used to assess intensities for peaks assigned to putatively identified saponins across all extracts. The assignment of putative IDs required a DB score >= 70 and a mass tolerance of +/− 0.05 Da

2.2.4. Interfacial Behaviour of Saponin-Rich Extracts

The dynamic surface tension of the air–solution interface was measured using the Profile Analysis Tensiometer PAT-1 M (SINTERFACE Technologies, Berlin, Germany). The instrument accessories were cleaned thoroughly as follows: 20 mL of 2% Mucasol™ (Sigma Aldrich, Bayswater, Australia) in Milli-Q water and 20 mL of AR grade ethanol was flowed through the tubing, gas-tight syringe (ILS®, Dortmund, Germany), and capillary. Each accessory was rinsed thoroughly with Milli-Q water after the surfactant and solvent flushing steps, followed by drying with high-purity N_2 (99.999%, BOC, North Ryde, Australia). The optical glass cuvette (Hellma Analytics, Müllheim, Germany) was immersed in 2% Mucasol™ in Milli-Q water for 30 min then immersed in 1M KOH in Milli-Q for a further 30 min. The cuvette was rinsed with copious amounts of Milli-Q following each immersion before drying in a glassware oven at 55 °C. The optical glass cuvette, gas-tight syringe, and capillary were all plasma-cleaned using a PDC-OD2 plasma cleaner (Harrick, Pleasantville, NY, USA) prior to mounting.

The PAT was fitted with a U-shaped stainless-steel capillary with a diameter of 2.935 mm, which was immersed into the aqueous solution within the optical glass cuvette. Several air bubbles were detached from the capillary tip before a bubble of fixed volume (18 µL for water/ethanol extracts and 30 µL for water extracts) was formed at t = 0. The software-controlled dosing system maintained the droplet volume for the duration of the experiment. The experiment ended once the system reached a quasi-equilibrium, which we defined as a change in surface tension of 0.1 mN/m per 2000 s. For the water extracts, the duration of experiments was 30,000 s, and for the ethanol/water extracts, the experimental duration was 18,000 s.

Dilatational rheology was performed via the oscillating bubble method, where sinusoidal volumetric perturbations were applied to a bubble over the frequency range 0.01–0.20 Hz at a fixed amplitude of 10%.

2.2.5. Antibacterial Studies

American Type Culture Collection (ATCC) strains of bacteria were obtained from stock cultures in glycerol broth preserved at −80 °C at the Clinical and Health Sciences, University of South Australia, Adelaide, South Australia. Bacteria were grown overnight on Mueller Hinton (MH) agar plates (Thermo Fisher Scientific, Melbourne, Australia) at 37 °C. Plant extracts were prepared as stock solutions in DMSO. The MIC method of the CLSI [27] was used with some modifications. Duplicate, 2-fold serial dilutions of extracts were prepared in sterile round bottom 96-well plates (Sarstedt, Mawson Lakes, Australia) in Cation-Adjusted Mueller–Hinton Broth (CAMHB, BD, Murdoch, Australia) to contain a final concentration of 4% v/v DMSO. Column 11 of the plate served as a no-treatment control. Levofloxacin was also included in each assay as a positive control.

A colony of bacteria was added from an agar plate to prewarmed broth and incubated with shaking. After the required incubation time, the A_{630} was determined and adjusted, if necessary, to give a McFarland standard of 0.5. This bacterial suspension was diluted 100× with CAMHB. To each well containing the test sample dilutions or no-treatment control, a bacterial cell suspension (100 µL) corresponding to 1×10^6 CFU/mL was added. Wells in column 12 of the plate received no bacteria, and instead 200 µL/well of the broth with DMSO only served as a media sterility control. In the assay, the final CFU/mL for the bacteria was 5×10^5 CFU/mL and the final concentrations of extract samples and DMSO were 1 to 512 µg/mL and 2% v/v, respectively. Additionally, a plate was prepared with the same dilutions of the extracts, but with 100 µL/well of CAMHB added at the final step instead of the bacterial suspension (extract absorbance control plate). Plates were incubated overnight at 37 °C for 20 ± 2 h in air then the A_{630} was recorded using a plate reader (BioTek, Potton, Bedfordshire, UK). The MIC was determined as the lowest concentration at which no visual growth was observed in the duplicate wells and for which there was at least 95% inhibition, as measured by the change in absorbance compared with the no-treatment control after subtracting the absorbance values for the extract absorbance control. The entire experiment was repeated in duplicate.

3. Results and Discussion

3.1. Extract Characterisation

The extract yield was the first parameter characterised following the completion of the extraction process. The yield values for each extract (expressed as a percentage of solid, dried plant material) are presented in Table 1. The yields for the BG and CY samples were similar when water was used as the extraction solvent. The ethanol/water mix resulted in increased yields. This is likely due to the destructive effect of ethanol on the plant cell walls, enabling the cell contents to leach into the extraction solution. A study by Naz showed slightly higher yields for methanolic extracts over water extracts of *A. excelsa* [5] and the extracts displayed a similar visual appearance to the ones obtained in our study.

Table 1. The solid yield extract following freeze drying for each sample is expressed as a percentage of dry leaf mass. The TSC is expressed as g (oleanolic acid equivalents) per 100 g of extract.

Sample ID	Extract Yield (% of Dry Weight)	Appearance	TSC (g OE/100 g)
CYP-Et	22.86		62.63 ± 0.67
CYP-H2O	13.28		36.68 ± 3.55
BG-Et	18.22		36.21 ± 3.88
BG-H2O	15.37		24.07 ± 3.74

The extraction yield is not an indication of the amount of saponins within each extract, so the TSC of each extract was analysed using the vanillin–sulphuric spectrophotometric method. According to Navarro del Hierro et al., this spectrophotometric method should only be used as a point of comparison between different extracts, due to the nonspecific

nature of the reaction [20]. It is possible that other nonsaponin compounds present within the extract may undergo the desired reaction and alter the results. However, the vanillin–sulphuric method has been widely used as a method for TSC evaluation and is considered valid for comparison between extracts. The TSC values for each extract are presented in Table 1. The values suggest that for each plant sample, the presence of ethanol is beneficial for the extraction of saponins. Saponin content within the extract increases when ethanol is present in the extraction solution. Additionally, the sample obtained from Cape York contains a greater amount of saponins than the samples obtained from the Botanic Gardens, for both the water and water/ethanol extractions.

3.2. LC-MS Analysis

LC-MS data were acquired from replicate analyses of BG-H2O, BG-Et, CYP-H2O, and CYP-Et, and a pooled control was processed. The feature list was exported as a MetaboAnalyst file and loaded as a *.csv into MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/). The 'Statistical Analysis' module was used to process the features for each group (i.e., BG-H2O, CYP-H2O, BG-Et, CYP-Et, and pooled). Missing values were replaced by 1/5 of the minimum positive value of each variable, and the data were filtered using an interquartile range. The data were normalised against the pooled control LCMS analyses, log-transformed, and scaled using autoscaling. Principal component analysis (PCA) of the LCMS analyses showed distinct groups forming for each group (Figure 1), showing the largest variation being due to the source of the extract (i.e., BG vs CYP) rather than the extraction type.

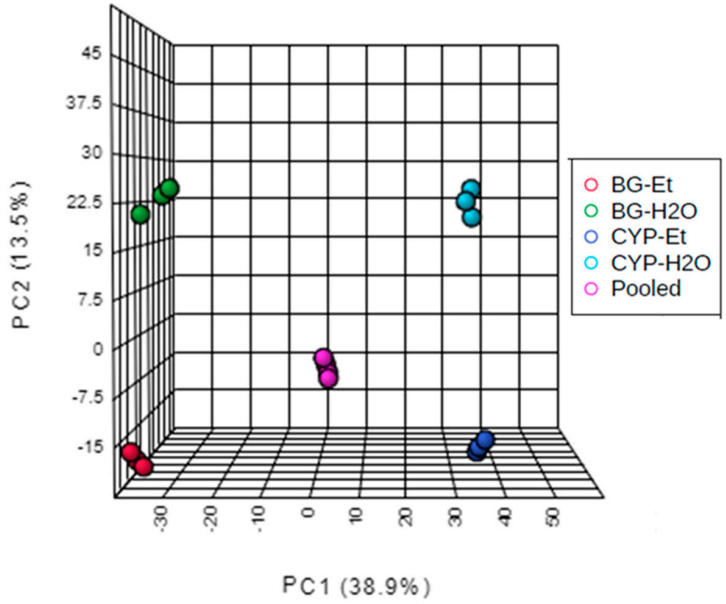

Figure 1. Principal component analysis scores plot between the selected PCs from LC-MS analysis *A. excelsa* extract solutions. The explained variances are shown in brackets. The CYP-H2O sample is represented by light blue, BG-H2O by green, CYP-Et by dark blue, BG-Et by red, and the pooled sample by pink spheres.

As anticipated, the pooled samples are clustered between the CYP and BG samples, and the pooled samples were used to normalise all the identified features. Hierarchical clustering (Figure 2A) of the LCMS data from each group highlights the similarity of the observed features being dependent on the source of the extracts rather than the extraction protocol adopted. The heat map (Figure 2B) shows the top 50 feature abundance, accounting for the clustering observed. LC-MS/MS data acquired from each extract were used to search against the METLIN Mass Spectral Database for putative identification of saponins in each extract and to determine if these constituted the features accounting for the clustering observed in Figure 2. Extracted ion chromatograms (EICs) from the LC-MS analyses were

generated for each putatively identified saponin, and the abundance was calculated for each abstract. The bar chart in Figure 2C shows the change in normalised abundance relative to the pooled control.

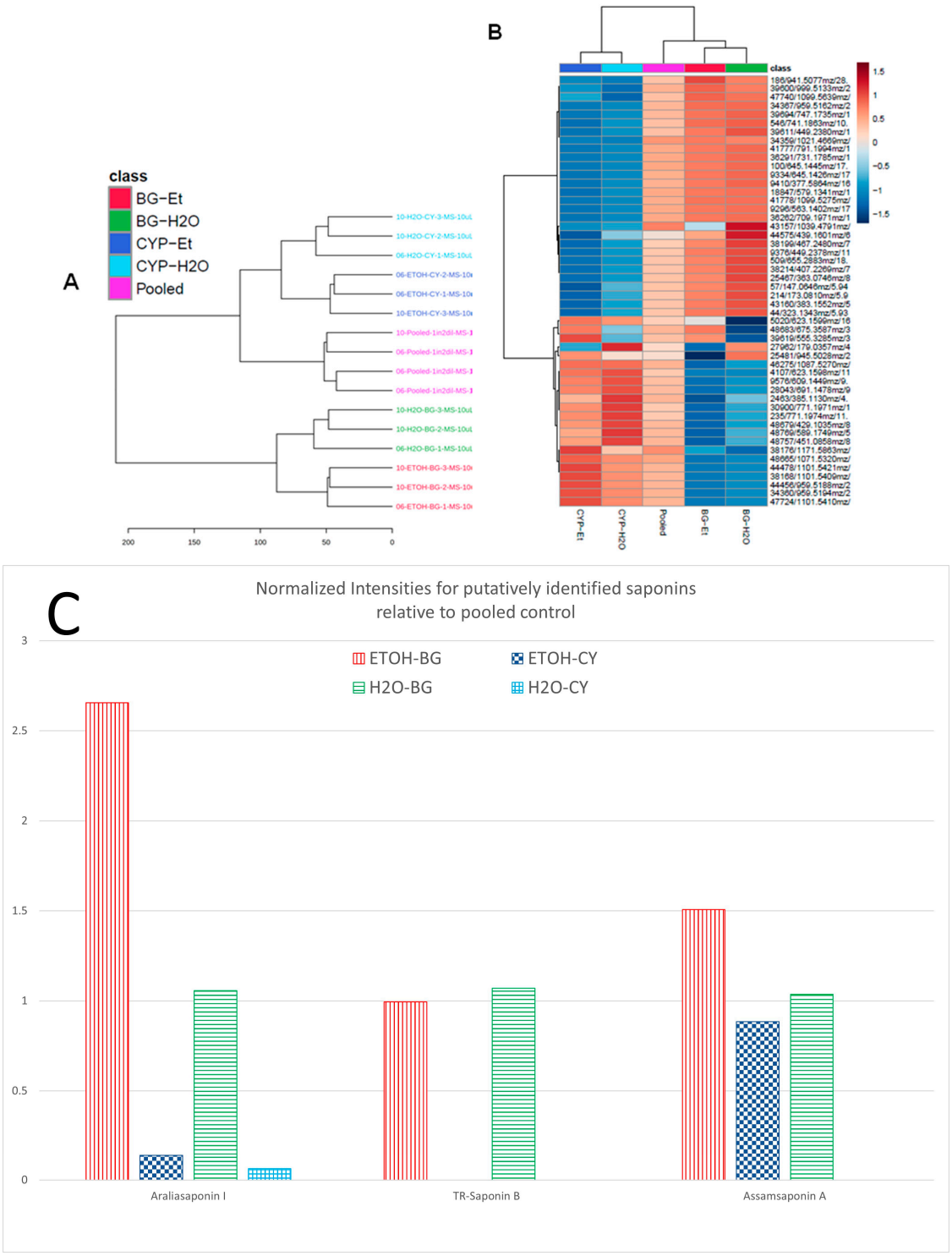

Figure 2. Hierarchical clustering analysis from LC-MS analysis *A. excelsa* extract solutions: (**A**) is the dendrogram generated using Euclidean distance and Ward clustering. The CYP-H2O sample is represented by light blue, BG-H2O by green, CYP-Et by dark blue, BG-Et by red, and the pooled sample by pink spheres. (**B**) shows the clustering result, shown as a heatmap of the top 50 features (distance measure using Euclidean and clustering algorithm using Ward). (**C**) shows the normalised abundance of the putatively identified saponins relative to the pooled control for the different extracts.

3.3. Interfacial Characterisation

To evaluate the suitability of *A. excelsa* extracts as stabilising additives, profile analysis tensiometry (PAT) was used to monitor the surface tension of an air bubble immersed in aqueous extract solutions. The majority of existing studies analyse the surface tension of pendant droplets of saponin solutions [2,11,13,28]; however, air bubbles in aqueous solutions have also been studied [17]. Due to the timescales required to reach equilibrium (up to 30,000 s), evaporation of the surface water molecules was of concern, so the captive bubble method was used.

In our analyses, a concentration of 1.0 mg/mL was used for the water extracts, and 0.5 mg/mL was used for the water/ethanol extracts. Figure 3A shows the surface tension vs time (linear scale) for each extract. There is no significant difference in the equilibrium surface tensions of the BG and CYP water extracts at 30,000 s. The average drop in surface tension for the BG-H2O samples is 13.2 ± 1.2 mN/m, compared with the CYP-H2O sample, which is 11.9 ± 0.9 mN/m. This suggests that the overall surface activity of the extracts is similar, given that the kinetics of adsorption are well aligned. However, the TSC data show a significant discrepancy in the amount of saponins between the two water extracts, with the CYP-H2O sample containing 1.5x greater saponin content than the BG-H2O sample. This points toward the exact nature of the individual saponins having a greater influence on extract surface activity, rather than simply the total saponin content.

The ethanol/water extracts have significantly lower surface tension profiles than those of the water extracts, with the drop in surface tension for the CYP sample being 28.0 ± 2.9 mN/m and the BG sample being 20.3 ± 1.3 mN/m. Once again, the surface tension and TSC data are not in agreement. The TSC for the BG-EtOH and CYP-H2O are no different, yet there is a significant difference in the equilibrium and surface tension. The CYP-Et sample has the highest TSC but a smaller drop in surface tension than the BG-Et extract. Again, the LC-MS data point toward the likely reason for the noncorrelation between TSC and interfacial tension; different saponins have varying degrees of interfacial activity.

Figure 3B displays the same data on a $\log_{10}$ scale in order to highlight the adsorption behaviour in the early stages of film formation. The kinetics in terms of initial adsorption rate appear to be very similar up to 10 s, at which point the rate of change for the BG-Et extract starts to slow, until approximately 1000 s when the surface tension begins to drop at a greatly increased rate.

In order to further understand the interfacial activity of *A. excelsa* extracts, the interfacial dilatational rheology of the extract solutions was investigated. Previous studies have reported high dilatational elasticity of adsorbed saponin layers [13,16], which has been linked to foam stability through the prevention of Ostwald ripening and film drainage [29–33]. The study by Stanimirova et al. showed that the oscillating droplet method yields inaccurate surface elasticity values due to wrinkle formation at the droplet neck [13]. Their work used purified *Quillaja* saponin extracts and gave elasticity values of 280 ± 30 mN/m. However, the oscillating droplet method is still used in the determination of dilatational viscoelastic parameters [2,11]. The oscillating droplet method is useful for making comparisons between our extracts, but comparisons with other systems may be inaccurate.

In this instance, there is no appreciable difference in the dilatational elasticity (Figure 4A) and viscosity (Figure 4B) for the CYP-H2O and BG-H2O samples, similar to that for the dynamic surface tension. Both systems show a very slight increase in dilatational elasticity with an increase in oscillation frequency. The BG-H2O system shows an increase from 11.0 ± 3.3 mN/m to 16.4 ± 8.4 mN/m, while the CYP-H2O system experiences an increase from 10.0 ± 1.0 mN/m to 16.4 ± 1.4 mN/m. Similarly, the dilatational viscosity decreases for the CYP and BG H2O systems in a consistent manner. The BG-H2O system decreases from 40.9 ± 13.6 s.mN/m to 4.1 ± 3.0 s.mN/m, and the CYP-H2O system decreases from 38.1 ± 3.3 s.mN/m to 3.7 ± 0.3 s.mN/m.

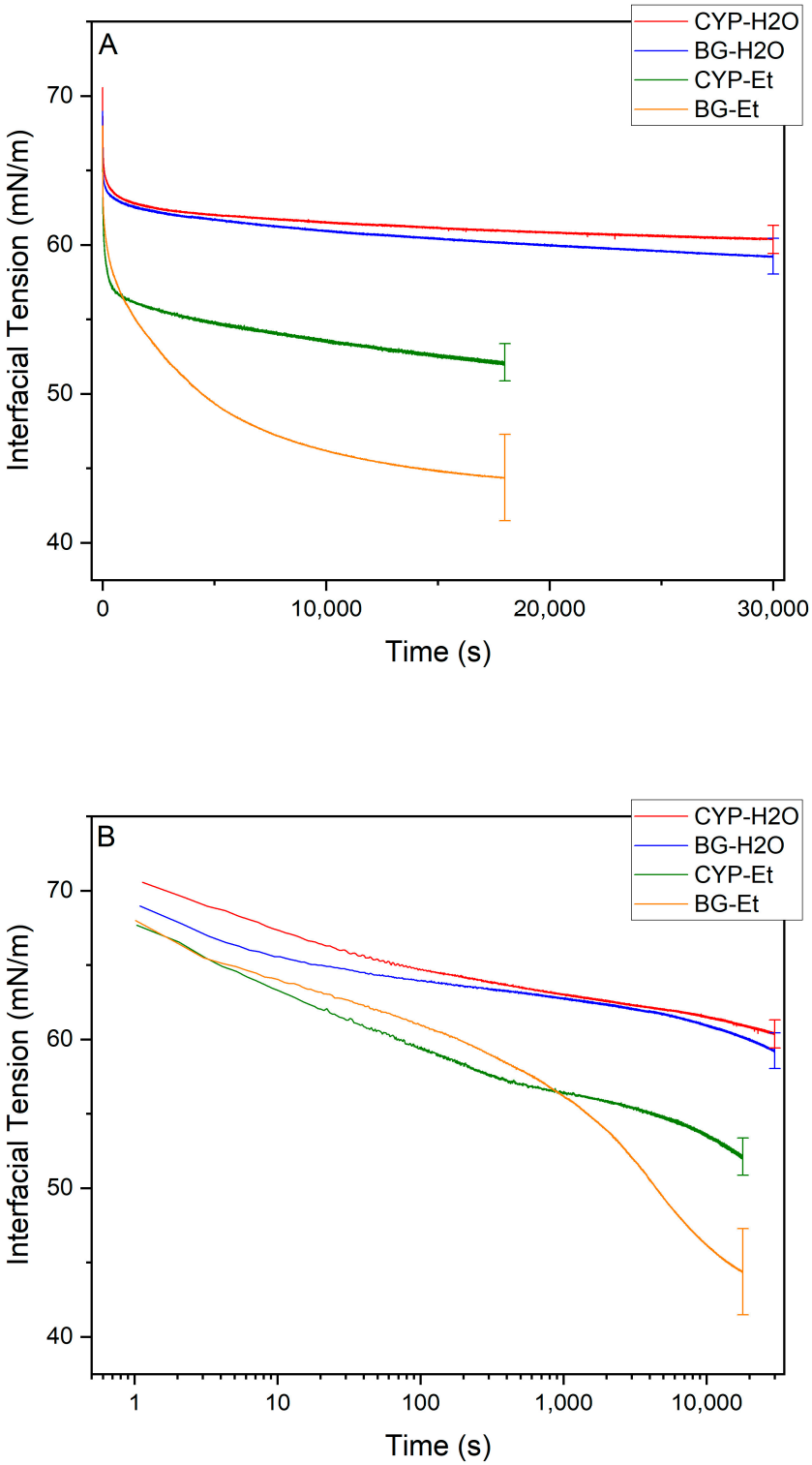

Figure 3. Surface tension vs time for the four extract systems, as a function of time in both linear (**Panel A**) and logarithmic (**Panel B**) scales.

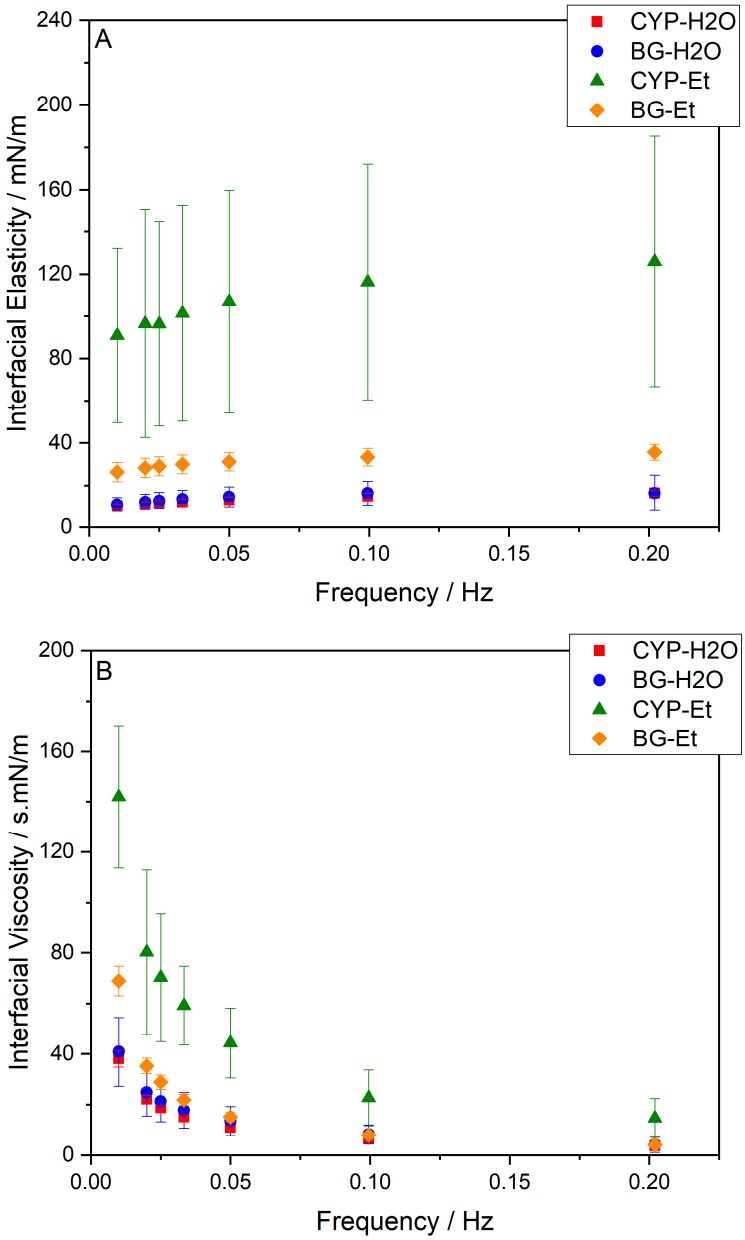

Figure 4. The interfacial dilatational elasticity (**Panel A**) and interfacial dilatational viscosity (**Panel B**) for *A. excelsa* extract solutions over the range 0.01 to 0.20 Hz. The CYP-H2O sample is represented by squares, BG-H2O by circles, CYP-Et by triangles, and BG-Et by diamonds.

The dilatational elasticity for both the CYP and BG-Et samples is significantly higher than for the water-extracted samples. The BG-Et sample has an increase in dilatational elasticity from 26.3 ± 4.5 mN/m to 35.6 ± 3.8 mN/m with an increase in frequency, while the CYP sample has an increase from 90.9 ± 41.2 to 126.0 ± 59.3 mN/m. The standard deviation seen for the CYP-Et sample is very large, suggesting there may be some heterogeneity within the sample, leading to different results between analyses. However, the CYP-Et has a significantly higher dilatational elasticity than all other samples. Similarly, the dilatational viscosity is significantly higher for the CYP-Et extract, decreasing from 142.0 ± 28.4 s.mN/m to 14.7 ± 7.7 s.mN/m with increasing frequency, while the BG-Et extract results in a decrease from 68.9 ± 5.8 s.mN/m to 4.0 ± 0.6 s.mN/m.

The high values of dilatational elasticity and viscosity seen with the CYP-Et sample are likely the result of the higher saponin content (Table 1). The overall drop in surface tension for the CYP-Et sample was not as large as the BG-Et sample, but the interfacial

film strength was greater. This suggests the BG-Et sample contains larger amounts of the saponin components with greater affinity for the air–solution interface.

3.4. Antimicrobial Effects of A. excelsa Extracts

Plant extracts were tested for antibacterial activity against different bacterial species that can cause spoilage of pharmaceutical or cosmetic formulations or food contamination. These included the Gram-positive species, *Staphylococcus aureus* and *Bacillus subtilis*, and Gram-negative species, *Escherichia coli* and *Salmonella typhimurium*. As shown in Table 2, the extracts lacked antibacterial activity against the two Gram-negative strains at the maximum tested concentration (512 µg/mL). The CYP-EtOH extract showed some modest activity against the Gram-positive *Staphylococcus aureus* with a MIC of 512 µg/mL. *S. aureus* is a common microbial contaminant of used and unused topical creams and cosmetic formulations [34], as well as a potential cause of food poisoning following contamination of food.

Table 2. Minimum inhibitory concentration (MIC) of *A. excelsa* extracts against Gram-positive and Gram-negative bacterial strains.

	GRAM-POSITIVE		**GRAM-NEGATIVE**	
Extract	***Staphylococcus aureus* ATCC 29213 MIC µg/mL**	***Bacillus subtilis* ATCC 11774 MIC µg/mL**	***Escherichia coli* ATCC 25922 MIC µg/mL**	***Salmonella typhimurium* ATCC 13311 MIC µg/mL**
BG-Et	>512	>512	>512	>512
BG-H2O	>512	>512	>512	>512
CYP-Et	512	>512	>512	>512
CYP-H2O	>512	>512	>512	>512
Levofloxacin (pos. control)	0.31	0.16	0.02	0.02

MIC is the minimum concentration causing ≥95% inhibition of overnight bacterial growth measured as absorbance at 630 nm compared with no-treatment control (after subtracting an extract and media-only blank).

The finding of antibacterial activity against *S. aureus* agrees with previous studies examining extracts of *A excelsa* leaves. A study of medicinal plants used by the Yaegl Aboriginal community in New South Wales [5] found EtOAc and MeOH extracts showed activity at concentrations of 500–1000 µg/mL against antibiotic-sensitive and -resistant strains of *S. aureus* using a tetrazolium-based microdilution assay but lacked activity against Gram-negative bacteria including *Pseudomonas aeruginosa* and *E. coli*. In another recent study [35], a MeOH extract of *A. excelsa* grown in Brisbane, Australia, displayed antibacterial activity against a clinical isolate of *S aureus* with a MIC of 927 µg/mL. However, in contrast to our findings, the MeOH, water, and EtOAc extracts reported in the later study also displayed some antibacterial activity against some Gram-negative bacteria, including *E. coli* and *Pseudomonas* species. The study used a disk diffusion method to determine the MIC in contrast to the broth microdilution method used in our study, and all bacterial strains tested were clinical isolates, in contrast to the ATCC strains reported here. Differences in the reported activities may also be due to the different extraction solvents used and variations in the chemical composition of the plant materials grown in different locations, a conclusion that is supported by the difference in chemical composition between the two extracts highlighted in the MS analysis.

The CYP-Et extract that showed activity against *S. aureus* was also the extract displaying the highest saponin content. While the activity of the crude extract reported here is modest, it falls below a cut-off active concentration of 1000 µg/mL, which has been suggested by some authors for plant extracts warranting further investigation [36]. Thus, further studies to isolate and identify the active constituents of the ethanol extract are needed. Triterpenoids isolated from another *Alphitonia* species, *A. xerocarpus* Baill. have been shown to exhibit good antibacterial activity against *S. aureus* and another Gram-positive bacterium *Enterococcus faecalis*, with MIC values of 4–16 µg/mL [37].

4. Conclusions

Two samples of leaves from *Alphitonia excelsa* plants, each from different geographical locations in Australia, were sourced for the purpose of extracting bioactive components, including saponins. Both water and water/ethanol extractions were acquired, with differential extraction between the two samples seen for saponin content, with the leaves from the wild-harvested sample yielding greater amounts of saponin than the cultivated sample. The influence of the extracts on the interfacial characteristics of the air–water interface (examined for *Alphitonia excelsa* for the very first time) did not completely align with total saponin content, either in terms of absolute interfacial tension drop relative to a bare interface or with dilatational rheology, with the relative amounts of the different saponin molecules present in each extract most likely being the dominant factor in interfacial activity, highlighting that extraction method needs to be carefully considered and tailored for a given application. Antimicrobial experiments yielded promising results only for the sample (Cape York) that gave the highest amount of saponin, a result that is in agreement with the existing literature on the topic, which has highlighted the need to have high concentrations of saponin present to provide antimicrobial efficacy.

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