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Research Article

Effects of Extraction Methods on the Physicochemical Properties and Biological Activities of heteroglycans from *Hibiscus sabdariffa* calyx

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Abstract

The hot-water extraction method has been employed to enhance the diffusion rate of heteroglycans, thereby augmenting the extraction efficiency. This study investigates the antioxidant activities and immunological properties of water-soluble polysaccharides derived from the *Hibiscus sabdariffa* calyx (HWPF and LWPF). These polysaccharides were isolated through two distinct extraction methods involving hot water followed by ethanol precipitation. The physicochemical characterization was conducted through a series of experiments encompassing monosaccharide composition, ultraviolet-visible spectrum analysis, and Fourier-transform infrared (FT-IR) spectroscopy. The evaluation of antioxidative properties employed various techniques, including DPPH and ABTS radical scavenging activities, Ferric Reducing Antioxidant Power, ferrous chelating power, total reducing power, and protection against DNA damage. The assessment of anti-inflammatory potential involved the inhibition of hemolytic activity induced by a hypotonic solution and heat. Furthermore, the impact of the polysaccharides on the production of pro-inflammatory cytokines was analyzed through Luminex assay and RTPCR assay in PBMCs and LPS-stimulated RAW 264.7 cells, respectively. The findings revealed that heteroglycans (LWPF and HWPF) exhibit a content of Ara-Man-Rha-GluA-GalA (41.2:17.2:15.7:9.2:8.8) and Ara-Glu-Rha-Xyl-GluA-Man (31.5:23.8:12.16:10.4:7:6) with molecular weights of approximately 4.5 KDa and 41.3 KDa, respectively. HWPF demonstrated robust metal chelating and scavenging activities. Additionally, each fraction exhibited substantial protection of DNA against damage induced by H2O2. LWPF was observed to significantly down-regulate the expression of pro-inflammatory cytokines IL-6 and COX-2 at both transcriptional and translational levels, along with inhibiting NO production. In summary, these results indicate that the choice of extraction methods influences both the structure and biological activities of heteroglycans, providing valuable insights into the structure-activity relationship.

Keywords: *Hibiscus sabdariffa*, heteroglycans, hot water extraction, physicochemical properties, Antioxidant activity, Pro-inflammatory cytokines.

1. INTRODUCTION

Heteroglycans are complex carbohydrates (polysaccharides) found in various plant foods, including vegetables, fruits, grains, and mushrooms. Their unique chemical structure imparts diverse biological properties, encompassing potential antioxidative, anticancer, and anti-inflammatory effects. Due to their therapeutic effects and favorable biocompatibility, these polysaccharides find applications in the food industry, cosmetics production, and pharmaceuticals [1].

They have demonstrated a notable impact on the immune response by specifically influencing immune cells and the generation of cytokines associated with immunological responses. Numerous studies suggest that specific polysaccharides isolated from plants can modulate inflammation and the production of proinflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-alpha) [2-6]. These cytokines play a pivotal role in the inflammatory response. Certain polysaccharides can modulate the activation of immune cells, such as macrophages and lymphocytes, and inhibit the production of inflammatory mediators, including prostaglandins, by blocking enzymes such as cyclooxygenase COX-2 [7,8]. Additionally, polysaccharides from plants may act as antioxidants, neutralizing reactive oxygen species, regulating NO secretion by macrophages, and reducing oxidative damage

associated with inflammation. They can also modulate the expression of genes involved in the regulation of inflammation in macrophages and peripheral blood mononuclear cells (PBMCs) [9-12].

Polysaccharides derived from higher plants are considered valuable, causing minimal side effects and being relatively non-toxic compared to bacterial polysaccharides and synthetic compounds [13]. Most studies on bioactive polysaccharides have gathered information from traditional practitioners regarding the use of medicinal plants and their related bioactivities, guiding the selection of plants for further study.

Hibiscus sabdariffa L. (Roselle or "Bissap") belongs to the Malvaceae family and has been traditionally used to treat various ailments, such as respiratory tract infections, colds, fever, hypertension, and malaria [14]. Previous studies have reported that the plant contains numerous compounds and possesses antioxidant, hypotensive, hypocholesterolemic, anti-inflammatory, immunostimulatory, antinociceptic, hepatoprotective, renoprotective, diuretic, anti-obesity, antiurolithic, antidiabetic, antimicrobial, and anticancer properties without significant genotoxic effects [15-20]. Polysaccharides isolated from its flowers stimulated proliferation and differentiation of human keratinocytes and displayed immunoregulatory activities on RAW264.7 macrophages [21-23]. Nevertheless, some studies have noted differences in the polysaccharides obtained based on the extraction rate, structure, and activity method due to various extraction process factors such as temperature, solid–liquid ratio, and extraction medium (solvent) [24]. Reports indicate that polysaccharides obtained through different extraction processes significantly differ in their physicochemical properties, affecting their antioxidant and immunomodulatory activities [1,25,26]. The typical extraction method for polysaccharides includes hot water extraction adopted to accelerate the diffusion rate of polysaccharides and enhance extraction efficiency [27]. Previous works on water-soluble polysaccharides of *Hibiscus sabdariffa* have involved acidic (TCA) treatment [21] and D354FD resin pretreatment [22] before purification.

The present study aimed to investigate the effect of two hot water extraction methods (hot water and hot water-ethanol) followed by ethanol precipitation on the antioxidant and anti-inflammatory activities of heteroglycans isolated from *Hibiscus sabdariffa* Linn calyx.

2. MATERIALS AND METHODS

2.1. Plant materials

Hibiscus sabdariffa (Voucher specimen number 25776/SRF/Cam) Linn calyces were harvested in August 2015 in the Adamawa regions of Cameroon, respectively. They were identified at the Cameroon National Herbarium, air-dried and ground into powder (0.3 mm) using a grinding.

2.2. Extraction of water-soluble Polysaccharide (WSP)

The water soluble polysaccharides of *Hibiscus sabdariffa* Linn calyx were isolated through hot-water extraction followed by ethanol precipitation, as described by Hua et al [28] with slides modifications. Plant powder (100 g) was mixed with 1000 mL of 60 % methanol (1:10, w/v) and stirred at room temperature for 24 h to remove fats, pigments and some oligosaccharides. This treatment was repeated twice. After filtration, the pellets were air-dried and used for the isolation of water soluble polysaccharides.

For LWP extraction, the air-dried pellet was mixed with distilled water 1:20 (v/w) and heated at 90 °C for 2 h. The mixtures were filtered and centrifuged (5000 rpm for 15 min). The collected supernatant was treated with two volumes of 95 % ethanol at 4 °C for 48 h followed by centrifugation (3000 rpm, 30 min at 4 °C).

For HWP extraction, the air-dried pellet was extracted twice with tenfold volume of 50 % ethanol at 70 °C for 2 h. The extracts were then centrifuged at 5000 g for 15 min to remove the contaminants. The combined extraction solutions were concentrated and precipitated with 20 %, 50 % and 70 % ethanol at 4 °C for 48 h followed each time by centrifugation (3000 rpm, 30 min at 4 °C).

The isolated polysaccharides (LWP and HWP) were solubilized in distilled water and deproteinized with isoamyl alcohol and chloroform (1:4) and intensively dialyzed against distilled water for 72 h (MWCO 6 000 and MWCO 14 000). The dialyzed solution was centrifuged at 2500 rpm for 20 min to remove insoluble substances and the supernatant was freeze-dried to obtain the heteroglycans of *Hibiscus sabdariffa* Linn coded as LWPF and HWPF respectively.

2.3. Partial characterization of polysaccharides extracts

2.3.1. Quantification of protein content, total sugar, uronic acid, phenolic compounds and total saponin content

Protein Content Assay: The quantity of proteins present in each polysaccharide extract was determined by the Bradford method using Bovine Serum Albumin (BSA) as standard [29]. Briefly, each sample (1 mL) was added to the same volume of Bradford reagent freshly prepared. After incubation in the darkness for 30 min, the absorbance of the mixture was measured at 595 nm using a UV-VIS 1605 Shimadzu spectrophotometer.

Total sugars assay: Total sugars were determined using phenol-H_2SO_4 as described by Dubois et al [30] where neutral monosaccharides were heated in acid medium and transformed into dehydrated derivatives of furfural. Practically, 200 µL of sample were mixed with 0.2 mL of 5 % phenol. Then, 1 mL of concentrated sulfuric acid was added quickly and stirred. The mixture was placed at 100 °C for 10 min until it developed a yellow colour. The absorbance was read at 485 nm. The amount and level of sugar present were calculated using glucose as standard and expressed as µg equivalent of glucose (GE) per mg of dry polysaccharides.

Uronic acid content assay: Uronic acids content was done using the method of Blumkrantz et Asboe-Hansen [31]. 200 µL of sample were placed on an ice bath at 4 °C then were added 1.25 mL of 12.5 mM sodium tetraborate solution prepared in concentrated sulfuric acid. After homogenization by vortexing, the tubes are placed in a water bath at 100 °C for 5 min then cooled in an ice bath. After cooling, 20 µL of a solution 0.15 % meta-hydroxydiphenyl prepared in 0.5 % NaOH solution (m/v) are added. The mixture is homogenized before being placed in the dark for 30 min. The quantities of uronic acids can be established in comparison with a standard range of galacturonic acid which underwent the same steps as the sample. Absorbance is measured using a UV-VIS 1605 Shimadzu spectrophotometer at 520 nm.

Phenolic compounds assay: Phenolic compounds were estimated by the Folin Ciocalteu method [32]. Briefly, 750 µL of extract solution (0.3 mg/mL) of sample were added to 75 µL of Folin-Ciocalteu reagent. After 3 min, 750 µL of Na_2CO_3 (20 %) were added. The absorbance was measured at 760 nm using a UV-VIS 1605 Shimadzu spectrophotometer after 30 min in the dark. Phenolic compound amounts were calculated using ferulic acid as standard and expressed as µg equivalent of ferulic acid equivalent (FAE)/mg of dry polysaccharide.

Total saponin content assay: Total saponin content was determined through spectrophotometry, as described by Medina-Meza et al [33]. Briefly, 25 µL of each sample solution

were added to 1 mL of glacial acetic acid/sulfuric acid (1:1 v/v). The mixture was vortexed and reacted at 60 °C in a water bath for 30 min and then cooled. The absorbance of the sample was measured at a wavelength of 527 nm using a spectrophotometer (UV-1605 Shimadzu). Oleanolic acid was used as a standard (0–1000 µg/mL). Total saponin content was expressed as g /100 g of oleanolic acid equivalents.

2.3.2. Analysis of monosaccharide composition by Gas chromatography

Gas chromatography (GC) was used for identification and quantification of the monosaccharide units of water soluble polysaccharide extracts of *Hibiscus sabdariffa* leaves. Neutral glycosyl composition of their alditol acetate and their trimethyl-silyl-glycoside derivatives respectively were determined (York et al [34]). As references, the following standard monosaccharides were derivatized and analyzed accordingly: D-Mannose, D-ribose, L-rhamnose, D-glucose, D-xylose, D-galactose, D-glucuronic acid, D-galacturonic acid, L-arabinose, D-fucose. Inositol (1 mg/mL) was used as internal standard.

2.3.3. UV Absorption Peak Detection

UV-visible spectra of WSP were determined using UV spectrophotometer V-670 (Serial No. B12736115) at 25 °C in the wavelength range of 200-800 nm [35]. The sample was dissolved in ultrapure water to a final concentration of 0.05 %.

2.3.4. Fourier-Transformed Infrared Spectroscopy (FTIR) Analysis

The structure groups of the WSP of *Hibiscus sabdariffa* leaves were identified using Fourier-transformed infrared spectrophotometer (FT/IR-4200 type A Serial Number B066661018) equipped with a horizontal attenuated total reflection (ATR) accessory [36]. 1 mg of dried sample was grounded with KBr powder and then pressed into 1 mm pellets for FTIR measurement from 4000 to 400 cm^{-1}.

2.4. Antioxidant potential assay

2.4.1. DPPH radical scavenging assay

The DPPH radical scavenging activity of water-soluble polysaccharides was determined according to Fan et al [37]. In brief, 300 µL of DPPH (0.1 mM) in methanol solution were added to 100 µL of methanol (blank) or polysaccharides preparative solution at varying concentrations (50-1000 µg/mL). The mixture was incubated at 37 °C for 30 min in the dark and the absorbance was measured at 517 nm using a spectrophotometer (Shimadzu UV-1605, ThermoFisher Scientific). Vitamin C was used as a positive control. The percentage of DPPH scavenging activity was calculated using the following equation:

$$Inhibition\ (\%) = \frac{AOC - AOT}{AOC} \times 100 \quad (1)$$

Where, AOC is absorbance of control, and AOT, absorbance of test sample.

2.4.2. ABTS radical scavenging assay

The ABTS radical scavenging activity was determined as described by Katalinic et *al.* with slide modifications [38]. In this respect, 2.5 mL of ABTS diammonium salt (7 mM) were mixed with 0.5 mL of potassium persulfate (15 mM). After storage in the dark for 24 h, the solution was diluted tenfold with distilled water. The diluted solution (0.2 mL) was added to 100 µL sample solution (50-1000 µg/mL). The absorbance was then measured at 734 nm after incubation for 15 min at 20 °C using a spectrophotometer (Shimadzu UV-1605, ThermoFisher Scientific). Vitamin C was used as a positive control and distilled water was used as the blank. The ABTS radical-inhibitory percentage was calculated using equation (1).

2.4.3. Ferrous ion chelating assay

The metal chelating effect of the water-soluble polysaccharides was determined using the ferrous ion chelating assay from the method of Wang et al with some modifications [39]. A reaction solution composed of 100 µL of sample at different concentrations (50–1000 µg/mL) and 0.1 mL of $FeCl_2$ (2 mM), was well homogenized and allowed to stand for 30 s. This was activated by the addition of 0.2 mL of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine, referred to as ferrozine (5 mM) and 2.7 mL of distilled water. After mixing with a vortex mixer, the reaction mixture was incubated at room temperature for 30 min and the absorbance was measured at 562 nm using a spectrophotometer (Shimadzu UV-1605, ThermoFisher Scientific). In the control, the sample was substituted with EDTA and blank contained the sample only without $FeCl_2$. The Chelating ability percentage was calculated using equation (1).

2.4.4. Reducing power assay

The reducing power was determined as described by Kong et al with slide modifications [40]. Briefly, 1 mL of different concentrations of samples (50–1000 µg/mL) in phosphate buffer (0.2 M, pH 6.6) was mixed with 2 mL potassium ferricyanide (1 %, w/v), and incubated at 50 °C for 20 min. Afterwards, 2 mL trifluoroacetic acid (10 %, w/v) was added to the mixture to stop the reaction and centrifuged at 2000 rpm for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 1 mL of ferric chloride (0.1 %). The absorbance of the reaction mixture was read at 700 nm using a spectrophotometer (Shimadzu UV-1605, ThermoFisher Scientific). Vitamin C was used as a positive control. Increased absorbance of the reaction mixture indicated increased reducing power.

2.4.5. DNA damage protective effect

The DNA damage protective activities of polysaccharides were assayed with genomic DNA, isolated from buffy coat from whole blood by using a Zymo research kit. DNA samples were damaged by H_2O_2 and $FeCl_2$ treatment using the method described by Mediesse et al [41] Different structural or conformational forms of genomic DNA were separated by electrophoresis. The reaction system contained 2 µL of tris-buffer (50 mmol/L, pH 7.4) 5 µL of human genomic DNA, 5 µL of WSP solution prepared at different concentrations (50-1000 µg/mL), 12 µL of H_2O_2 (30 %) and 6 µL of $FeCl_2$ (500 µM). The reaction mixture was incubated at 37 °C for 30 min and the samples were loaded with loading buffer (10x and separated by electrophoresis on a horizontal agarose gel (0.8 %) in Tris–borate–EDTA buffer for 30 min (105 V). The gel was visualized and photographed. The damaged and undamaged genomic DNA was used as negative and blank controls, respectively.

2.5. Anti-inflammatory activity assay

2.5.1. Preparation of erythrocytes suspension and isolation of human Peripheral Blood Mononuclear Cells (PBMCs)

Ethical clearance N° 2015/12/613/CE/CNERSH/SP was obtained from the National Ethics Committee on Research and Human Health (Cameroon). Signed informed written consent was obtained from each person enrolled. For the preparation of erythrocytes suspension, fresh blood samples were collected in anticoagulant EDTA tubes from healthy volunteers who had not taken NSAIDS for 2 weeks prior to the experiment, and thoroughly mixed. The blood samples were then centrifuged at 3000 rpm for 10 min at room temperature. The supernatant was collected and diluted to 40 % (v/v)

suspension with isotonic buffer solution (pH 7.4). The packed red blood cells (RBC) were washed three times using saline solution (0.9 %) followed by centrifugation (1500 rpm for 10 min). The suspension finally collected constituted the stock of erythrocytes (RBC) suspension. For Peripheral Blood Mononuclear Cells (PBMCs) isolation, the blood was collected from healthy volunteer men and diluted with 10 mL of incomplete Roswell Park Memorial Institute (RPMI-1640) medium. 15 mL of this mixture was added to Ficoll in 2/3–1/3 (v/v) and centrifuged at 10,000 rpm for 25 min. PBMCs suspended in 4 mL of incomplete RPMI 1640 medium were washed and centrifuged at 800 rpm for 5 min. After separation, PBMCs was finally diluted in complete RPMI 1640 medium containing type AB human serum, HEPES, 200 mM L-glutamine and 50 mg/mL Gentamicin and counted with Neubauer chamber.

2.5.2. Hemolytic assay

Hemolytic assay was performed using human erythrocytes suspension as reported earlier [42]. 160 µL of the erythrocytes suspension (2 %) were mixed with 40 µL of polysaccharide extracts. Saponin (50 µg/mL) was used as standard. Mixtures were incubated at 37 °C for 60 min, centrifuged (2500 g, 20 min) and 150 µL of the supernatant were transferred to 96-well microtiter plates for spectrophotometric quantitation of hemoglobin in the supernatant at 560 nm. Parallel measurements of extract dilutions without erythrocytes, erythrocytes without extract dilutions, and saline without both erythrocytes and extracts dilutions were also made. Results were expressed as percentage of activity and compared to a positive control containing erythrocytes in distilled water. Saponin was used as standard.

2.5.3. Hypotonicity induced hemolysis

The membrane stabilizing activity of the polysaccharide extracts was evaluated by using hypotonic solution induced human erythrocyte hemolysis designed by Sikder et al. with minor modification [43]. The test sample, consisted of stock erythrocyte (RBC) suspension (0.50 mL), was mixed with 5 mL of hypotonic solution (50 mM NaCl) in sodium phosphate buffered saline (pH 7.4; 10 mM) containing either the extracts (50-1000 µg/mL) or ibuprofen (0.1 mg/mL). The control sample, consisted of 0.5 mL of RBCs, was mixed with hypotonic-buffered saline alone. The mixture was incubated for 10 min at room temperature, centrifuged for 10 min at 3000 g and the absorbance of the supernatant was measured at 540 nm using UV spectrophotometer. The percentage inhibition of either hemolysis or membrane stabilization was calculated using the following equation:

$$\% \text{ inhibition of hemolysis} = 100 \times (OD_1 - OD_2)/OD_1$$

Where OD_1 is the Optical density of the hypotonic-buffered saline solution alone (control) and, OD_2 is the Optical density of the test sample in hypotonic solution.

2.5.4. Heat induced hemolysis

The membrane stabilizing activity of the polysaccharide extracts was evaluated by using heat induced human erythrocyte hemolysis [44]. Aliquots (0.5 mL) of the isotonic buffer, containing different ranges of plant extract concentrations (50-1000 µg/mL), were put into two sets of centrifuge tubes. The carrier, in the same amount, was added to another tube as control. Erythrocyte suspension (3 mL) was added to each tube and gently mixed by inversion. One pair of the tubes was incubated at 54 °C for 20 min in a water bath and the other pair was maintained at 4 °C. The reaction mixture was centrifuged for 3 min at 1300 g and the absorbance of the supernatant was measured at 540 nm using UV spectrophotometer. The percentage inhibition or acceleration of hemolysis in the tests was calculated using the following equation:

$$\% \text{ inhibition of hemolysis} = 100 \times [1 - (OD_2 - OD_1)/(OD_3 - OD_1)]$$

Where, OD_1 is the unheated test sample, OD_2 is the heated test sample and OD_3 is the heated control sample.

2.6. Multiplex Bead-Based Cytokine Assay

This essay was performed according to Boudjeko et al [9]. The human PBMCs culture ($2x10^5$ cells/well/100 µL) was incubated with 100 µL of different polysaccharide extracts (200 µg/mL) or MSP1 (20 µg/mL) for 16 h at 37 °C with CO_2 (5 %) at 90 % relative humidity and used to test the production of pro-inflammatory cytokines. The treatment of cells with both polysaccharide fractions and MSP1 was performed to see the effect of these polysaccharides in the presence of an antigen. After incubation in the same conditions, the mixture was centrifuged. Supernatants were used for detection and quantification of IL-1β, IL-6 and TNF-α pro-inflammatory cytokine levels using Luminex kit (Human Premixed Multi-Analyte kit) of R&D Systems, Inc. Minneapolis. MN 55413, USA. The experiments were performed following the manufacturer's protocol (Luminex kit HCYTOMAG-60K-05). The plates were read with the *Luminex* 100 cytometer equipped with a *Luminex* MAGpix Analyzer.

2.7. Effect on Macrophage Cell Proliferation

2.7.1. Cell culture and treatment

The murine macrophage cell line RAW 264.7 was purchased from the National Centre for Cell Science (NCCS) Pune, India. RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Inc., NY, USA) supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL) and fetal bovine serum (10 %) (FBS; GIBCO, Inc., NY, USA). The cells were incubated in an atmosphere of CO_2 (5 %) at 37 °C and were subcultured every 3 days. The assays were carried out on cells with 80 % confluence. The cell viability was tested after culture with 100 µL of different polysaccharide extracts (100 µg/mL) using Trypan Blue dye and at least 95 % of viability was confirmed to carry out the assay.

2.7.2. Determination of nitric oxide (NO) production

RAW 264.7 cells (1.5×10^5 cells/mL) were treated with non-toxic concentrations of polysaccharide extracts (100 µg/mL) for 30 min, and then stimulated with lipopolysaccharide (1 µg/mL) or without for 24 h at 37 °C in a CO_2 (5 %) incubator. After incubation, the quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production. Briefly, 100 µL of cell culture medium were mixed with 100 µL of Griess reagent (1 % sulfanilamide and 0.1 % naphthyl ethylene diamine dihydrochloride in 2.5 % phosphoric acid), the mixture was incubated at room temperature for 10 min, and the absorbance at 540 nm was measured in a microplate reader (Molecular Device Spectra M4, USA). Fresh culture medium was used as a blank in every experiment. The quantity of nitrite was determined from a sodium nitrite standard curve.

2.8. Measurement of Gene Expression of pro-inflammatory cytokines by RT-PCR analysis

The expression of selected cytokine and related genes in LPS-stimulated RAW 264.7 macrophage cells was determined by real-time quantitative RTPCR using SYBR Green technology. Total RNA from treated and non-treated RAW 264.7 cells was prepared with Tri-Reagent, according to the manufacturer's protocol. RNA was stored at -70 °C until use. One µg RNA was reverse transcribed, oligo dT- 18 primer, dNTP (0.5 µM) and 1 U RNAse inhibitor. After this, reaction cocktail was incubated

at 70 °C for 5 min, 25 °C for 5 min, and 37 °C for 60 min in series, and the reverse transcriptase was inactivated by heating at 70 °C for 10 min. Polymerase chain reaction (PCR) was performed on reversed-transcribed cDNA products for determination of IL-1β, IL-6, TNF-α and COX-2 expression according to the manufacturer's instructions using a DNA gene cycler (BIO-RAD, USA) with amplification by 30 cycles of 94 °C for 45 sec (denaturing), 60-65 °C for 45 sec (annealing) and 72 °C for 1 min (primer extension). The primers used in this experiment are indicated in Table 1 (F: forward, R: reverse). For quantification, the target gene was normalized to the internal standard β-actin gene.

Table 1 : The primer sequences.

Genes		Primers sequences	Size (bp)
β-actin	F R	5'-GTGGGCCGCCCTAGGCACCAG-3' 5'-GGAGGAAGAGGATGCGGCAGT-3'	603
TNF-α	F R	5'-TTGACCTCAGCGCTGAGTTG-3' 5'-CCTGTAGCCCACGTCGTAGC-3'	364
IL-1β	F R	5'-CAGGATGAGGACATGAGCACC-3' 5'-CTCTGCAGACTCAAACTCCAC-3'	447
IL-6	F R	5'-GTACTCCAGAAGACCAGAGG-3' 5'-TGCTGGTGACAACCACGGCC-3'	308
COX-2	F R	5'-CACTACATCCTGACCCACTT-3' 5'-ATGCTCCTGCTTGAGTATGT-3'	696

2.9. Statistical analysis

The data obtained were analyzed using SPSS version 18.0 (SPSS Inc. Chicago, IL. USA) and values were expressed as mean ± SD. The data were analyzed by one-way ANOVA and significant differences were analyzed using paired t- test. Data obtained from cytokines/chemokines level determination were analysed using Luminex xPONENT® multiplex assay software. $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Characterization of different fractions

The characterization of the heteroglycans of interest revealed an absence of various metabolites. The **table 2** presents the quantitative analysis of total sugars, proteins, total polyphenols, starch, and saponin content in the polysaccharide extracts. Carbohydrates constituted the predominant components, accounting for 82.11 % and 89.5 % in LWPF and HWPF, respectively. The extraction yield of LWPF exceeded that of HWPF by more than fourfold (4.18 % compared to 0.94 5%). Quantitative estimation indicated non-detectable levels of proteins, phenolic compound, and saponin. Additionally, the uronic acid content was found to be 10.18 % for HWPF and 16.97 % for LWPF.

UV spectroscopy is typically employed to identify proteins or nucleic acids in polysaccharides, while FT-IR analysis is used for characterizing the basic groups in the polysaccharide structure. Notably, there was no absorption peak at 260 to 280 nm in the UV spectra, indicating the absence of contamination by peptides or proteins in these water-soluble polysaccharides (WSP). This finding confirms the exclusive presence of polysaccharides in the fractions (**Figures 1 A-B**).

As shown **in Figures 1 C-D**, the FT-IR spectra gives the vibration of different functional group. The strong absorption around 3400.9 cm^{-1} was due to the stretching vibration of the hydroxyl groups in the sugar residue. The weak absorption around 2920 cm^{-1} represented the asymetric stretching vibration of C-H in the sugar ring. The peak at approximately 1746 cm^{-1} was assigned to the C=O stretching vibration of esterified groups. The strong absorption peak around 1611 cm^{-1} was due to the C=O asymetric stretching of COO^-, signifying the presence of uronic acids, corroborated by monosaccharide composition analysis. The absorption peak around 1100 was attributed to the vibration of C-O-C of pyranose and the band around 783 cm^{-1} indicated the presence of D-glucopyranose ring. The appearance of peak around 811 cm^{-1} should corresponded to the presence of β-type rhamnoside.

The sugars identified in the heteroglycans fractions of *Hibiscus sabdariffa* calyx comprised neutral sugars (mannose, ribose, rhamnose, glucose, xylose, galactose, and arabinose) and uronic acids (glucuronic acid and galacturonic acid). Several sugars were below the detection limit (ribose, galactose, and galacturonic acid for HWPF; ribose, glucose, xylose, and galactose for LWPF). **Table 3** illustrates that arabinose, mannose, rhamnose, glucuronic acid, galacturonic acid, glucose, and xylose were quantified, with arabinose, rhamnose, mannose, glucose, and xylose being the most abundant sugars. These results indicate that LWPF and HWPF consist of Ara-Man-Rha-GluA-GalA (41.2:17.2:15.7:9.2:8.8) and Ara-Glu-Rha-Xyl-GluA-Man (31.5:23.8:12.16:10.4:7:6), respectively, with molecular weights around 4.5 KDa and 41.3 KDa.

Table 2 : Biological compounds contents

Extracts	Yield (%)	Total sugar (%)	Uronic acid (%)	Total proteins (%)	Total phenolic compound (%)	Saponin (%)
HWPF	0.945	89.50	10.18	/	/	/
LWPF	4.18	82.11	16.97	/	/	/

Table 3 : Monosaccharide composition

Monosaccharides (%)	**Mannose**	**Ribose**	**Rhamnose**	**Glucuronic acid**	**Galacturonic acid**	**Glucose**	**Xylose**	**Galactose**	**Arabinose**
HWPF	**6**	**<2**	**12.16**	**7**	**<2**	**23.8**	**10.4**	**<2**	**31.5**
LWPF	**15.7**	**<2**	**17.2**	**9.2**	**8.8**	**<2**	**<2**	**<2**	**41.2**

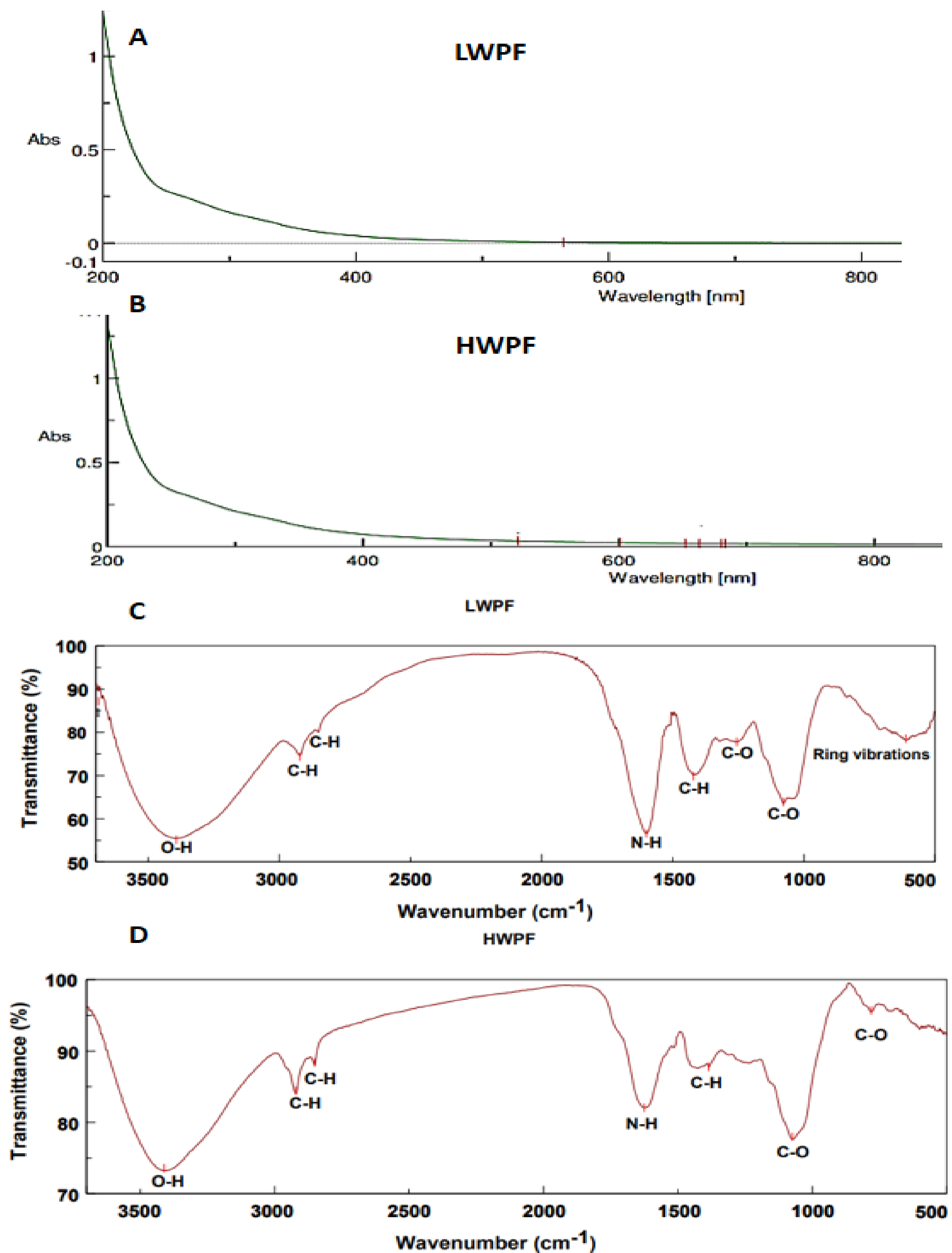

Figure 1: Chemical characterization of water-soluble polysaccharides isolated from *Hibiscus sabdariffa* Linn calyx. (**A, B**): UV visible spectra; (**C, D**): FTIR spectra of LWPF and HWPF respectively.

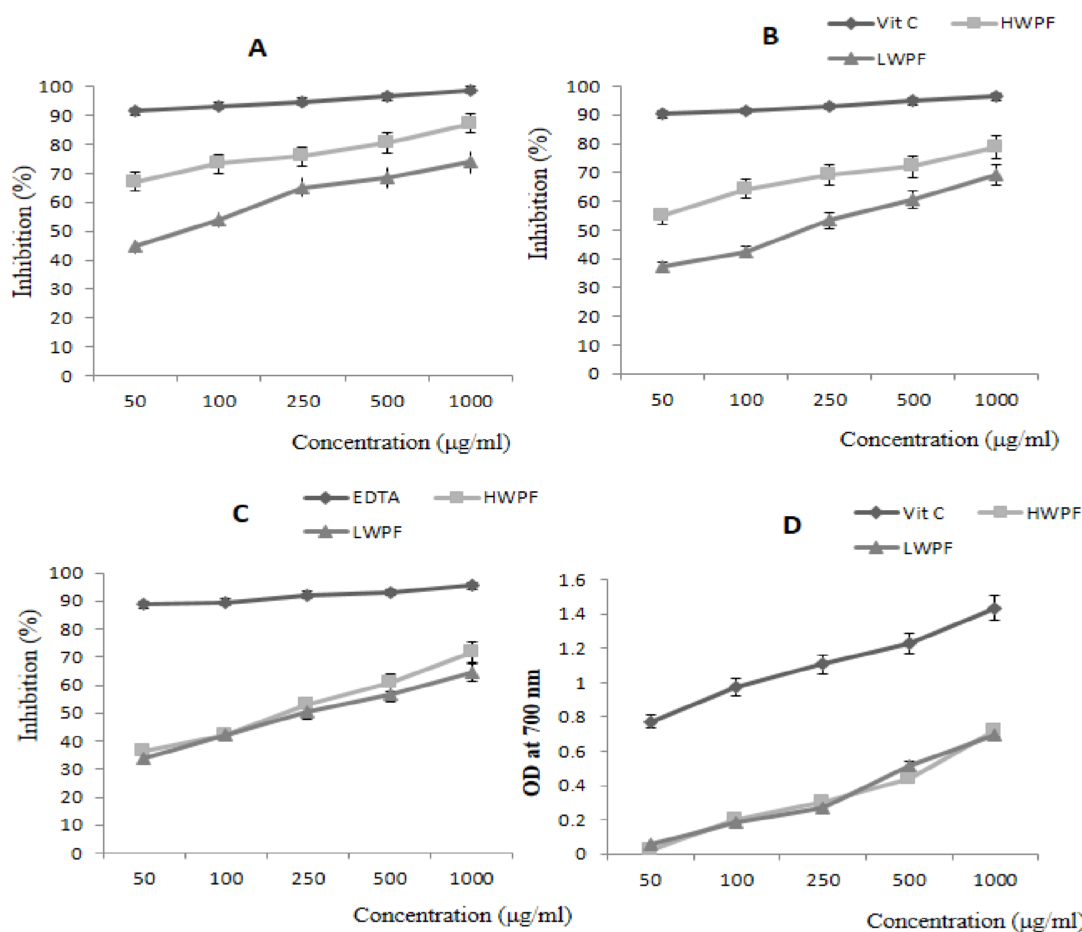

Figure 2: Antioxidant activity of polysaccharides extracted from *Hibiscus sabdariffa* Linn calyx. (**A**) DPPH radical scavenging activity; (**B**) ABTS radical scavenging activity; (**C**) Chelating ability of ferrous ion; (**D**) Reducing power ability. Data are presented as mean ± SD (n = 6).

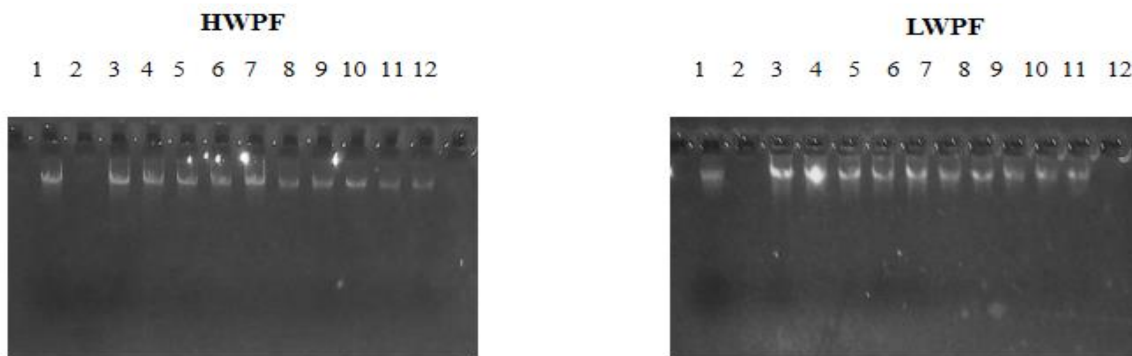

Figure 3: Protective effect of heteroglycans (HWPF and LWPF) isolated from *Hibiscus sabdariffa* Linn calyx against oxidative damage on DNA induced by hydrogen peroxide. 1: DNA; 2: DNA+H_2O_2+$FeCl_2$; 3: DNA+H_2O_2+$FeCl_2$+Extract 50 µg/mL; 4: DNA+H_2O_2+$FeCl_2$+Extract 100 µg/mL; 5: DNA +H_2O_2+$FeCl_2$+Extract 250 µg/mL; 6: DNA+H_2O_2+$FeCl_2$+Extract 500 µg/mL; 7: DNA+H_2O_2+$FeCl_2$+Extract 1000 µg/mL; 8: DNA+ Extract 50 µg/mL; 9: DNA+ Extract 100 µg/mL; 10: DNA+ Extract 250 µg/mL; 11: DNA+ Extract 500 µg/mL; 12: DNA+ Extract 1000 µg/mL.

3.2. Antioxydant potential effect

3.2.1. DPPH radical Scavenging ability

The DPPH free-radical scavenging effect of the heteroglycans is presented in **Figure 2A**. The results showed that the different samples inhibit DPPH radical. HWPF and LWPF concentration-dependently displayed DPPH scavenging effects of 67.12 to 87.27 %; and 44.63 to 73.79 %, respectively at the tested concentrations of 50 to 1000 µg/mL. However, none of the samples had stronger activity than vitamin C (reference compound).

3.2.2. ABTS radical scavenging ability

The results showed that the different samples inhibit the radical ABTS in a concentration-dependent manner. The ABTS scavenging activity HWPF and LWPF presented in **Figure 2B** varied of 50.95 to 78.76 % and 37.06 to 69.09 % respectively at the tested concentrations. The scavenging effect of vitamin C was greater than that of the samples, and ranged from 90.24 % to 96.31 % at the concentration between 50-1000µg/mL. The Scavenging activities of HWPF is significantly higher than LWPF.

3.2.3. Ferrous ion chelating activity

As shown in **Figure 2C**, HWPF and LWPF exhibited a potent inhibition metal chelating in a range from 33.75-77.04 % at the tested concentrations between 50-1000 µg/mL. However, EDTA (positive control) exhibited 88.68 % chelating ability and this effect is higher than extracts.

3.2.4. Reducing power

Figure 2D shows the reduction capability of HWPF and LWPF. The increase of absorbance at 700 nm indicate the reducing power of sample. The polysaccharides displayed a reduction ability but the effect is low than the positive control (vitamin C).

3.2.5. DNA damage protective effect

Genomic DNA isolated from buffy coat of whole blood showed bands on agarose gel. **Figure 3** reveals that H_2O_2+$FeCl_2$ effectively induced damage on DNA as shown in lane 2 where the DNA band totally disappeared. It can equally be seen that when HWPF and LWPF were associated to H_2O_2/$FeCl_2$, the DNA was protected at all the tested concentrations (lane 3-7). The protective effect of the extracts seems to be dose independent for the concentrations used. In addition, when the polysaccharide samples were alone in contact with DNA (lane 8-12), no DNA damage was found at the concentration of 50-1000 µg/mL.

3.3. Anti-inflammatory properties evaluation

3.3.1. Hemolytic assay

The heteroglycans isolated from *Hibiscus sabdariffa* Linn calyx were tested for hemolytic activity against human Red Blood Cells. As shown in **Figure 4A**, HWPF and LWPF protect hemolysis of Red Blood Cells (RBCs) at low concentrations. The highest concentrations exhibited around 10 % of RBCs. This activity is low than the effect of saponin which cause around 80% of RBCs hemolysis at tested concentrations. In hypotonic solution hemolysis, the polysaccharides were found to inhibit lysis of erythrocyte membranes with percentages varying from 10.22 to 58.74 %. It was noted that all the samples showed dose dependent membrane stabilizing activity over all the concentration ranges. Among the samples, HWPF showed high percentage of inhibition (56.50 %) hemolysis of RBC, while the minimum inhibition capacity was observed for LPWF **(Figure 4B**). Compared to Ibuprofen used as standard with a percentage inhibition of 71.21 %, all the extracts showed relatively lower values.

In heat-induced hemolysis conditions, the samples were found to inhibit lysis of erythrocyte membrane with percentage inhibition in the range of 27.03 to 60.28 % (**Figure 4C**). The inhibitory capacity of RBC hemolysis increased with the concentration, but not significantly different among HWPF and LWPF. However, the inhibition activities of all the samples were lower than Ibuprofen used as positive control

3.3.3. Effect on Macrophage Cell Proliferation

This work reports the effect of heteroglycans of *Hibiscus sabdariffa* Linn calyx on Raw 264.7 cells. The cytotoxic effects of HWPF and LWPF increase in a dose-dependent manner. At 1600 µg/mL, toxicity percentages ranged from 77.6 to 85.05 % (**Figure 6A**). Analysis of macrophage cytotoxicity by the MTT assay indicates that at 100 µg/mL do not significantly affect cell viability after 24 h of incubation. In addition, the effect of polysaccharide extracts at 100 µg/mL on the viability of macrophages exposed to LPS 100 ng/mL was evaluated. It was found that macrophages pretreated with HWPF and exposed to LPS had a viability greater than 85 %. While those pretreated with LWPF have a viability of less than 80 % (79.3 %) (**Figure 6B**).

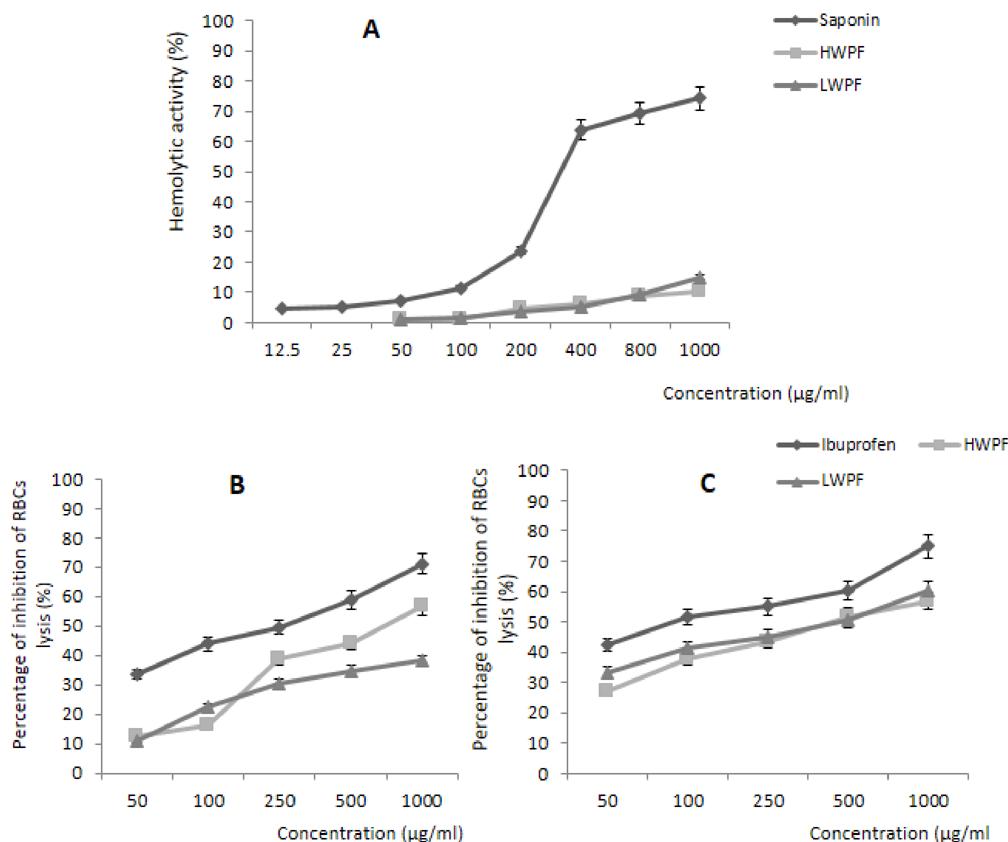

Figure 4: Effect of heteroglycans isolated from *Hibiscus sabdariffa* Linn calyx on: (**A**): Hemolytic activity; (**B)**: Hypotonic solution induced hemolysis of erythrocyte membrane. (**C):** Heat induced hemolysis of erythrocyte membrane. Values represent the mean ± SD of three independent experiments. *P < 0.05; compared with control.

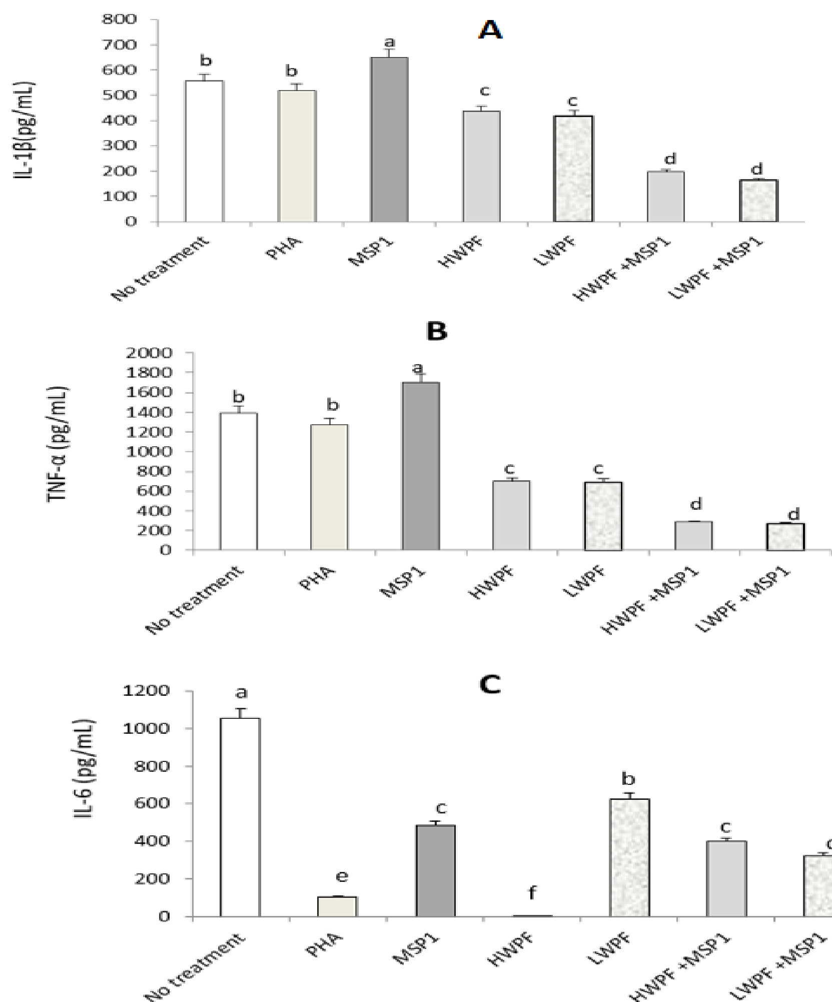

Figure 5: Concentration of pro-inflammatory cytokines produced by PBMCs after treatment with polysaccharides extracts of **A**: IL-1β; **B**: TNF-α; **C**: IL-6. PHA: phytohemagglutinin, MSP1: Merozoite Surface Protein 1, P < 0.05 with a > b > c > d > e.

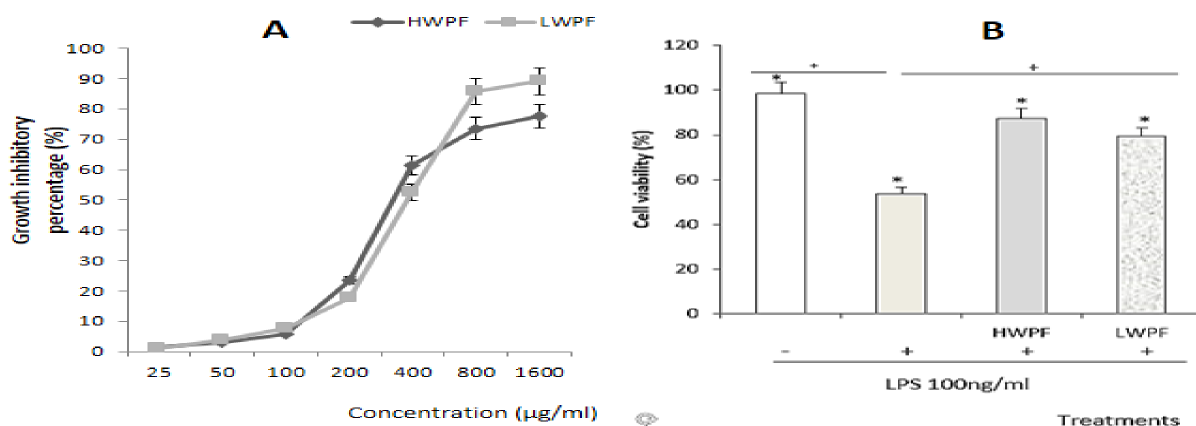

Figure 6: (A): Cytotoxicity of polysaccharide extracts of *Hibiscus sabdariffa* Linn calyx on RAW 264.7 macrophages cell lines. (**B**): Effect of polysaccharide extracts of *Hibiscus sabdariffa* Linn calyx on the viability of Raw 264.7 macrophages induced or not by LPS Values represent the mean ± SD of three independent experiments. *P < 0.05; compared with control, +P < 0.05 as indicated.

3.11. Determination of nitric oxide (NO) production

The effect of heteroglycans of *Hibiscus sabdariffa* Linn calyx on LPS-induced NO secretion was investigated by measuring nitrite accumulation in the culture medium and estimated by the Griess reaction. After 24 h of exposure to LPS (100 ng/mL), the concentration of nitrite in the culture medium varies in a dose-dependent manner. When RAW 264.7 cells are treated with HWPF and LWPF one hour prior to LPS exposure, significant inhibition (P< 0.01) is observed from 25 µg/mL (**Figure 7**) while significant inhibition is observed from 100 µg/mL after treatment with HWPF. The concentrations of nitrite secreted by cells treated with the plant extract (100 µg/mL) and exposed to LPS 100 ng/mL were 45.375 and 35.29 µM for HWPF and LWPF respectively. It's relevant to note that LWPF exhibited significant inhibition of NO production at each tests concentration.

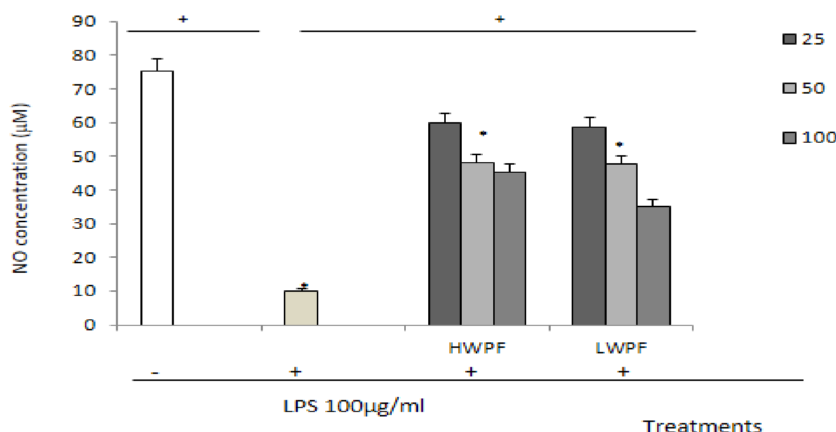

Figure 7: Effects of polysaccharide extracts isolated from *Hibiscus sabdariffa* Linn calyx on nitrite (NO) production in LPS-stimulated RAW264.7 macrophages for 24 h. The normal group was treated with media only. The control group was treated with LPS (100 ng/mL) alone. Values represent the mean ± SD of three independent experiments. *P < 0.05; compared with control, +P < 0.05 as indicated.

3.12. Measurement of Gene Expression of pro-inflammatory cytokines by RT-PCR analysis

TNF-α, IL-1β, IL-6, and COX-2 are pro-inflammatory cytokines that play critical roles in the pathology of many inflammatory conditions. The transcription of TNF-α, IL-1β, IL-6 and COX-2 was quantified in RAW 264.7 cells in response to treatment with extracts (100 µg/mL) and LPS (100 ng/mL) using real-time PCR. **Figure 8** shows that the gene transcription of IL-1β decreased by 5 and 3 times respectively for the batches treated with HWPF, LWPF compared to that of LPS. Regarding TNF-α, it decreased by 7.5 and 6.5 times for HWPF and LWPF respectively, compared to LPS. COX-2 gene transcription decreased by 1.75 and 4.5 for HWPF and LWPF respectively, compared to LPS while that of IL-6 decreased by 1.8 and 3.5 times respectively for HWPF and LWPF compared to LPS. It clear LWPF reduced significantly the gene expression of IL-6 and COX-2.

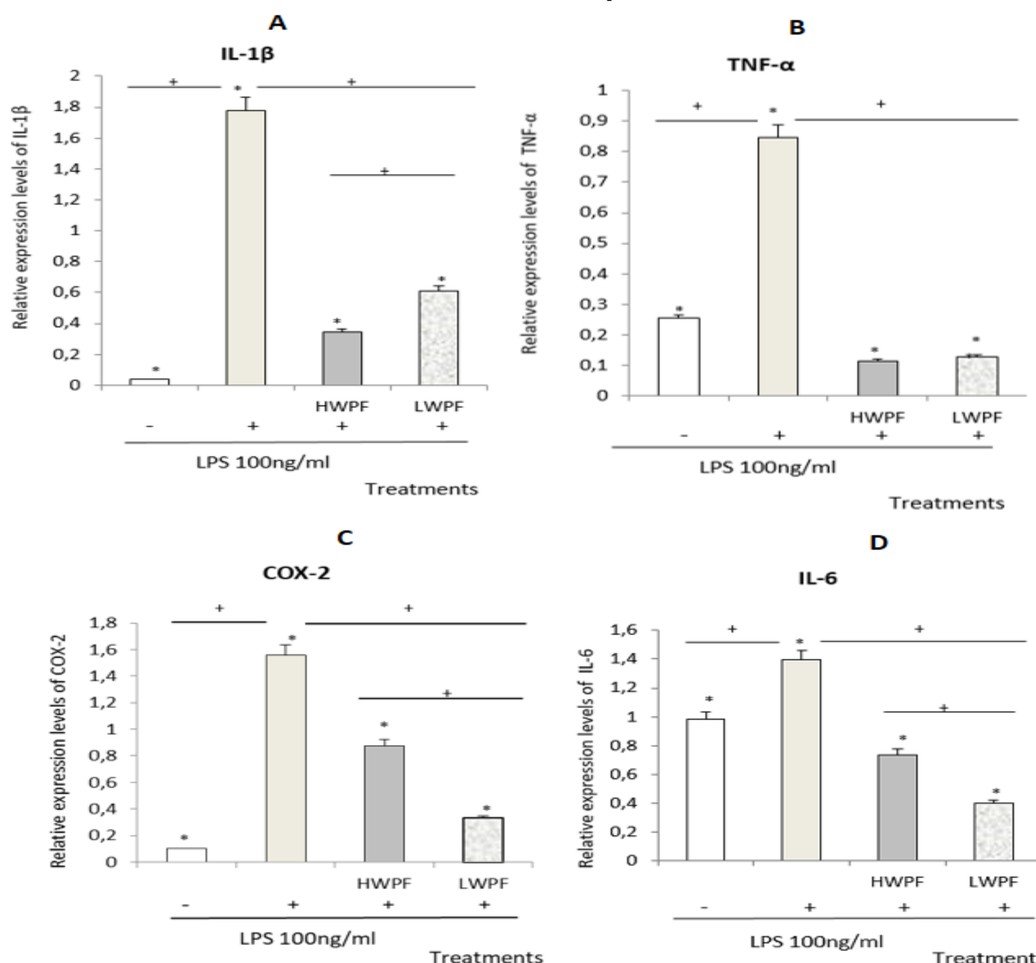

Figure 8: Effect of heteroglycans isolated from *Hibiscus sabdariffa* Linn calyx on Gene Expression of pro-inflammatory cytokines by RT-PCR analysis in LPS-stimulated RAW264.7 macrophages for 24 h. The normal group was treated with media only. The control group was treated with LPS (100 ng/mL) alone. Values represent the mean ± SD of three independent experiments. *P < 0.05; compared with control, +P < 0.05 as indicated

DISCUSSION

The heteroglycans were extracted using hot water. During this process, dialysis was done to eliminate the residual pigments and polyphenols after maceration with methanol. Additionally, treatment with isoamyl alcohol and chloroform was conducted to remove proteins. It can be hypothesized that the observed biological activities of HWPF and LWPF during this study may solely be attributed to the presence of polysaccharides in the samples.

The qualitative analysis of the functional group structure of WSP was based on absorption peaks and chemical bonds of specific atomic groups [36]. Notably, typical protein bands at 1651 cm^{-1} and 1555 cm^{-1} were not detected, indicating the very low amount of proteins. These results aligned with those obtained using Bradford's method. Polysaccharides, with distinct monosaccharide compositions, exhibit varying biological activities and therapeutic capacities and have been extensively studied for their functions, structures, and structure-function relationships [45].

The antioxidant activity of plant-derived polysaccharides is influenced by factors such as uronic acid content, monosaccharide composition, types of glycosidic linkage, and conformation. [46]**.** In this context, the reducing property is often linked to the presence of reductones, contributing to antioxidant ability by breaking free radical chains. Uronic acids (GluA and GalA) have been reported to display significant antioxidant activity due to the presence of carboxyl groups, acting as efficient electron or hydrogen donors. [47]. While Ara and Rha play crucial roles in radical-scavenging abilities [48,49]. Chen et al [50], demontrated that GlcA, Gal, Ara, Rha, and Fuc mainly contributed to the antioxidant activity of polysaccharide isolated from Cassia Seed (*Cassia obtusifolia*). It was also reported that polysaccharides contained uronic acid and having complicated monosaccharide composition exhibited stronger antioxidant effects [35,51]. But it is not the case in the present study. LWPF had a higher uronic acid content and a very complex monosaccharide composition (GlcA, GalA, Ara and Rha) than HWPF. the complex monosaccharide composition of heteroglycans of *Hibiscus sabdariffa*, contrasts with the findings of Zheng et al and Shen et al [21,22].

It is relevant that the heteroglycans isolated from *Hibiscus sabdariffa* Linn calyx protect the human erythrocyte membrane from lysis induced by hypotonicity and heat. The inhibition of lysis of red blood cell membrane was taken as a measure of the mechanism of anti-inflammatory activity of many plant extracts, because human red blood cell membranes are considered similar to lysosomal membrane components [52,53]. The possible mode of action of HWPF and LWPF could be the binding with the erythrocyte membranes with subsequent alteration of the surface charges of the cells. Falade et al [54] indicated that the decoction of *Hibiscus sabdariffa* exhibited moderate membrane stabilizing activity on the lysis of the bovine erythrocyte membrane at a dose of 3mg.ml^{-1} and this stability could be biphasic especially at high concentration of the extracts [55].

According to published data, the monosaccharide composition could enhance the immunomodulatory activity of heroglycans fractions [50]. In this respect, the presence of Mannose, is suggested to contribute to immunomodulatory activity ; The higher proportion (15.7) exhibited by LWPF could explain its potential [27].

5. CONCLUSION

Hibuscus sabdariffa calyx contain an important amount of heteroglycans with effective antioxidant and anti-inflammatory properties. Despite the fact that the methods of extraction affected the yield, the monosaccharide composition and the molecular weight of the different fractions isolated, it's appeared that LWPF presented better biological properties as compared to HWPF. They exhibited free radicals scavenging activities, reducing and antioxidative power. The heteroglycans protect human DNA from damage induced by $H_2O_2/FeCl_2$, protect the membranes of human red blood cells and reduce inflammatory injury by modulation of the production of IL-1β, IL-6, TNF-α and COX-2 by PBMCs and RAW264.7 cells. These findings support the medicinal use of heteroglycans of *Hibiscus sabdariffa* calyx and underscore their potential for further exploration in the pharmaceutical and nutraceutical industries..

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Author's contributions

The study conception and design were done by Judith Emery Kanemoto Ngomoyogoli, Thaddée Boudjeko, and Nicolas Njintang Yanou. Material preparation, data collection and analysis were performed by Judith Emery Kanemoto Ngomoyogoli, Fils Armand Ella, and Alice Louise Woguia. The methodology was done by Judith Emery Kanemoto Ngomoyogoli and Stanley Olivier Kanemoto. The first draft of the manuscript was written by Judith Emery Kanemoto Ngomoyogoli, Fils Armand Ella, Judith Laure Ngondi and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript**.**

Competing interest

The authors declare that they have no competing interests.

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