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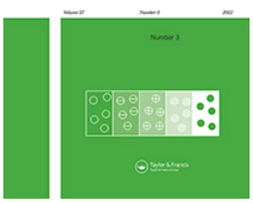

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Subcritical Ethanol-Water and ionic liquid extraction systems coupled with multi-frequency ultrasound in the extraction and purification of polysaccharides

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ABSTRACT

This study obtained crude sorghum leaf sheath polysaccharide (39.99% wet matter (wm)) by subcritical ethanol-water (40% v/v) extraction (180°C, 40 min). The subcritical extraction solution was transformed into an ionic liquid aqueous two-phase extraction system and subsequently coupled with ultrasound extraction to obtain partially purified polysaccharides (PPP). PPP yields of 20.89%, 27.38%, and 36.49% (wm) were obtained using 60 kHz, 20/60 kHz, and 20/40/60 kHz ultrasound frequencies, respectively. Polysaccharide functional groups such as hydroxyl, aldehyde, and amide were detected using Fourier Transform Infrared Spectroscopy (FT-IR). Amylose contents of 15%, 18%, and 25% were obtained for PPP under single, dual, and tri-frequencies, respectively. Amylose contents were associated with aggregation of PPP particles sizes after heat exposure (70°C for 1 h 50 min). Triple-frequency extracted polysaccharides with the highest uronic acid (1.51%) and polyphenolic (27.79%) contents had an IC50 of 1.37 mg/mL in an in-vitro hydroxyl scavenging activity assay. Three interesting co-extracted bioactive phytochemicals; 2-amino-5[(2-carboxy) vinyl]-Imidazole, N-[4-bromo-n butyl]-2-Piperidinone, and 3-Trifluoroacetyl Pentadecane were detected. The PPP extract showed antioxidant activity and contained phytochemicals with potential antimicrobial and antiviral activities, and thus may be useful in food, nutraceutical, and pharmaceutical applications.

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Introduction

Sorghum bicolor (sorghum) is a grass species that originates from Northeastern Africa. It is mainly cultivated to obtain its grain for food, animal feed, and ethanol production.[1] Nutritional analysis of the alcohol extract of forage sorghum or leaf sheath showed the presence of carbohydrates (75.39 g), proteins (4.87 g) and dietary fiber (50.30 g).[2] Research has also shown that consumption of diets prepared with the sorghum leaf sheath provides natural antioxidants and essential fatty acids that can fight cardiovascular-related diseases.[3] Bioactive polysaccharides present in such natural sources are responsible for antioxidation and other biological activities.[4] Thus, novel extraction techniques to increase the extraction yield and purity of such bioactive compounds have become a paramount interest to researchers.

For a good extraction yield of moderately polar and nonpolar targets, a less polar medium driven by elevated temperature is required.[5] The viscosity of the extraction solvents decreases with an increase in temperature[6]. This is due to the reduction in strength of its intermolecular forces allowing better matrix particle penetration. Also, at elevated temperatures, surface tension decreases allowing an excellent coat of feedstock by solvent. This leads to an increase in the rate of extraction. Fortunately, subcritical water extraction operates at such elevated temperatures (100–374°C) and pressure, high enough to maintain the liquid state.[7] However, water used as a solvent in this form of extraction has high polarity. The ethyl (C_2H_5) group of ethanol is nonpolar and can therefore serve as an adjunct in water to achieve a less polar medium. This can facilitate the easy dissolution of nonpolar substances (water-insoluble polysaccharides). However, polysaccharides solubility in ethanol-water solutions was found to decrease at 80% ethanol content.[8] In previous work, a ratio of 40%: 60% (v/v) ethanol-water used in the formation of an ionic liquid aqueous two-phase system (ILATPS) was established to be the most appropriate volume ratio for remarkable extraction yield of sorghum leaf sheath polysaccharides.[9] Thus, this work employed

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40% ethanol as an adjunct in the water for subcritical extraction of crude polysaccharides from sorghum leaf sheath.

Ultrasound sonication can disrupt cell wall structure via cavitaton and thus accelerate the diffusion of cell content through membranes.[10] Ultrasound generates cavitation bubbles through the propagation and interaction of ultrasound pressure. When cavitation bubbles collapse, microturbulence, perturbation, liquid circulation, and rotational flow structure occur and it is known as eddy.[11] Interparticle collision together with eddies ensures a dramatic eddy diffusion and internal diffusion and also speeds up mass transfer of solvent from continuous phase into plant cells. Furthermore, a quick moving stream of liquid passes through the cavity at the surface whenever there is a collapse of the bubble near the liquid-solid interface. Particles of the surfaces are impacted and cell walls are disrupted which promotes the release of intracellular components into solvents.[12–15] Ultrasound extraction has been established to be an alternative to conventional extraction methods, and is effective even at low temperatures. It has the advantage of avoiding the use of organic solvent and has reduced extraction time[16]

As described by Otu et al. (2018), [17] crude polysaccharides water extract was separated, freeze dried and subsequently incorporated into an Ionic Liquid Aqueous Two-Phase System (ILATPS) coupled with ultrasound treatment to partially purify extract within a short time. The novelty of this study was the use of ethanol-water in a subcritical extraction and shortening the processing time by skipping protocol for the precipitation of crude polysaccharides extract. Ionic liquid and salt were then introduced into the extraction solution to produce an ethanol-adjunct ionic liquid aqueous-two phase system. This system with its crude polysaccharides content was coupled with ultrasound treatment to produce partially purified polysaccharides with much higher yield within a short time.

Research has established that the method of extraction has a direct influence on the amount and type of biomolecules obtained.[18] Therefore, the specific objectives of this work were: 1) determine the effect of subcritical ethanol-water extraction system on yield of polysaccharides; 2) determine the effect of subcritical and frequency-varied ultrasound-assisted extraction system on yield of partially purified polysaccharides; 3) characterize the particle size, morphological structure, primary structural changes, molecular weight, and rheological properties of partially purified polysaccharides using dynamic light scattering, scanning electron microscopy, Fourier transform-infrared spectroscopy, high-performance size-exclusion chromatography and rheometer, respectively; and 4) investigate the antioxidant activity and phytochemical constituents of the polysaccharides extract.

Materials and methods

Sample preparation

Dried *S. bicolor* leaf sheath was collected from Ghana. The leaf sheath was pulverized into powder, sieved, and defatted using the method described by Otu *et al.*, (2018).[17]

Chemicals and reagents

Ionic liquid, 1-octyl-3-methylimidazolium chloride [C_8 mim]Cl, potassium carbonate (K_2CO_3), ethanol, methanol, sulfuric acid (H_2SO_4), phenol, glucose, coomassie G-250, bovine serum albumin (BSA), phosphoric acid (H_3PO_4), potassium bromide (KBr), gold palladium, Fe(II) sulfate ($FeSO_4$), salicyclic acid, hydrogen peroxide (H_2O_2), ABTS [2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)], potassium peroxidate, and carbazole. All chemicals were purchased from Sigma (St. Louis, MO, USA).

Subcritical ethanol-water extraction (SEWE)

Research has demonstrated that ethanol combined with subcritical water treatment can improve the recovery efficiency of by-products from raw sample material.[19] Therefore, 40% (v/v) of the ethanol-water solution was prepared. Subcritical ethanol-water extraction was similarly carried out as described by Yabalak and Ahmet (2012) with little modification.[20] To a 25 mL Teflon cup, 120 mg of dried sample powder and 14.4 mL of the prepared ethanol-water solution were added (Fig. 1). The Teflon cup was then fitted into a pressure cell, screwed tight, and placed into a hot-air oven. Crude polysaccharides from sample powder were then extracted using designed single factors; temperature (100, 120, 140, 160,180, 200°C) and time (10, 20, 30, 40, 50, 60 min). The polysaccharide content was measured using the phenol-sulfuric method.

Ionic liquid aqueous two-phase system (ILATPS)

Three (3) sets of sorghum leaf sheath crude polysaccharides solutions were obtained using subcritical ethanol-water extraction methodology as described above (section 2.2). The polysaccharide solutions were poured from the Teflon cups into 50 mL plastic tubes. Ionic liquid, [C_8mim]Cl (10.5 g), K_2CO_3 salt (5.1 g) were

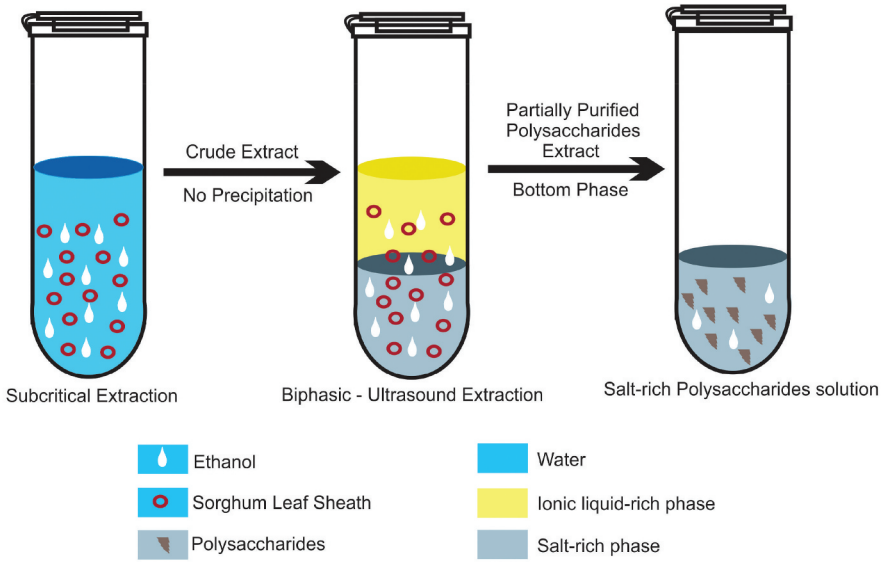

Figure 1. Schematic diagram of subcritical ethanol-water & biphasic ultrasound extraction of sorghum leaf sheath polysaccharides. Experimental conditions; 40% (v/v) ethanol solution at 180 °C for 40 mins, and ultrasound treatment with 60 or 20/60 or 20/40/60 KHz, 120 W/L for 30 min at 35 °C.

added and vortexed to complete a 30 mL ILATPS formation. Each sample set was exposed to a different ultrasound frequency and therefore assigned the names; subcritical ethanol-water extraction – mono (SEWE-M), subcritical ethanol-water – dual (SEWE-D), and subcritical ethanol-water – triple (SEWE-T). Partially purified polysaccharides were finally obtained by treating the formed ILATPS with ultrasound waves. Purifed samples were desalinated using a recyclable dialysis membrane (D44 mm, MWCO 8000–14000) enabling filtration of the salt and IL. It was then freeze dried for further analysis.

Single-factor design for ultrasound-assisted purification

As described by Yan et al. (2018), [21] a triple-band ultrasound water bath (KQ-300 DE, Kunshan Ultrasonic Instruments Co., Ltd., China) was used in this work. Three major parameters, extraction frequencies (Mono – 20, 40, 60 kHz, Dual – 20/40, 40/60, 20/60 KHz, and Tri – 20/40/60 kHz), extraction temperature (25, 30, 35, 40, and 45°C), and extraction time (15, 20, 25, 30, 35, and 40 min) were explored. A power of 240 W and power density of 120 W/L was used in this work.

Characterization

Preliminary characterization

The three sets of partially purified samples (SEWE-M, SEWE-D, and SEWE-T) were desalinated using a dialysis membrane and thereafter freeze-dried. The carbohydrate and protein content was determined using the phenol-sulfuric[22] and Bradford method .[23] The apparent amylose content was measured using the iodometric method.[24] The phenolic contents were measured as described by Kumar et al. (2008).[25] The Carbazole-sulfuric acid method was adopted for the measurement of uronic acid in the samples.[26] All experiments were conducted in triplicate.

Particle size (dynamic light scattering, DLS)

The hydrodynamic diameters of the three polysaccharides samples (SEWE-M, SEWE-D, and SEWE-T) were measured. Using the dynamic light scattering method as described by Ren et al. (2015).[27] Briefly, the hydrodynamic diameters of the partially purified samples in water (2 mg/mL) were examined using dynamic scattering on a Litesizer 500 (Anton Paar, UK). The solutions containing the samples were filtered through a 0.45 µm syringe filter. The solutions were measured initially at room temperature (25°C) and subsequently heated at

70°C for 1 h 50 min and then cooled down to a temperature of 25°C and again measured. Each sample was measured 10 times under each condition.

Scanning electron microscopy (SEM)

The Scanning Electron Microscope (S-3400 N, Tokyo, Japan) was used to observe the morphological characteristics of the three polysaccharide samples. Each sample was coated with a conductive layer of gold-palladium.

Fourier transform-infrared (FT-IR) spectroscopy

The infra-red spectra of the samples were determined using the FT-IR spectrophotometer (Nicolet, Nexusn670) as described by Otu et al. (2018).[17] Briefly, IR spectra were determined between an absorbance mode of (4000– 400 $cm^{-1)}$ and a resolution of (4 cm^{-1}).

Molecular weight (HPSEC)

High-performance size-exclusion chromatography was used to determine the molecular weight of samples as described by Otu et al. (2018).[17]

Rheological properties

The steady shear and oscillatory tests of samples at a concentration of 5 mg/mL were conducted with a DHR-1 rheometer (TA Instruments, Surrey, UK) using a cone – and – plate geometry. Conditions used were as described by Otu et al. (2018).[17]

Antioxidant activity in-vitro

Hydroxyl radical scavenging assay. The hydroxyl radical assay was performed as described by Jen et al. (1998).[28] Briefly, samples were dissolved in distilled water (5– 30 mg/mL). The sample solution (1.0 mL) was mixed with $FeSO_4$ (9 mM, 1.0 mL) and 9 mM salicylic acid solution (1 mL, 50% ethanol). Then, 8.8 mM H_2O_2 (1.0 mL) was added to start the reaction. The mixture was kept in a water bath at 37°C for 1 h. The background was mixed as described above except 50% ethanol (1.0 mL) was used in place of the salicylic acid solution. For the control, distilled water (1.0 mL) was substituted for the polysaccharide solution. After warming in a water bath, the absorbance of the mixture was measured at 510 nm. All measurement was done in triplicate.

The hydroxyl radical scavenging rate was calculated using the following formula in Eq. (1). The half-maximal inhibitory concentration, $IC_{50,}$ was calculated using Eq. (2).

$$ScavengingRate = \left(1 - \frac{A_1 - A_2}{A_0}\right) x100\% \quad (1)$$

Where A_0 is the absorbance of the control group (without polysaccharides), A_1 is the absorbance of the test group, A_2 is the absorbance of the background group.

$$IC_{50} = \%Max.inhibition - 50\%x(\%Max.inhibition - \%Min.inhibition) \quad (2)$$

ABTS radical scavenging assay. The ABTS radical scavenging assay was measured using the method described by Zhou et al. (2011)[29] with slight modification. Briefly, ABTS (50 mL, 7 mM) was mixed with 140 mM potassium peroxydisulfate (890 µL), then kept in the dark at room temperature for 12–16 h before use. The samples were prepared in a variety of concentrations (5–30 mg/mL). The polysaccharides solution (0.2 mL) was added to the $ABTS^{\cdot+}$ solution (5 mL). The absorbance of the mixture was measured at 734 nm after holding at room temperature for 6 min. All measurements were made in triplicate.

The ABTS radical scavenging rate was calculated using the following formula in Eq. (3). Again, the IC_{50} was calculated using Eq. (2).

$$ScavengingRate = \left(1 - \frac{A_1 - A_2}{A_0}\right) x100\% \quad (3)$$

Where A0 is the absorbance of the control group (without polysaccharides), A_1 is the absorbance of the test group, A_2 is the absorbance of the background group.

Phytochemical constituent analysis of extracted polysaccharides (GC-MS/MS)

Polysaccharides extract was dissolved in methanol (1 mL) and filtered through a 0.2 µm syringe filter and carefully kept at 4°C for 24 h before analysis using the GC-MS/MS .[30] To identify the compounds observed, the mass spectra of the analytes were screened against the NIST mass spectral database. The retention indices were also compared either with literature values or authentic compounds.[31,32]

Phytochemical constituents were identified using GC-MS/MS (Agilent 789A) equipped with a DB-5 MS column (30 m × 0.25 mm i.d., 0.25 um film thickness, J&W Scientific, Folsom, CA).[33] Helium was used as the carrier gas at the rate of 1.0 mL/min. The effluent of the GC column was introduced directly into the source of the MS via a transfer line (250°C). The ionization voltage was 70 eV and the ion source temperature was 230°C. Scan range was 41– 450 amu.

Statistical analysis

All experiments were conducted in triplicate and means determined. The statistical difference in means was determined using one-way ANOVA (OriginPro 2015). Statistical difference between means was considered significant if ($p < 0.05$).

Results and discussion

Single-factor design for subcritical adjunct water extraction

Effect of temperature on polysaccharides extraction

At a fixed extraction time of 30 min and different extraction temperatures of 100, 120, 140, 160,180, and 200°C, polysaccharides from dried sample powder were extracted (Fig. 2a). Extraction at 180°C gave the highest yield of polysaccharides (44.96% wet matter basis, $p < .05$). A significant decrease ($p < .05$) was recorded at 200°C. Similarly, the optimum temperature of 140°C in a subcritical extraction attained a 12.28% (dry matter basis) pectic polysaccharides in a 10%(v/v) ethanol-water.[34] Though higher yield can be obtained at a much higher temperature, thermal degradation of polysaccharides has been found to occur at the third stage of a mass loss experiment.[35] The temperature 180°C was therefore selected for this work.

Effect of time on polysaccharides extraction

The extraction temperature was adjusted to 180°C and polysaccharides were extracted at different times (10, 20, 30, 40, 50, 60 min) (Fig. 2b). The extraction time of 40 min gave the highest polysaccharides yield of 42.60% (wet matter basis) ($p < .05$). A significant decrease ($p < .05$) in polysaccharides yield was recorded at 50 and 60 min. Within 43.65 min at 210°C, 25.1% (wet matter basis) of *Grifola frondosa* polysaccharides were obtained by subcritical water extraction .[36] The much higher extraction yield in this work within a similar extraction time may be attributed to the nonpolar effect of ethanol on the extraction water medium and the ultrasound treatment. An extraction time of 40 min was thus selected for this work. The yield of crude polysaccharides extract obtained based on selected temperature (180°C) and time (40 min) has been presented in (Fig. 3).

Single-factor design for ultrasound-assisted polysaccharides purification

Effect of ultrasound temperature on polysaccharides extraction

Ultrasound time was fixed at 30 min throughout the experiment. In a stepwise manner, ultrasound extraction frequencies were adjusted to obtain a mono-frequency (60 kHz), dual-frequency (20/60 kHz), and tri-frequency (20/40/60 kHz). Different ultrasound temperatures (25, 30, 35, 40, and 45°C) were used for the extraction of partially purified polysaccharides (PPP) under each ultrasound extraction frequency (Table 1). The highest polysaccharide extraction yields of 27.19%, 30.29%, and 42.69%, ($p < .05$) were recorded at 35°C temperature for mono, dual, and tri extraction frequency, respectively. The highest extraction yield for *Aloe vera* polysaccharide was attained at room temperature (25°C) using ILATPS[37] . It was explained that at a certain extraction temperature, water is transferred from the top-to-bottom phase of ILATPS. The transferred water decreases the salting-out effect at the bottom phase and subsequently decreases polysaccharides yield. Again, transient cavitation filled bubbles undergoes irregular oscillation and implodes, producing high local increase of temperature and pressure that in turn disintegrate the cells for mass transfer.[38] Ultrasound temperature directly impacts the formation of transient cavitaional bubbles. Nagalingam and Yeo (2018)[39] showed that at 10°C, bubble population under the horn of an ultrasound was very low. Hence, very less mass loss values were recorded. At 30°C and 50°C, high bubble population was observed, which in turn increased the mass loss during machining. At 70°C and 90°C, very higher bubble population was observed, but it was noted that the bubbles did not implode but instead settled near the horn and on the sides of the workpiece and the test chamber. This may be the reason for the observed reduction in polysaccharides yield for experimental temperatures beyond 35°C. Thus, the temperature 35°C was selected for this work.

Effect of ultrasound frequencies on polysaccharides extraction

The ultrasound irradiation temperature and time were adjusted to 35°C and 30 mins, respectively. Variation of frequencies included: mono-frequency (20, 40, 60 kHz) and dual-frequency (20/60, 20/40, 40/60 kHz) and triple-frequency (20/40/60 kHz). Triple frequency gave the best results. The highest polysaccharide extraction yields of 27.71%, 29.39%, and 37.52% ($p < .05$) were recorded for mono – 60 kHz, dual – 20/60 KHz, and triple – 20/40/60 kHz ultrasound frequencies, respectively (Table 1). The results agree with the report that multi-frequency gives a much higher extraction yield than single frequency.[40,41] In an experimental report, Guo and Zhu (2017)[42] observed that, as ultrasound frequency increased, maximum radius (R_{max}) of cavitation bubble was found to have slightly reduced and oscillation interval was shortened. This implied that completion of bubble growth was made difficult whilst bubble collapse became

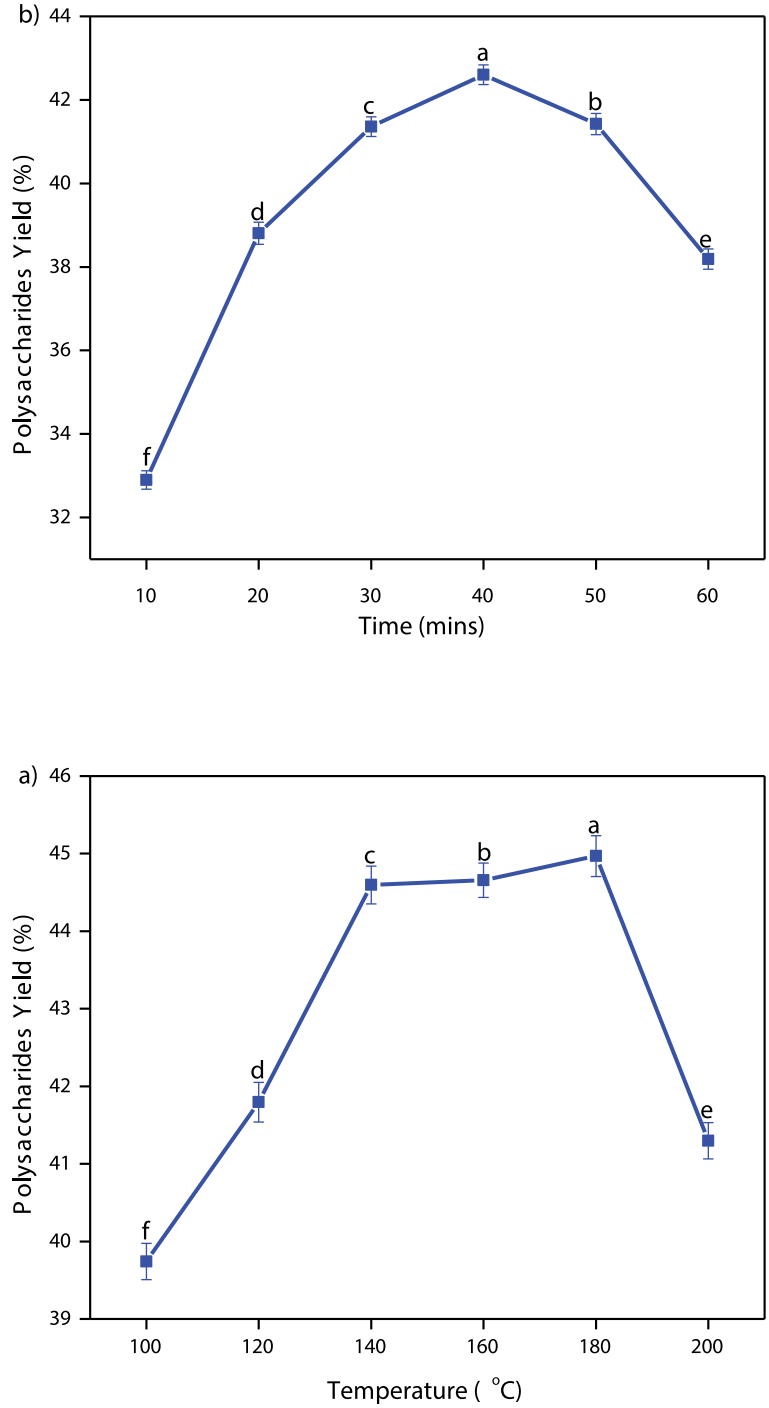

Figure 2. Effect of temperature a) for 30 min under (100, 120, 140, 160,180, and 200 °C) and time b) at 180 °C under (10, 20, 30, 40, 50, 60 min) on the yield (Mean ± SEM) of sorghum leaf sheath crude polysaccharides under subcritical ethanol-water extraction. Means with different letters are significantly different (Turkey's HSD, $p < .05$).

an easy occurance at a high frequency. Therefore, frequent cavitation bubble collapse, possibly aided by multi-frequency obsviously increased the rate of mass transfer. Thus, the selection of highest frequencies in this work (60 kHz, 20/60 KHz, and 20/40/60 KHz) for mono, dual, and triple ultrasound frequencies, respectively.

Effect of ultrasound time on polysaccharides extraction

Adjustment of ultrasound operational parameters to obtain a temperature of 30°C was made. Secondly, step-wise adjustments to obtain (i) 60 KHz mono frequency, (ii) 20/60 KHz dual-frequency, and (iii) 20/40/60 kHz triple frequency were made. Varying ultrasound time

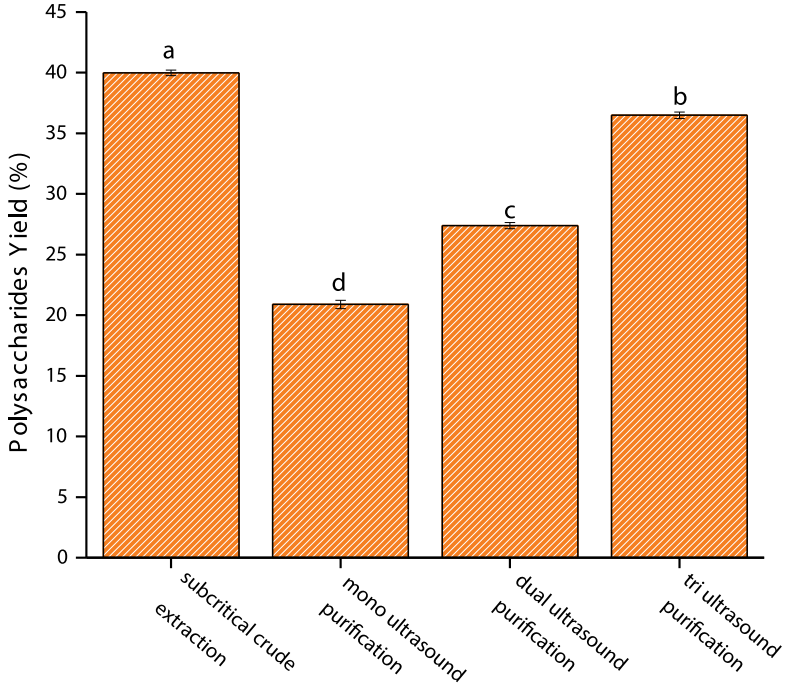

Figure 3. Yield (mean ± SEM) of sorghum leaf sheath polysaccharides (crude and partially purified) under selected best conditions for subcritical (180 °C and 40 min) and ultrasound extractions (60 kHz or 20/60 kHz or 20/40/60 kHz, 35 °C, time 30 min, 120 W/L) respectively. Means with different letters are significantly different (Turkey's HSD, $p < .05$).

(15, 20, 25, 30, 35, and 40 min) extraction of the purified polysaccharides under each ultrasound frequency were carried out (Table 1). The highest polysaccharide extraction yield of 26.03%, 29.13%, and 41.52% ($p < .05$) were recorded at 30 min time for mono, dual, and tri ultrasound extraction frequency (Table 1). In a work by Otu et al. (2018), [17] a notable yield of sorghum leaf sheath polysaccharides was similarly achieved by coupling dual-frequency ultrasound and ILATPS within 30 min. Therefore, an extraction time of 30 min was selected for this work.

Table 1. Effect of single factors temperature, time, and frequency on yield of *Sorghum bicolor* leaf polysaccharides under ultrasound extractions under constant experimental conditions of (30 min, 30°C, and 60 or 20/60 or 20/40/60 KHz).

	Polysaccharides Yield (%)		
Temperature (°C)	Mono frequency	Dual frequency	Triple frequency
25	20.99 ± 0.30	22.28 ± 0.22	31.19 ± 0.26
30	23.19 ± 0.21	22.54 ± 0.22	31.97 ± 0.26
35	27.19 ± 0.23	30.29 ± 0.26	42.69 ± 0.27
40	20.86 ± 0.25	25.00 ± 0.23	38.42 ± 0.31
45	18.02 ± 0.21	22.54 ± 0.21	38.17 ± 0.30
Polysaccharides Yield (%)			
Time (mins)	Mono frequency	Dual frequency	Tri frequency
15	20.73 ± 0.23	17.76 ± 0.30	31.06 ± 0.26
20	24.09 ± 0.24	19.18 ± 0.21	36.23 ± 0.26
25	24.74 ± 0.23	20.60 ± 0.23	37.91 ± 0.27
30	26.03 ±0.25	29.13 ± 0.26	41.52 ± 0.31
35	24.35 ± 0.21	27.58 ± 0.26	32.61 ± 0.30
40	23.70 ± 0.22	24.09 ± 0.28	30.55 ± 0.21
Polysaccharides Yield (%)			
Frequency (KHz)	Mono frequency	Dual frequency	Tri frequency
20	17.63 ± 0.25	-	-
40	20.09 ± 0.21	-	-
60	27.71 ± 0.22	-	-
20/40	-	19.05 ± 0.30	-
40/60	-	17.63 ± 0.21	-
20/60	-	29.39 ± 0.23	-
20/40/60	-	-	37.52 ± 0.21

The yield of purified polysaccharides extracted based on selected ultrasound frequencies (mono - 60 kHz, dual - 20/60 kHz, and triple - 20/40/60 kHz), temperature (35°C), and time (30 min) under this part of work has also been presented in (Fig. 3).

Preliminary characterization

The total carbohydrates, proteins, apparent amylose, uronic acid, and polyphenolic content of the lyophilized polysaccharides have been presented in Table 2.

Swamy and Narayana (2001)[43] discovered that the mechanism of combining two frequencies of ultrasound gave better efficiency when compared to single-frequency ultrasound sonication. Following this discovery, Manickam et al. (2014)[44] found that cavitation effects were higher for the triple frequency operation than with dual and single frequency. The mode of operation of the mono frequency in this work allowed cavitation bubble formation, growth, and collapse at 60 kHz frequency continuously without pulse time. The dual and the triple frequency on the hand started with the

Table 2. Preliminary characterization of SEWE-M, SEWE-D, and SEWE-T polysaccharides.

Composition	SEWE-M	SEWE-D	SEWE-T
Carbohydrate (%)	60.17 ± 0.15	69.21 ± 0.17	80.83 ± 0.16
Protein (%)	0.085 ± 0.16	0.050 ± 0.14	0.034 ± 0.14
Uronic acid (%)	1.04 ± 0.12	1.12 ± 0.20	1.51 ± 0.22
Amylose (%)	15.35 ± 0.16	18.67 ± 0.29	25.27 ± 0.19
Polyphenol (%)	24.23 ± 0.12	26.22 ± 0.06	27.79 ± 0.08

lowest frequency (20 kHz) and switched to a much higher frequency in the next cycle without pulse time. This effected continuous production of low to high level turbulent cavitation throughout the cycles and may have caused better disruption of plant cells for mass transfer. This mechanism explains the observed pattern of increase in total carbohydrates and polyphenolic recorded values for single, dual, and triple frequency ultrasound-extracted polysaccharides in this work (Table 2).

There was a close content correlation observed between the primary metabolite (carbohydrates) and secondary metabolites (polyphenolic). Primary and secondary metabolites are usually not thoroughly separated during extraction. This may explain the observed close content correlation. Total proteins associated with extracted polysaccharides were lowest with dual and triple frequency ultrasound extraction. Sugar molecules have been found to possess OH groups that can destroy the natural hydrogen bond network of water. Eventually, new hydrogen bond networks between water molecules and sugars are formed. This reduces "free" water molecules in the bottom phase of ILATPS, and therefore forces proteins to be transferred to the top phase[45] . As the carbohydrate content increased, the associated proteins were effectively transferred into the top phase. This explains why the protein content of extracted polysaccharides reduced with an increase in carbohydrate content.

Oxidation at the carbon-6 of sugars produce uronic acids.[46] This may explain the observed increase in the uronic acid content of samples as the total carbohydrates increased. That is, as the availability of carbohydrates rise, possible oxidation of carbohydrate into uronic acid also increases. Similarly, apparent amylose content was found to have increased with an increase in carbohydrate content. A report by Kumari et al. (2007), [47] indicated an increase in apparent amylose content with increased resistant starch content. The higher cavitational effect may have aided in the extraction of resistant starch and therefore the increase in amylose content.

Fourier transform-infrared (FT-IR)

The FT-IR analysis of all samples in this work has been presented in (Fig. 4). The objective was to investigate any possible primary structural changes. A peak was observed at 3,196 cm^{-1} which is known to be the hydroxyl stretching vibration.[48] This first peak is usually associated with carbohydrate compounds confirming the extracted samples to be carbohydrate. A second peak was also observed at 2989 cm^{-1} which is the alkyl C-H vibration. Its intensity relative to other peaks indicates the size of the alkyl group.

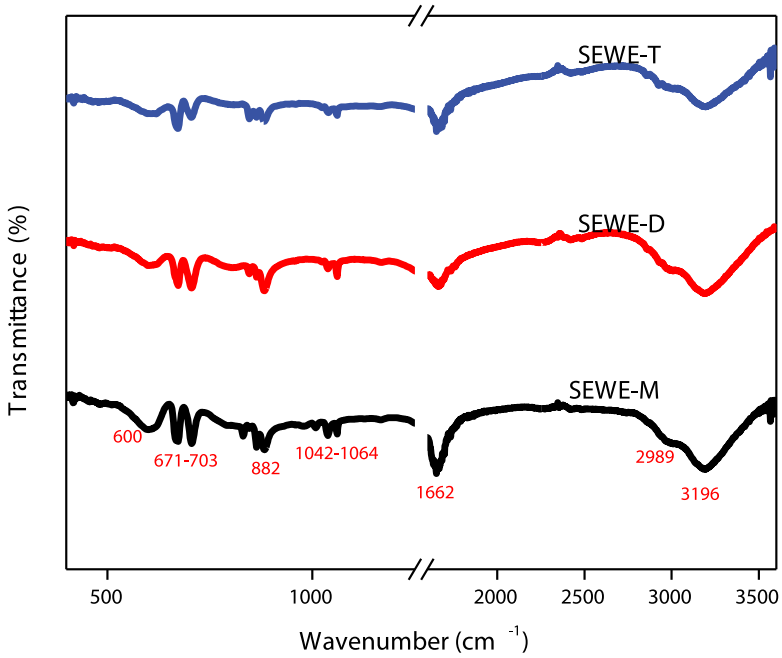

Figure 4. FT-IR spectra of SEWE-M, SEWE-D, and SEWE-T polysaccharides.

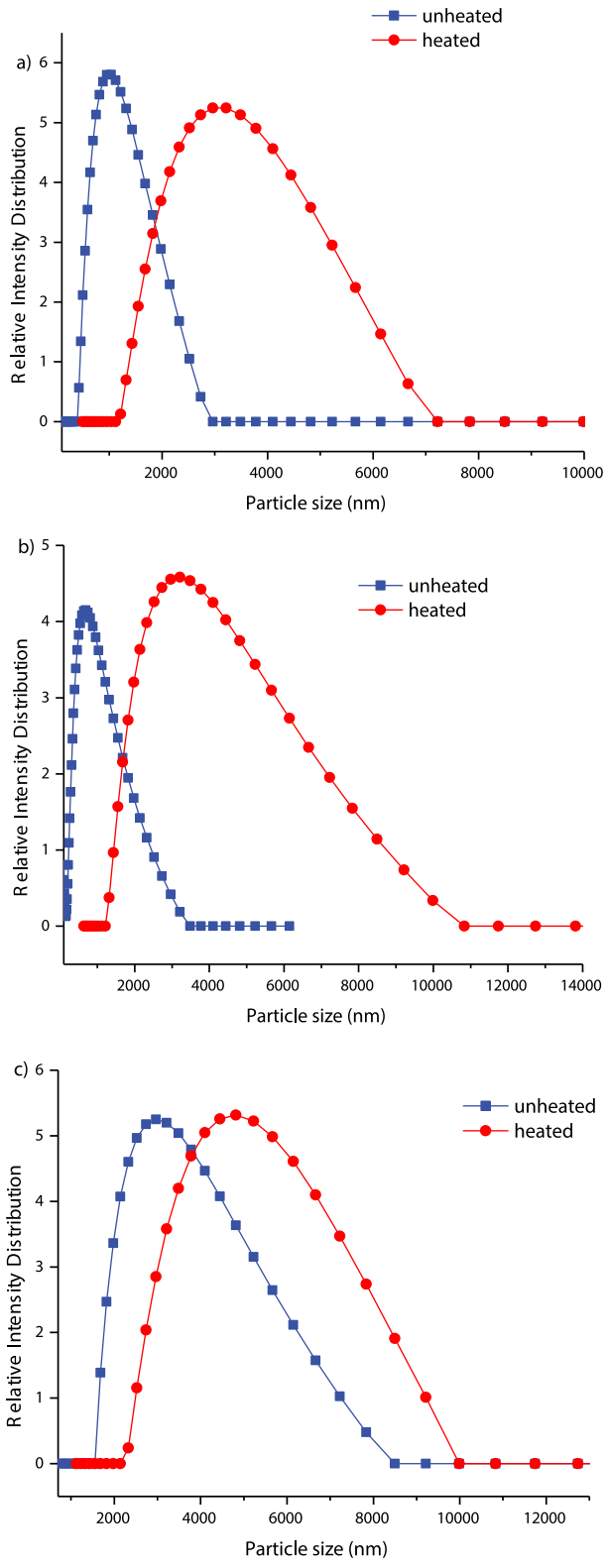

Figure 5. Particle size distribution of polysaccharides: a) SEWE-M, b) SEWE-D c) SEWE-T.

The third peak observed at 1662 cm^{-1} is referred to as Amide I stretching which depicts the presence of some soluble proteins in polysaccharides.[49] Another peak ranged at the band 1,042– 1,064 cm^{-1} known to be C-O stretching vibration[50] was observed. A fifth peak was found at the band 882 cm^{-1} also known as β anomer[51] denotes the presence of a dehydrated form of a salt or organic derivative. Finally, a weak absorption band between 600 and 703 cm^{-1} was attributed to O-H stretching vibrations.[52,53]

Particle size (dynamic light scattering, DLS)

The particle size distribution of the three polysaccharides SEWE-M, SEWE-D, and SEWE-T in aqueous solutions has been displayed in (Fig. 5). When the ultrasound single frequency extracted polysaccharide SEWE-M was dispersed in water, the particle size distribution with diameters that ranged (94.30–1754.44 nm) and main peak diameter at (919.99 nm) was observed (Fig. 5a). Holding the aqueous solution under a temperature of 70°C for 1 h 50 min, the particle size distribution increased to (233.92– 4535.64 nm) and displayed peak diameter at (2329.46 nm).

A similar trend of particle size distribution was observed for the multi-frequency extracted polysaccharides SEWE-D and SEWE-T. Dispersing these two samples in water, SEWE-D recorded a particle size distribution with diameters in the range of 106.34–1585.78 nm and a main peak diameter at 625.02 nm (Fig. 5b). The sample SEWE-T recorded a particle size distribution with diameters in the range 167.54–4946.67 nm and peaked at 2936.99 nm (Fig. 5c). Application of temperature of 70°C for 1 h 50 min similarly increased particle size distribution of SEWE-D and SEWE-T. The sample SEWE-D recorded a particle size distribution with diameters in the range 362.67– 5806.10 nm and a main peak diameter at 2611.65 nm. The sample SEWE-T recorded a particle size distribution with diameters in the range 319.29– 6552.50 nm and peaked at 3379.21 nm.

The aggregation of particles observed after heat exposure can be attributed to retrogradation which refers to the reformation of a more ordered structure.[54] It is affected by the presence of sugars or other hydroxyl-containing molecules.[55] It is known to mostly favor amylose chains.[56] The percentage of amylose in polysaccharides increased with an increase in carbohydrate content (Table 2). Therefore, more intense retrogradation or aggregation was observed for multi-frequency extracted samples.

SEM and molecular weight of polysaccharides

The morphological structure of polysaccharides SEWE-M, SEWE-D, and SEWE-T have been presented in (Fig. 6). The sample SEWE-M presented a grain morphological structure that was round and loosely packed. The multi-

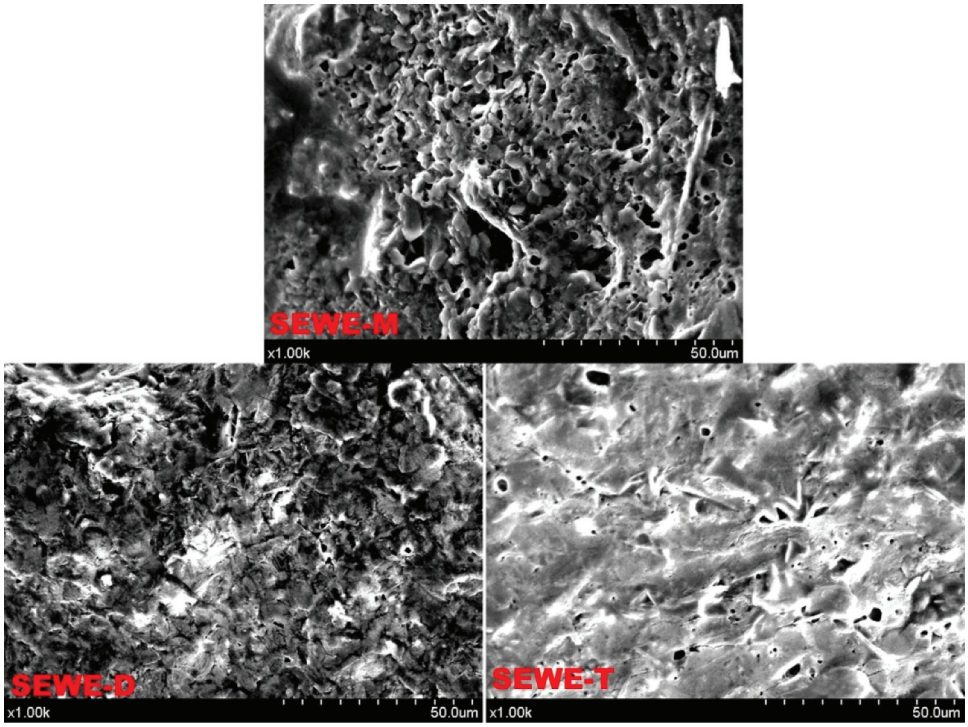

Figure 6. SEM of extracted polysaccharides under different ultrasound frequencies.

Table 3. Molecular weight of *Sorghum bicolor* leaf polysaccharides.

	Molecular Weight (g/mol)				
Samples	Mn	Mp	Mw	Mz	Mw/Mn
SEWE- M	1,160,000	1,285,000	1,202,000	1,243,000	1.037
SEWE- D	48,500	39,720	55,140	70,860	1.137
SEWE- T	2,688	2,090	3,133	4,671	1.166

frequency extracted samples SEWE-D and SEWE-T presented a densely packed morphology. The multi-frequency ultrasound extracted polysaccharides showed a dramatic deformation of cells compared to the mono-frequency extract (Fig. 6). The triple-frequency ultrasound treatment showed the most deformed and compact cells.

Molecular weight reduced with the use of multiple frequencies of ultrasound extraction (Table 3). This can be ascribed to the vigorous cavitational effect of multiple ultrasound frequencies on the sizes of extracted polysaccharides. The mono-frequency ultrasound extracts presented the highest molecular weight (1,202,000 g/mol) due to the use of cavitation with lowest intensity.

Rheological properties

Viscoelasticity and steady shear flow properties of all three polysaccharides have been displayed (Fig. 7). The three polysaccharides samples proved to be elastic in nature. As frequency increased from (1–10 Hz), G' values increased consistently (Fig. 7a).

However, there were clear differences in viscosity. The sample SEWE-M with smaller particle size distribution recorded the highest G" values (Fig. 7b). The samples, SEWE-D and SEWE-T with much larger particle size distributions, recorded lowest G" values. This indicates that SEWE-M was the most viscous. In a similar report by Afoakwa et al. (2008), [57] an increase in particle size distribution gave rise to a significant reduction in viscosity.

All three polysaccharide samples, however, showed dominance in elasticity since tan δ was (< 1) throughout the experiment (Fig. 7c). The samples SEWE-M, SEWE-D, and SEWE-T displayed a near-Newtonian flow behavior, with constant records throughout the shear rate experiment (Fig. 7d).

Antioxidant activity

Scavenging effect on hydroxyl radical

The hydroxyl scavenging activity of ascorbic acid and the three polysaccharides samples within the final calculated concentration range 1.25–7.5 mg/mL have been displayed in (Fig. 8). The sample SAW-T displayed a very close scavenging rate as ascorbic acid and recorded the same value (79%) at the final concentration of 7.5 mg/mL. The IC_{50} of SEWE-M, SEWE-D, SEWE-T, and ascorbic acid were found to be 2.25, 1.87, 1.37, and 1.25 mg/mL, respectively. An interesting report by Noda et al. (1997)[58] revealed a reduction in hydroxyl

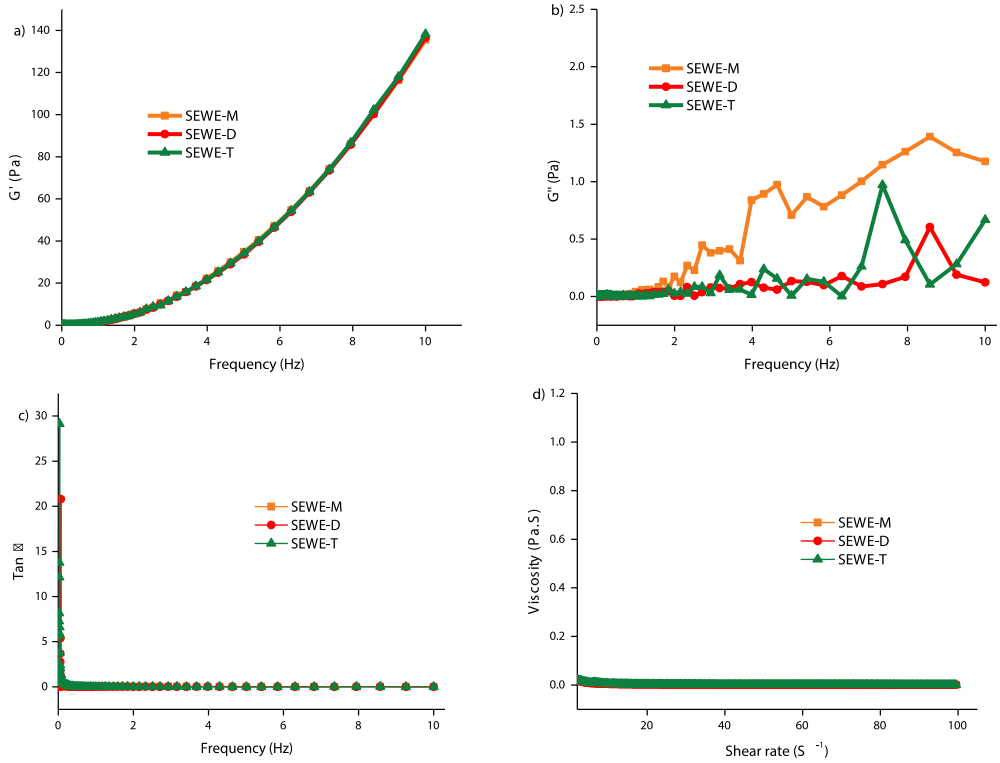

Figure 7. Rheological properties of SEWE- M, D and T polysaccharides: a) storage modulus G′ b) loss modulus G″ c) tangent tan δ in dynamic frequency test d) steady shear flow curves.

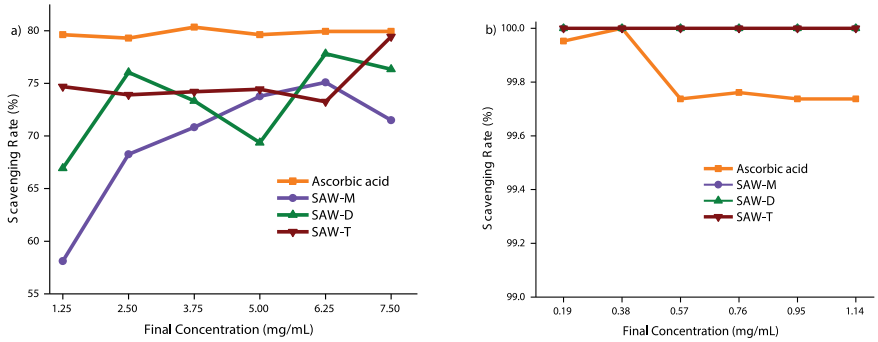

Figure 8. Scavenging effects of ascorbic acid and polysaccharides: a) Hydroxyl radical and b) ABTS radical.

scavenging inhibition of various plant extracts after ascorbate oxidase treatment. The extraction technique used to obtain polysaccharides may therefore have a direct impact on the inhibition ability. The high efficiency of triple frequency ultrasound gave rise to low molecular weight polysaccharides. Low molecular weight polysaccharides are known to give rise to high

Table 4. Phytochemical Constituents of *Sorghum bicolor* Leaf Sheath (SEWE- T).

No.	RT	Name of Compound	Molecular	Molecular Weight	Area Sum (%)
1	5.89	Hexadecane, 1-chloro	$C_{16}H_{33}Cl$	260	3.83
2	6.82	Imidazole, 2-amino-5-[(2- carboxy)vinyl]-	$C_6H_7N_3O_2$	153	10.87
3	7.97	3- Trifluoroacetoxypentadecane	$C_{17}H_{31}F_3O_2$	324	5.57
4	8.62	3-Butoxy-1,1,1,7,7,7- hexamethyl-3,5,5- tris(trimethylsiloxy)tetrasiloxa	$C_{19}H_{54}O_7Si_7$	591	7.05
5	11.28	2-Piperidinone, N-[4-bromo-nbutyl]-	$C_9H_{16}BrNO$	234	9.49
6	13.51	Oxalic acid, allyl pentadecyl ester	$C_{20}H_{36}O_4$	340	9.95
7	29.60	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13, 13-tetradecamethyl	$C_{14}H_{44}O_6Si_7$	505	5.74
8	30.77	Tris(tertbutyldimethylsilyloxy)arsane	$C_{18}H_{45}AsO_3Si_3$	468	7.06

hydroxyl activity. Furthermore, phenolic content of the polysaccharides in this work was observed to increase with increase in inhibition activity (Table 1) (Fig. 8a).

Scavenging effect on ABTS radical

The ABTS scavenging activity of ascorbic acid and the three polysaccharides samples within the final calculated concentration range of 0.19– 1.14 mg/mL have been displayed in (Fig. 8b). Similar to the ascorbic acid scavenging rate (99–100%), polysaccharides extracted in this work displayed 100% scavenging rate at all experimental concentrations. An IC_{50} of 0.19 mg/mL was therefore recorded for ascorbic acid, SEWE-M, SEWE-D, and SEWE-T. Similarly, analysis of some plant ethanolic extracts revealed higher antiradical and antioxidant activity with ABTS scavenging activity among other scavenging activities conducted. This was attributed to a greater quantity of phycobilin pigments and phenolics extracted.[59] The high ABTS activity observed may be further attributed to the appreciable amount of uronic acid content of all samples. Higher uronic acid content has also been discovered to give rise to high ABTS activity .[60]

Phytochemical constituents of extracted Polysaccharides (GC-MS/MS)

A total of 8 different phytochemical compounds were co-extracted with the triple frequency extracted polysaccharides and identified based on peak area, molecular weight, and molecular formula (Table 4). Bioactive compounds, 2-amino-5-[(2- carboxy)vinyl]-Imidazole and N-[4-bromo-n butyl]-2-Piperidinone with peak areas of 10.87% and 9.49% at RT of 6.82 and 11.28, respectively, have antimicrobial potentials.[61,62] Again, 3-Trifluoroacetyl Pentadecane with a peak area of 5.57% at RT of 7.97 has been reported to have an anti-viral ability.[63] Other phytochemicals identified were Hexadecane 1-chloro, 3-Butoxy-1,1,1,7,7,7- hexamethyl-3,5,5- tris(trimethylsiloxy)tetrasiloxane, Oxalic acid allyl pentadecyl ester, Heptasiloxane 1,1,3,3,5,5,7,7,9,9,11,11,13, 13-tetradecamethyl, and Tris(tert butyldimethylsilyloxy) arsane.

Conclusion

Subcritical ethanol-water generated approximately 40% crude polysaccharides on a wet matter basis. Synergized ILATPS with triple-frequency ultrasound also generated 36.49% partially purified polysaccharides on a wet matter basis. Again, the triple-frequency extracted polysaccharides upon dialysis and freeze-drying recorded a total of 80.83% carbohydrate content, 0.034% total proteins, 25.27% apparent amylose content, 1.51% uronic acid content, and 27.79% polyphenolic content. Polysaccharides samples obtained displayed aggregation after heat exposure. The triple frequency extracted polysaccharides presented the most deformed and compact cells. Though the polysaccharides displayed strong elasticity, the viscosity decreased with an increase in particle size distribution. The hydroxyl and ABTS radical scavenging ability of triple-frequency ultrasound extracted polysaccharides was as strong as ascorbic acid. Under ABTS radical scavenging rate analysis, both triple-frequency ultrasound extracted sample and ascorbic acid recorded an IC_{50} of 0.19 mg/ml. Some of the co-extracted phytochemical constituents, Imidazole 2-amino-5-[(2- carboxy)vinyl]-, 2-Piperidinone N-[4-bromo-n butyl]- and 3-Trifluoroacetyl Pentadecane are potentially bioactive. The combined thermal and non-thermal extraction method, therefore, gave a high yield of bioactive polysaccharides that can be useful for the food, nutraceutical, and pharmaceutical industry.

Disclosure statement

No conflict of interest.

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