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Immunomodulatory and Hemagglutinating Activities of Acidic Polysaccharides Isolated from *Combretum racemosum*

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Abstract

Extracts of leaves of different species of the genus *Combretum* have been used historically to treat a variety of medicinal problems. However, little is known about the active components conferring therapeutic properties to these extracts. In the present studies, we evaluated biochemical properties and immunomodulatory activity of polysaccharides isolated from the leaves of *Combretum racemosum*. Water-soluble polysaccharides from leaves of *C. racemosum* were extracted and fractionated by DEAE-cellulose and Diaion HP-20 to obtain a Diaion-bound fraction, designated *Combretum* polysaccharide-acidic bound or **CP-AB**, which was eluted with methanol, and an unbound fraction, designated as **CP-AU**. Molecular weight determination, sugar analysis, and other physical and chemical characterization of the fractions were performed. Fraction **CP-AU** (mol. weight 5.0 kDa) contained type II arabinogalactan and had potent immunomodulatory activity, inducing the production of interleukin (IL)-1 β , -6, -10, and tumor necrosis factor- α (TNF- α) by human peripheral blood mononuclear cells (PBMC) and MonoMac-6 monocytic cells. Likewise, intraperitoneal administration of **CP-AU** increased *in vivo* serum levels of IL-6 and monocyte chemoattractant protein-1 (MCP-1) in mice. **CP-AU**-induced secretion of TNF- α in PBMC was prevented by Toll-like receptor 4 (TLR4) antagonist LPS-RS. Treatment with **CP-AU** induced phosphorylation of Akt2, Akt3, GSK-3 β , HSP27, mTOR, and all p38 MAPK isoforms (α , β , δ , and γ), as well as stimulation of AP-1/NF- κ B transcriptional activity. In addition, **CP-AU** effectively agglutinated erythrocytes from several species, including human, mouse, and rabbit. In contrast, fraction **CP-AB** was inactive in all biological tests, including cytokine production and hemagglutination. These data suggest that at least part of the beneficial therapeutic effects reported for the water extracts of leaves from *C. racemosum* are due to modulation of leukocyte functions.

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Keywords

Combretum racemosum; polysaccharide; macrophage; cytokines; hemagglutination; immunomodulation

1. Introduction

Plants represent a source of medicines useful in treatment of various human ailments and conditions. Indeed, the World Health Organization estimates that 80% of the population of developing countries relies on traditional medicine for their primary care needs [1]. In Africa, traditional remedies made from plants play an important role in the health of millions of people [2], and novel bioactive natural products with intriguing chemical structures and pharmacological properties have been identified in medicinal plants found in Africa [3]. For example, many of these plant materials are known to enhance the innate immune system and modulate host defense responses [4–6]. On the other hand, our understanding of the active principles of plant-derived medicines used widely in African countries is not extensive. Thus, the optimal therapeutic potential of these materials remains unrealized.

Combretum racemosum (P. Beauv.) belongs to the family of Combretaceae, which is comprised of about 370 species of trees and shrubs, roughly 300 of which are native to tropical and southern Africa. Extracts of *Combretum* species have been reported extensively in African or Indian traditional medicine [7]. For example, extracts of *C. racemosum* leaves have been traditionally for treating ulcers, helminthic infections, trypanosome infections, and bacterial infections of the genitourinary and gastrointestinal systems [8–10]. Water or organic extracts of *C. racemosum* leaves also showed significant antimicrobial activities against various pathogenic microorganisms [10, 11]. Furthermore, the antiinflammatory, vasorelaxant, and trypanocidal properties of the *C. racemosum* extracts have been validated in recent pharmacological studies [12, 13]. Preliminary phytochemical screening of *C. racemosum* extracts indicated the presence of alkaloids, steroids, cardiac glycosides, saponins and tannins [9]. It should be noted that combretastatins, found in the South African bush willow *Combretum caffrum* are currently under clinical studies for cancer therapy [14], although the presence of these compounds in *C. racemosum* was not evaluated. In addition, the biological activities of polysaccharides extracted from *C. racemosum* have not yet been investigated. Indeed, botanical polysaccharides represent great potential for natural product drug discovery and for the identification of novel therapeutic agents [15].

Here, we fractionated water-soluble polysaccharides from leaves of *C. racemosum* and evaluated their immunomodulatory and hemagglutinating activities. We found that the most active *Combretum* polysaccharide fraction (CP-AU)s contained type II arabinogalactan and had potent immunomodulatory activity *in vitro* and *in vivo*. To investigate the molecular mechanism responsible for the polysaccharide-induced immunomodulatory responses, the roles of signaling pathways including mitogen-activated protein kinase (MAPK) phosphorylation and transcription factors activating protein-1 (AP-1) and nuclear factor κ B (NF- κ B) were evaluated. Our results suggest that Akt and p38 signaling pathways may participate in stimulation of cytokine production by acidic polysaccharide fraction (CP-AU). In addition, fraction CP-AU effectively agglutinated erythrocytes from various species. Thus, we suggest that at least part of the beneficial therapeutic effects of *C. racemosum* extracts are due to modulation of leukocyte immune functions.

2. Materials and Methods

2.1. Fractionation of *Combretum* polysaccharides

Leaves of *C. racemosum* were collected in the Memni areas of Cote d'Ivoire and were taxonomically confirmed. Dried and ground leaves (500 g) were extracted with 3 L boiling distilled H₂O for 1 hr, and the aqueous extracts were centrifuged at $2,500 \times g$ for 15 min. A four-fold volume of ethanol was added to each supernatant to precipitate the polysaccharides overnight at 4°C. The precipitates were pelleted by centrifugation, dissolved in distilled H₂O, centrifuged at $80,000 \times g$ for 1 hr, and re-precipitated with a four-fold volume of ethanol. After centrifugation, the polysaccharide pellets were re-dissolved in distilled H₂O, filtered through a 0.22 µm filter, and concentrated in an Amicon concentrator with a 1 kDa PLAC membrane (Millipore, Billerica, MA) to obtain crude extracts. The crude polysaccharide extracts were further purified using ion-exchange chromatography on a DEAE-cellulose column equilibrated with 0.05 M Tris-HCl buffer (pH 8.0). For each fractionation, the column was washed with equilibration buffer to obtain the crude neutral polysaccharide fraction. The bound material (acidic fraction) was eluted with equilibration buffer containing 2 M NaCl. The eluates were concentrated in an Amicon concentrator with a 1 kDa PLAC membrane to obtain the crude neutral and acidic polysaccharide fractions. The acid fraction was further fractionated using Diaion HP-20 absorbent resin (2.5×20 cm). The Diaion column was eluted with distilled H₂O and lyophilized to obtain the unbound fraction (designated as **CP-AU**). Bound polysaccharides were eluted with methanol and dried to obtain fraction **CP-AB**. For analysis of biological activity, the fractions were diluted in Hank's balanced-salt solution without Ca²⁺ and Mg²⁺ (HBSS-) to a concentration of 5 mg/ml and filtered through sterile 0.22 µm filters.

2.2. Characterization of polysaccharide fractions

For nuclear magnetic resonance (NMR) analysis, samples (5 mg) were dissolved in deuterium oxide (0.5 ml), and ¹H NMR spectra were recorded on a Bruker DRX-600 spectrometer (Bruker BioSpin, Billerica, MA) at 20°C using 3-(trimethylsilyl)-propionic 2,2,3,3,3-*d*₅ acid sodium salt as an internal reference (δ 0.00 ppm).

Fluorescence measurements were performed using an LS50 luminescence spectrometer (Perkin Elmer). Samples were dissolved in H₂O, and synchronous fluorescence spectra were recorded from 300 to 600 nm at a scan rate of 240 nm/min. The excitation–emission wavelength difference (δλ) was 20 nm.

The homogeneity and average *M_r* of the polysaccharide fractions were determined by high performance size-exclusion chromatography (HP-SEC) using a Shimadzu Class VP HPC and Shodex OHPak SB-804 HQ column (8 mm × 300 mm) eluted with 50 mM sodium citrate buffer, pH 7.5, containing 0.15 M NaCl and 0.01% NaN₃ at a flow rate of 0.3 ml/min. Peaks were detected using a refractive index detector (RID-10A; Shimadzu, Torrance, CA). Average molecular weights of the polysaccharide fractions were estimated by comparison with retention times of pullulan standards P-100, 50, 20, 10, and 5 (Phenomenex, Torrance, CA), which have molecular weights of 112, 47.3, 22.8, 11.8, and 5.9 kDa, respectively. Reproducibility of the retention times was typically >98%.

The presence of arabinogalactan in the samples was detected by single radial gel diffusion in 1% agarose gels containing 100 µg/ml β-glucosyl Yariv reagent, which selectively interacts with and precipitates compounds containing type II arabinogalactan structures. Four µl of polysaccharide samples (10 mg/ml; w/v) were loaded into the wells, and the samples were incubated at 25°C for 24 hr in a humid atmosphere. A positive reaction was identified by a

reddish circle (halo) around the well, and arabic gum (4 mg/ml) (Fluka Chemie GmbH, Germany) served as a positive control.

Protein content was measured using the Bradford method with bovine serum albumin as the standard (Bio-Rad Protein Assay: Bio-Rad; Hercules, CA). The total amount of polyphenols in the polysaccharide fractions was determined by the Folin-Ciocalteu assay [16]. Briefly, 250 μ L of Folin's phenol reagent was added to the samples dissolved in 500 μ L distilled water. After 3 min at room temperature, 1.25 mL of 20% sodium carbonate was added, the mixture was allowed to stand for 40 min, and the absorbance was measured at 750 nm in a SpectraMax Plus microplate reader. Tannic acid (TA) from *Rhus semialata* (Sigma-Aldrich, St. Louis, MO) was used to generate a standard curve.

The Limulus Amebocyte Lysate (LAL) assay was used to estimate the amount of endotoxin contained in the polysaccharide fractions (LAL kit, GenScript, Piscataway, NJ) using a SpectraMax Plus microplate reader. To further evaluate the possible role of endotoxin, polysaccharide fraction **CP-AU** was applied to a column containing Detoxi-Gel Endotoxin Removing Gel (Pierce, St. Louis, MO) and eluted with 0.05 M phosphate buffer containing 0.5 M NaCl to decrease ionic interactions of sample molecules with the affinity ligand. The concentration of polysaccharides in the eluted sample was adjusted to match that of the untreated fraction, as determined by the phenol-H₂SO₄ method, and both treated and untreated samples were analyzed for biological activity.

For monosaccharide analysis, the polysaccharide fractions were lyophilized and submitted for analysis to the Oklahoma Center for Glycobiology Analytical Core Lab (Oklahoma City, OK). The polysaccharide samples or background blanks were subjected to methanolysis (methanolic 2 M HCl, 16 hr, 80°C), followed by acid hydrolysis (2 M trifluoroacetic acid, 4 hr, 100°C), and the resulting monosaccharide mixtures were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex DX-600 HPAEC system equipped with an ED50 detector (Dionex Corporation, Sunnyvale, CA). The samples were separated on a Dionex CarboPac PA20 column eluted isocratically with 12 mM NaOH at a flow rate of 1 ml/min at 22°C. For analysis of uronic acids, the column was eluted with 10 mM NaOH for 20 min, followed by a gradient of 100 mM NaOH/150 mM sodium acetate (0–100% in 45 min). Background signals were subtracted from all samples, and individual components were quantified based on electrochemical detection relative to known standards.

2.3. Cell cultures

Human MonoMac-6 monocytic cells (DSMZ, Germany) were grown in RPMI 1640 supplemented with 10% (v/v) endotoxin-free FBS, 10 μ g/ml bovine insulin, 100 μ g/ml streptomycin, and 100 U/ml penicillin. Human monocytic THP1-Blue cells obtained from InvivoGen (San Diego, CA) were cultured in RPMI 1640 medium supplemented with 10% (v/v) endotoxin-free FBS, 100 μ g/ml streptomycin, 100 U/ml penicillin, 100 μ g/ml zeocin, and 10 μ g/ml blasticidin S. These cells are stably transfected with a secreted embryonic alkaline phosphatase gene that is under the control of a promoter inducible by transcription factors AP-1 and NF- κ B. Human peripheral blood mononuclear cells (PBMC) were purified from the blood using dextran sedimentation, followed by Histopaque 1077 gradient separation and hypotonic lysis of erythrocytes. The blood was collected from healthy donors in accordance with a protocol approved by the Institutional Review Board at Montana State University. All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂. Cell number and viability were assessed microscopically using trypan blue exclusion.

2.4. Animal studies

Male 6–8 week old Balb/c mice were used for *in vivo* analysis of polysaccharides. Mice were maintained at the Montana State University Animal Resources Center under pathogen-free conditions in individual ventilated cages under HEPA-filtered barrier conditions and were fed sterile food and water *ad libitum*. All animal care and procedures were in compliance with a protocol approved by the Montana State University Institutional Animal Care and Use Committee.

2.5. Measurement of cytokine production

Cells were incubated for 24 hr in culture medium supplemented with 3% (v/v) endotoxin-free FBS, with or without polysaccharide fractions or bacterial lipopolysaccharide (LPS) from *Escherichia coli* K-235 (Sigma Chemical Co., St. Louis, MO) as a positive control. Human PBMC and MonoMac-6 cells were plated in 96-well plates at a density of 2×10^5 cells in 100 μ L per well and incubated for 24 hr. Where indicated, cells were pretreated with or without TLR4 antagonist LPS-RS (LPS from *Rhodobacter sphaeroides*; Invivogen, San Diego, CA) for 30 min and subsequently treated with or without polysaccharide fraction CP-AU, LPS, or 200 nM of phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich, St. Louis, MO) for 24 hr. Enzyme-linked immunosorbent assay (ELISA) kits for human tumor necrosis factor- α (TNF- α), granulocyte-macrophage colony-stimulating factor (GM-CSF), or interleukin (IL) -6 (all from Biolegend; San Diego, CA) were used to test the protein levels in the cell supernatants. A human cytokine Multi-Analyte ELISArray Kit (SABiosciences Corporation; Frederick, MD) was utilized to evaluate various cytokines in supernatants of PBMC (*in vitro*) and murine serum (*in vivo*).

2.6. MAPK profile

Analysis of the phosphorylation states of all MAPKs was performed using a human phospho-MAPK array kit (R&D Systems, Minneapolis, MN). Human PBMC were incubated for 30 min with the selected polysaccharide fractions or negative control (HBSS). The cells were rinsed with HBSS and lysed with the buffer provided. Arrays were incubated overnight at 4°C with lysates obtained from 3×10^6 cells for each sample. The arrays were washed three times with 20 ml of the wash buffer provided and incubated for 2 hr with the provided detection antibody cocktail containing phospho-site-specific MAPK biotinylated antibodies. The wash steps were repeated, after which the arrays were exposed to chemiluminescent reagents, and the signal was captured with an Alpha Innotech FluorChem FC2 imaging system.

2.7. Analysis of AP-1/NF- κ B activation

Activation of AP-1 and NF- κ B was measured using an alkaline phosphatase reporter gene assay in THP1-Blue cells. The indicated polysaccharide fraction or LPS (positive control) was added, and the cells (2×10^5 cells/well) were incubated for 24 hr. Alkaline phosphatase activity was measured in cell supernatants using QUANTI-Blue mix (InvivoGen). Activation of AP-1/NF- κ B is reported as absorbance at 655 nm and compared with positive control samples (LPS).

2.8. Cytotoxicity assay

Cytotoxicity was analyzed with a CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega; Madison, WI), according to the manufacturer's protocol. Briefly, cells were cultured at a density of 3×10^4 cells/well with polysaccharide fractions for 24 hr at 37 °C and 5% CO₂. Following treatment, the cells were allowed to equilibrate to room temperature for 30 min, substrate was added, and the samples were analyzed with a Fluoroscan Ascent FL.

2.9. Hemagglutination assay

The evaluation of hemagglutination activity was performed in U-shaped microtiter plates [17]. Polysaccharide fractions (50 μ l) were serially diluted in 0.15 M NaCl before addition of 50 μ l of a 2.5% (v/v) suspension of mouse or human erythrocytes (types A, B, AB, and O). After incubation at 37 °C for 30 min, the extent of agglutination was examined visually. The results are expressed as hemagglutination titer, which is an inverse of the last dilution of tested sample (5 mg/ml) with agglutinating activity. The specific hemagglutination activity is expressed as minimum concentration of the sample (μ g/ml) required for positive agglutination. If indicated, the tested erythrocytes were pretreated with neuraminidase (sialidase) from *Clostridium perfringens* (Sigma-Aldrich), pronase E from *Streptomyces griseus* (Sigma-Aldrich), or bovine trypsin (Worthington Biochemical Corp., Lakewood, NJ) for 1 hr at 37 °C. The plant-derived lectins, wheat germ agglutinin (WGA) from *T. vulgaris* (Sigma-Aldrich) and concanavalin A (Con A) from *C. ensiformis* (Calbiochem; San Diego, CA), were used as positive controls.

2.10. Statistical analysis

Statistical analysis was performed using Prism 5 (GraphPad Software, San Diego, CA). Statistically significant differences ($p < 0.05$ or better) are indicated.

3. Results

3.1. Preparation and partial characterization of polysaccharide fractions

The raw polysaccharides from *C. racemosum* obtained by ethanol precipitation were first fractionated using DEAE-cellulose into acidic and neutral fractions. The acidic fraction was further fractionated using Diaion HP-20 resin to obtain a bound fraction, designated *Combretum* polysaccharide-acidic bound or **CP-AB**, which was eluted with methanol and an unbound fraction, designated as **CP-AU**, with molecular weights of (4.7 and 5.0 kDa, respectively; Table 1). The presence of polysaccharides in the neutral fraction (not bound to DEAE-cellulose) fraction was minimal (<3% of total applied amount). Thus, this fraction was not fractionated or further studied.

Using the Folin-Ciocalteu assay, we found that polyphenols were a minor component of polysaccharide fractions **CP-AB** and **CP-AU**. Analysis of protein content indicated these fractions contained <3.0% protein (2.5 and 0.15% in **CP-AB** and **CP-AU**, respectively). Analysis of the fractions using the Yariv test showed that fraction **CP-AU** contained type II arabinogalactan, whereas fraction **CP-AB** tested negative for the presence of type II arabinogalactan (Table 1). The two fractions were also evaluated for possible endotoxin contamination using the LAL assay. The endotoxin-like activity in each polysaccharide solution in biological assays was less than 12.5 EU/mg, which is considered to be insignificant for various bioactive products [18, 19].

Sugar composition analysis revealed that fraction **CP-AU** consisted primarily of galacturonic acid, arabinose, galactose, rhamnose, glucose, and mannose (Table 2). Small amounts of glucuronic acids, fucose and xylose were also detected in this fraction. In comparison, fraction **CP-AB** contained 4-fold less galacturonic acid and ~2-, ~3-, and ~10-fold more galactose, glucose and xylose, respectively. A small amount of glucosamine was also found in fraction **CP-AB**.

Very-high-field (600 MHz) ^1H NMR was used to characterize the structure of native *C. racemosum* polysaccharides. The spectra from both acid fractions, **CP-AB** and **CP-AU**, were similar to each other in the range of 3.3–4.0 ppm, suggesting a common backbone structure (Figure 1). The strong signals at 3.6–3.9 ppm are consistent with the presence of β -

galactopyranose (β -Gal p) linkages [20]. The signal at 4.25 ppm found in fraction **CP-AU** could be assigned to H-2 of α -arabinofuranose (α -Ara f) [21]. Fraction **CP-AB** is enriched by aliphatic molecular fragments with terminal methyl groups, giving signals at 0.7–0.8 ppm, and acetyl groups (O -Ac or N -Ac) with resonances near 1.7–2.0 ppm. In contrast to **CP-AU**, the Diaion-bound fraction **CP-AB** contains glucosamine (Table 2), which together with specific ^1H -NMR signals for acetyl groups may indicate that N-acetyl-D-glucosamine (GlcNAc) is present in this polysaccharide fraction. In general, the spectra of both fractions resembled ^1H -NMR spectra of polysaccharides from tansy, which also had a high percent content of galacturonic acid [22].

The polysaccharide fractions **CP-AB** and **CP-AU** exhibited fluorescence emission peaks in the 390–420 nm and 430–470 nm regions of their synchronous fluorescence spectra. Fraction **CP-AB** exhibited a prominent peak at ~440 nm (Figure 2), suggesting enrichment of the Diaion-bound fraction with aromatic chromophoric groups. Indeed weak signals at 6.1 and 7.1 ppm were present in ^1H -NMR spectrum of this fraction (Figure 1).

3.2. Effects of Combretum polysaccharide fractions on monocyte/macrophage cytokine production in vitro

Using a cytokine ELISA semiquantitative array, we found that the profile of cytokine-stimulatory activity for fraction **CP-AU** was comparable that induced by LPS. Among the 12 cytokines analyzed, five were consistently induced in PBMC (>2-fold) by 250 $\mu\text{g}/\text{ml}$ of fraction **CP-AU** and 500 ng/ml of LPS, as compared with control (HBSS-treated) cells. As shown in Figure 3, fraction **CP-AU** induced production of IL-1 α [Fold increase (FI) = 3.4], IL-1 β (FI = 22.6), IL-6 (FI = 14.2), IL-10 (FI = 8.5), and TNF- α (FI = 3.8) (Figure 3). IL-8 production was inconclusive because of the high background production by PBMC (data not shown), a problem which has also been documented previously [e.g., [23]]. Fraction **CP-AB** was inactive and did not induce cytokine production by human PBMC (Figure 3).

To quantify dose-dependent effects of fraction **CP-AU** on cytokine production, levels of TNF- α , IL-6, and GM-CSF were determined by ELISA in supernatants from polysaccharide-treated human PBMC and MonoMac-6 cells. Incubation of PBMC and MonoMac-6 cells with fraction **CP-AU** enhanced TNF- α and IL-6 production in a dose-dependent manner, whereas cells treated with **CP-AB** produced negligible amounts of TNF- α and IL-6 (Figures 4 and 5), which is consistent with our cytokine array data. Fraction **CP-AU** significantly stimulated GM-CSF production in human PBMC and MonoMac-6 cells only at the highest tested concentration (500 $\mu\text{g}/\text{ml}$) (data not shown).

As described above, both fractions **CP-AU** and **CP-AB** contained <12.5 EU/mg endotoxin-like activity, but only fraction **CP-AU** was active in the cytokine-secretion assays. Although this endotoxin-like activity could reflect non-specific gelation of Limulus lysate by plant polysaccharides [24], it is possible that negligible endotoxin levels in fraction **CP-AU** could still activate cells. To address this issue, we pretreated polysaccharide fraction **CP-AU** by elution through a column of endotoxin-removing gel (fraction was designated as **CP-AU_{ER}**) and found that treated fraction was just as active as the untreated sample (at same polysaccharide concentration of 500 $\mu\text{g}/\text{ml}$) for activating TNF- α and IL-6 production by PBMC and MonoMac-6 cells (Figures 4 and 5). Thus, this control demonstrates that LPS-like contamination is not responsible for the responses observed.

3.3. Effect of Combretum polysaccharide fraction CP-AU on TLR4-dependent pathway, MAPK phosphorylation, and AP-1/NF- κ B transcriptional activity

LPS from the photosynthetic bacterium *Rhodobacter sphaeroides* (LPS-RS) is a potent TLR4 antagonist, and the primary mechanism of the antagonism consists of direct

competition with LPS from *E. coli* or other pathogenic bacteria for the same binding site on myeloid differentiation protein 2 (MD-2), an essential co-receptor in the LPS/TLR4 signaling pathway [25]. As shown in Figure 6, LPS-RS alone had a negligible effect on TNF- α production in PBMC over a concentration range from 3 ng/ml to 2 μ g/ml. LPS-RS at concentrations $\geq$ 50 ng/ml completely inhibited TNF- α secretion by PBMC induced by 250 μ g/ml of **CP-AU**. On the other hand, 2 μ g/ml of LPS-RS was necessary to completely inhibit TNF- α secretion by PBMC treated with 200 ng/ml of LPS. It should be noted that LPS-RS was unable to inhibit TNF- α secretion by PBMC activated with 200 nM of PMA (data not shown).

To evaluate effects of the fraction **CP-AU** on the activation status of the three major MAPKs, extracellular-signal regulated kinases (ERK1/ERK2), c-Jun N-terminal kinases (JNK 1–3), and different p38 isoforms (α , β , δ , and γ), as well as other intracellular kinases, such as MSK2, mTOR, CREB, HSP27, p53, Akt, glycogen synthase kinase β (GSK-3 β), p90 ribosomal S6 kinase (RSK)1/2, MAP kinase kinases (MKK3 and MKK6), and p70 S6 kinase 1 (p70S6K1) in PBMC, we utilized a human phospho-MAPK array (Proteome Profiler; R&D Systems), which simultaneously evaluates phosphorylation (i.e., the activation state) of these intracellular kinases. Of the signaling molecules screened, treatment with **CP-AU** was found to induce phosphorylation specifically of Akt2 (Ser⁴⁷⁴; fold increase (FI) = 1.6), Akt3 (Ser⁴⁷²; FI= 1.4), GSK-3 β (Ser⁹; FI=1.4), HSP27 (Ser⁷⁸/Ser⁸²; FI= 1.5), p38 α (Thr¹⁸⁰/Tyr¹⁸²; FI= 2.2), p38 β (Thr¹⁸⁰/Tyr¹⁸²; FI= 1.5), p38 δ (Thr¹⁸⁰/Tyr¹⁸²; FI= 1.3), p38 γ (Thr¹⁸³/Tyr¹⁸⁵; FI= 1.7), and mTOR (Ser²⁴⁴⁸; FI= 1.2) in human PBMC (Figure 7).

To evaluate activation of AP-1 and NF- κ B transcription factors by *Combretum* polysaccharide fraction **CP-AU**, we utilized a transcription factor-based bioassay in human THP-1Blue cells. Fraction **CP-AU** stimulated AP-1/NF- κ B transcriptional activity (Figure 8), but was less active than bacterial LPS (200 ng/ml). In contrast and consistent with our cytokine data, fraction **CP-AB** did not induce AP-1/NF- κ B activity (Figure 8).

3.4. Effect of Combretum polysaccharide fraction CP-AU on serum cytokine levels

We next determined whether fraction **CP-AU** could modulate cytokine levels *in vivo*. We found that after intraperitoneal administration of **CP-AU** (single dose of 250 mg/kg) only 2 of the 12 cytokines evaluated were consistently increased in serum (>2 -fold) at 2 hr post-injection, as compared with control, phosphate buffer saline (PBS)-treated mice. These cytokines included IL-6 (FI= 2.2) and monocyte chemoattractant protein-1 (MCP-1) (FI= 2.5) (Figure 9). This profile was quite different than that induced by LPS treatment (single dose of 500 μ g/kg), which also increased IL-10, TNF- α , macrophage inflammatory protein-1 α (MIP-1 α , CCL3), and MIP-1 β (CCL4). These cytokines were only slightly, but not significantly, increased after treatment with fraction **CP-AU** (Figure 9).

3.5. Hemagglutination activity of Combretum polysaccharide fractions

Of the two polysaccharide fractions isolated from *C. racemosum*, only fraction **CP-AU** caused agglutination of mouse, rabbit, and human erythrocytes (Tables 3 and 4). Hemagglutination activity was independent of human donor type, as the specific activity was the same in groups A, B, and O ($\sim$ 155 μ g/ml for each blood group). This concentration is higher than some of the most potent plant lectins, such as ConA and WGA (Table 4), but was comparable with the hemagglutination activity of other plant lectins, such as lectin from *Tuber borchii* fruiting bodies [26]. It should be noted that fraction **CP-AU** was active even after treatment at 100 $^{\circ}$ C for 1 hr, which was used during the first step of polysaccharide preparation (see the section 2.1).

After human erythrocytes were treated with sialidase, the hemagglutinating activity of fraction **CP-AU** increased two-fold; however, when the erythrocytes were treated with pronase E or trypsin, the hemagglutinating activity of this fraction decreased by 8-fold. It should be noted that sialidase, trypsin, and pronase E treatments increased the hemagglutinating activity of reference lectins Con A and WGA (Table 3), as reported previously [27, 28]. Sucrose and several monosaccharides, including D-glucose, D-xylose, D-mannose, D-arabinose, D-galactose, and L-rhamnose were tested for inhibition of agglutination by fraction **CP-AU**; however, none of these small molecule carbohydrates was inhibitory (data not shown).

Polysaccharide fractions from six different plants were tested to determine if hemagglutinating activity could be correlated with average molecular weight of the polysaccharides, the presence of type II arabinogalactan type II, protein and polyphenol content, or plant species. We tested polysaccharides isolated from *O. polyacantha* [29], *A. tripartita* [30], *T. vulgarie* [22], *E. oleracea* [31], *A. cordifolia* (unpublished), and *C. splendens* (unpublished). Polysaccharides isolated from *E. oleracea*, *A. cordifolia*, and *C. splendens* were inactive, while polysaccharides isolated from *O. polyacantha*, *A. ripartita*, and *T. vulgarie* had low hemagglutinating activity compared to *Combretum* polysaccharide fraction **CP-AU**. We did not observe a correlation between the hemagglutinating activity and molecular weight or protein and polyphenol content, suggesting hemagglutination activity is not related to presence of protein and polyphenol structures in fraction **CP-AU** (Table 3). Although type II arabinogalactan is present in polysaccharide fractions **Acai-2**, **AP-AU1**, and **CS-NU1** from *E. oleracea*, *A. cordifolia*, and *C. splendens*, respectively, all were inactive in the hemagglutination assay. Thus, other unknown structural features of **CP-AU** from *C. racemosum* must be responsible for its potent hemagglutinating properties. Future experiments will be necessary to define this issue.

3.6. Effect of Combretum polysaccharide fractions on cell viability

Although our functional assays suggested that the polysaccharides extracted from *C. racemosum* were relatively non-toxic, we evaluated the potential cytotoxic effect of the polysaccharides to determine if the results might be influenced by background toxicity. Using a cytotoxicity assay, we determined that the two polysaccharide fractions **CP-AB** and **CP-AU** had no effect on the viability of PBMC (Figure 10) or MonoMac-6 cells (data not shown) when tested over a concentration range of 10–250 µg/ml, thereby verifying that these polysaccharides were not cytotoxic.

4. Discussion

Over the past several decades, research on the medicinal properties of natural products has increased significantly, and a large body of evidence suggests that plant extracts may represent a source of novel compounds with medicinal properties [3, 32]. Macromolecules, including polysaccharides and lectins, isolated from botanical sources such as mushrooms, algae, lichen, and higher plants have also attracted a great deal of attention in the biomedical arena because of their broad spectrum of therapeutic properties [33–37]. While *C. racemosum* has been used extensively in traditional African medicine for treating many diseases, essentially nothing is known regarding potential immunomodulatory properties of polysaccharides from this plant. In the present report, we evaluated the immunomodulatory and hemagglutinating activities of polysaccharides isolated from *C. racemosum*.

Most commonly, lectins or hemagglutinins are simple proteins or proteoglycans. In contrast, only few natural macromolecules with hemagglutinating activity are composed of polysaccharides with low protein content. For example, hemagglutinins isolated from chitin and the red alga *Gracilaria verrucosa* had protein contents of 8.5% and <8%, respectively

[38, 39]. However, this is the first report of hemagglutinating activity of polysaccharides from a higher plant. Furthermore, we found that the hemagglutinating polysaccharide **CP-AU** was not cytotoxic for primary PBMC or MonoMac-6 tumor cells. In contrast, hemagglutinating lectins have been reported to exhibit cytotoxicity for tumor cells [e.g., [40–42]] and have been utilized previously for alternative tumor therapy [33]. Thus, the polysaccharides described here represent a potentially novel class of hemagglutinins.

The sugar composition, positive Yariv reaction, and $^1\text{H-NMR}$ spectrum suggest that the most active fraction **CP-AU** with hemagglutinating activity is a typical rhamnogalacturonan with type II arabinogalactan side chains. We tested several additional polysaccharide fractions isolated from six different higher plants but did not find a correlation between protein content and hemagglutinating activity in polysaccharides from these plants (Table 3). Indeed, fraction **CP-AU** had essentially no protein (0.15%). We also found that pretreatment of human erythrocytes with pronase or trypsin decreased hemagglutinating activity of **CP-AU** (Table 4), whereas a similar treatment had no effect on hemagglutination by the control lectins (Con A and WGA). Thus, our data suggest that the polysaccharides from *C. racemosum* may recognize a protease-sensitive protein(s) on the erythrocyte surface, which is distinct from the epitope targets of these lectins [43, 44].

Recently it was reported that plant polyphenols could have a hemagglutinating activity [45]. However, we found that polyphenols were a minor component of fraction **CP-AU** (3.0%), while fraction **CP-AB** with a higher polyphenol content (6.1%) was inactive in the hemagglutination assay. In addition, there was no correlation between polyphenol content and hemagglutinating activity among the other polysaccharide fractions tested (Table 3). Thus, at least in the case of *Combretum* polysaccharides, polyphenols do not seem to play a role in their hemagglutinating activity.

The polysaccharide fraction **CP-AU** induced cytokine production in human PBMC and MonoMac-6 cells, resulting in increased IL-1 β , IL-6, IL-10, and TNF- α . Because the Diaion-bound fraction **CP-AB** did not stimulate cytokine production, it is possible that the cytokine-inducing activity of fraction **CP-AU** could be correlated with hemagglutinating activity. However, plant polysaccharide with even higher immunomodulatory activity than **CP-AU**, such as polysaccharides from *O. polyacantha* [29], *A. cordifolia*, and *C. splendens* (unpublished data), had low or no hemagglutinating activity. Thus, surface structure(s) responsible for hemagglutination and cytokine-stimulation could be different. Future experiments will be necessary to characterize structural features of the polysaccharide fraction **CP-AU** and the protease-sensitive proteins (receptors) on cell surface that are responsible for the different biological activities of these polysaccharides.

Differences in intracellular signaling were observed between *Combretum* polysaccharides and previously reported botanical polysaccharides. For example, polysaccharides from algae *Capsosiphon fulvescens* induced ERK1/2, but not p38 [46], polysaccharide SP1 from *Caulerpa lentillifera* increased the phosphorylation of p38 [47], whereas polysaccharides from *Platycodon grandiflorum* activated phosphorylation of all three MAPK [48]. Here we demonstrated that *Combretum* polysaccharide fraction **CP-AU** induced phosphorylation of Akt2, Akt3, GSK-3 β , HSP27, mTOR, and all p38 isoforms (α , β , δ , and γ), but not JNK1/2/3 or ERK1/2 in human PBMC. Thus, it is clear that various polysaccharides can exert their biological activities through different kinase signaling pathways, and a better understanding of these signaling events will be important as we expand our knowledge of such complementary and alternative medicines. We also found that the fraction **CP-AU** stimulated AP-1/NF- κ B transcriptional activity but was less active than *E.coli* LPS. These results indicate that AP-1 and NF- κ B are not the only transcription factors involved in the immunomodulatory activity of **CP-AU**. Indeed other transcription factors, such as STAT-1,

ATF2, ATF6, CREB, and GATA-3, were reported to be involved in molecular mechanisms of immunomodulatory activity of different plant polysaccharides (e.g., [49, 50]).

Plant polysaccharides can modulate leukocyte activity via different receptors, including TLR4 [for review [36]]. For example, polysaccharides ASP and G1-4A, which were isolated from *Acanthopanax senticosus* and *Tinospora cordifolia*, respectively, activate macrophages and B cells by interacting with TLR4 [51, 52]. TLR4 was also found to be one of the cellular receptors mediating TNF- α secretion induced by ZPF1, polysaccharide isolated from *Dioscorea batatas* [53]. Likewise, polysaccharide CWSP from *Chlorella pyrenoidosa* induced cytokine secretion in macrophages via TLR4-mediated signaling pathways [54]. In the present work, we found that the *Combretum* polysaccharide fraction **CP-AU** induced secretion of TNF- α in human PBMC mainly via TLR4, but binding affinity for *Combretum* polysaccharides was much lower than that of *E.coli*-derived LPS. Indeed, a 10-fold higher concentration of LPS-RS over LPS was necessary to completely inhibit TNF- α production in human PBMC. In comparison, a much lower relative concentration of LPS-RS versus **CP-AU** was needed to inhibit **CP-AU** activity. Likewise, the pharmacological properties of such low-affinity TLR4/MD-2 ligands appear to be different than those of bacterial LPS. Indeed, although the cytokine profiles for *in vitro* stimulatory activity by bacterial LPS and **CP-AU** were fairly similar (Figure 3), the profile was quite different in our *in vivo* experiments (Figure 9). Because several plant polysaccharides were recently found having therapeutic effects in different models of autoimmune diseases (e.g., [55–59]), we hypothesize that such natural low-affinity TLR4 agonists may have clinically beneficial effects on autoimmune diseases by their induction of immunosuppression [60]; however, future experiments will be necessary to address this hypothesis.

In summary, the present study demonstrates that polysaccharides from the leaves of *C. racemosum* are not cytotoxic and activate monocytes and PBMC, resulting in modulation of cytokine production. Our data provide a molecular basis to explain at least part of the beneficial therapeutic effects reported for extracts of *C. racemosum*, and suggest that leukocyte stimulation by the *Combretum* polysaccharides might enhance resistance to infection. Although immunologic effects of the *Combretum* polysaccharides following oral administration were not studied in the present work, numerous dietary polysaccharides appear to elicit diverse immunomodulatory effects in various animal tissues, including the blood, gastrointestinal tract and spleen (e.g., [61, 62]). Note, however, that our recent studies suggest that nasal application of some plant polysaccharides may be even more effective than oral administration for stimulation of innate immunity [63]. Direct application of polysaccharides to a tissue may also activate cutaneous wound healing processes. In any case, further studies are now in progress to determine the therapeutic potential of *Combretum* polysaccharide fraction **CP-AU** and its structural features conferring biological activity.

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Highlights

- The active *Combretum* polysaccharide fraction induced the production of interleukin (IL)-1 β , -6, -10, and tumor necrosis factor α by human mononuclear cells and MonoMac-6 cells.
- The active *Combretum* polysaccharide fraction induced AP-1/NF- κ B transcriptional activity and phosphorylation of Akt2, Akt3, GSK-3 β , HSP27, mTOR, and all p38 MAPK isoforms.
- Treatment of mice with the active *Combretum* polysaccharide fraction increased serum levels of IL-6 and monocyte chemoattractant protein-1.
- The active *Combretum* polysaccharide fraction effectively agglutinated erythrocytes from human, mouse, and rabbit.
- Our results show that *Combretum* polysaccharides exhibit novel immunomodulatory properties.

Figure 1.
 ^1H NMR spectra of the polysaccharide fractions **CP-AU** and **CP-AB** isolated from *C. racemosum*. The polysaccharide fractions were dissolved in D_2O at concentration 5 mg/ml, and spectra were recorded at 20 °C.

Figure 2. Synchronous fluorescence spectra of the polysaccharide fractions **CP-AU** and **CP-AB** isolated from *C. racemosum*. The polysaccharide fractions were dissolved in H₂O at concentration 250 µg/ml, and spectra were recorded at 20 °C.

Figure 3.

Effect of *Combretum* polysaccharide fractions **CP-AU** and **CP-AB** on cytokine production by human PBMC. PBMC were incubated for 24 hr with 250 μ g/ml of the indicated fractions or 500 ng/ml LPS (positive control), and production of cytokines in the supernatants was evaluated using a MultiAnalyte ELISArray kit. Cytokine expression is shown as fold increase (FI) compared to background measured in supernatants from cells treated with media alone. The data are presented as mean $\pm$ SD of duplicate samples from one experiment that is representative of two independent experiments. Statistically significant differences (* $p < 0.05$, or ** $p < 0.01$) between untreated cells (media alone) and cells treated with fraction **CP-AU** or LPS are indicated.

Figure 4. Effect of the *Combretum* polysaccharide fractions **CP-AU** and **CP-AB** on TNF- α production. Human PBMC (upper panel) or MonoMac6 cells (lower panel) were incubated for 24 hr with the indicated concentrations of **CP-AU** (●), **CP-AB** (○), 200 ng/ml LPS (positive control, ■), or 500 μ g/ml **CP-AU_{ER}** (▲). Cell-free supernatants were collected, and extracellular TNF- α was quantified by ELISA. Values are the mean $\pm$ S.D. of triplicate samples from one experiment, which is representative of three independent experiments. Statistically significant differences (* $p < 0.05$, ** $p < 0.01$) between untreated cells (media alone) and cells treated with fractions **CP-AU**, **CP-AB**, or LPS are indicated.

Figure 5.

Effect of the *Combretum* polysaccharide fractions **CP-AU** and **CP-AB** on IL-6 production. Human PBMC (upper panel) or MonoMac6 cells (lower panel) were incubated for 24 h with the indicated concentrations of **CP-AU** (●), **CP-AB** (○), 200 ng/ml LPS (positive control, ■), or 500 μg/ml **CP-AU_{ER}** (▲). Cell-free supernatants were collected, and extracellular IL-6 was quantified by ELISA. Values are the mean ± S.D. of triplicate samples from one experiment, which is representative of three independent experiments. Statistically significant differences (* $p < 0.05$, ** $p < 0.01$) between untreated cells (media alone) and cells treated with fractions **CP-AU**, **CP-AU_{ER}**, **CP-AB**, or LPS are indicated.

Figure 6.

Effect of TLR4 antagonist LPS-RS on TNF- α production by human PBMC induced with *Combretum* polysaccharide fraction **CP-AU**. PBMC were pretreated with indicated concentrations LPS-RS for 30 min, followed by treatment with media (negative control, □), polysaccharide fraction **CP-AU** (250 μ g/ml, ○), or LPS (200 ng/ml, ●) for 24 hr, and extracellular TNF- α was quantified by ELISA. Values are the mean $\pm$ S.D. of triplicate samples from one experiment, which is representative of three independent experiments. Statistically significant differences (* $p < 0.001$) between untreated cells (media alone) and cells pretreated with LPS-RS and agonist (**CP-AU** or LPS) are indicated.

Figure 7.

Effect of the *Combretum* polysaccharide fraction **CP-AU** on MAPK phosphorylation. PBMC were incubated for 30 min with 500 μ g/ml of fraction **CP-AU**, and levels of kinase phosphorylation were evaluated using a phospho-MAPK array kit. The data are presented as mean $\pm$ SD of duplicate samples from one experiment. Statistically significant differences (* $p < 0.05$, ** $p < 0.01$) between untreated cells (media alone) and cells treated with fraction **CP-AU** are indicated.

Figure 8.

Effect of the *Combretum* polysaccharide fractions **CP-AB** and **CP-AU** on AP-1/NF-κB activation. Human THP-1Blue monocytes (2×10^3 cells/well) were incubated for 24 hr with 250 μg/ml of fractions **CP-AB** and **CP-AU** or 200 ng/ml LPS (positive control). Alkaline phosphatase activity was analyzed spectrophotometrically (absorbance at 655 nm) in the cell supernatants, as described. Values are the mean $\pm$ S.D. of triplicate samples from one experiment, which is representative of three independent experiments. Statistically significant differences (* $p < 0.01$, ** $p < 0.001$) between untreated cells (media alone) and cells treated with fractions or LPS are indicated.

Figure 9.

Effect of the *Combretum* polysaccharide fraction **CP-AU** on serum cytokine levels *in vivo*. Balb/c mice were treated by intraperitoneal injection with fraction **CP-AU** (single dose 250 mg/kg in 100 μ l of PBS), LPS (single dose 0.5 mg/kg in 100 μ l of PBS), or PBS (100 μ l) as described. After 2 hr, the mice were sacrificed, and serum was collected. Cytokine levels in the serum were measured using a MultiAnalyte ELISArray kit. The cytokine levels are shown as fold increase (FI) compared to background levels measured in supernatants from PBS treated mice. The data are presented as mean $\pm$ S.D. of duplicate samples from one experiment that is representative of two independent experiments. Statistically significant differences (* $p < 0.05$, ** $p < 0.01$) between mice treated with PBS and fraction **CP-AU** or LPS are indicated.

Figure 10.

Effect of the *Combretum* polysaccharide fractions **CP-AU** and **CP-AB** on cell viability. PBMC were incubated for 24 hr with the indicated concentrations of **CP-AU** (●), **CP-AB** (○), and cell viability was determined using a luminescent cell viability assay kit, as described. Values are the mean $\pm$ S.D. of triplicate samples from one experiment that is representative of two independent experiments. No statistically significant differences between untreated cells (media alone) and cells treated with fractions **CP-AU** or **CP-AB** were found.

Table 1Chemical properties and molecular weights of *Combretum* polysaccharide fractions

Fractionation and Fraction Name		Mol. Weight (kDa)	Arabinogalactan Type II	Protein Content (%)	Polyphenol Content (%)
DEAE Cellulose	Diaion HP-20				
CP-A (parent fraction)	CP-AB	4.7	Negative	2.50 ± 0.20	6.08 ± 0.31
	CP-AU	5.0	Positive	0.15 ± 0.014	3.03 ± 0.08

Table 2Sugar composition of *Combretum* polysaccharide fractions

Monosaccharide	CP-AU	CP-AB
	Mol %	
Galacturonic Acid	31.6	7.8
Arabinose	29.1	22.6
Galactose	17.7	35.0
Rhamnose	8.1	4.4
Glucose	5.6	16.0
Mannose	4.3	6.8
Glucuronic Acid	2.2	1.3
Fucose	1.1	1.2
Xylose	0.4	4.2
Lyxose	0	0
Glucosamine	0	0.8

Table 3
Specific hemagglutinating activity induced by polysaccharide fractions isolated from different plants

Plant	Polysac- charide Fraction ^a	Molecular Weight (kDa)	Arabino- galactan Type II	Protein Content (%)	Polyphenol Content (%)	Hemagglutinating Activity (μg/ml) ^b
<i>Combretum racemosum</i>	CP-AU	5.0	Positive	0.15	3.0	155
<i>Combretum racemosum</i>	CP-AB	4.7	Negative	2.5	6.1	N.A.
<i>Opuntia polyacantha</i>	C-I	733	Negative	2.0	-	625
<i>Opuntia polyacantha</i>	C-IV	168	Positive	<1.0	-	625
<i>Artemisia tripartita</i>	A-II	251	Positive	1.0	-	625
<i>Artemisia tripartita</i>	A-III	126	Positive	1.5	-	625
<i>Artemisia tripartita</i>	A-IV	78	Positive	2.0	-	625
<i>Tanacetum vulgare</i>	T-III	64	Positive	7.0	-	800
<i>Euterpe oleracea</i>	Acai-2	26-60	Positive	0.4	2.8	N.A.
<i>Alchornea cordifolia</i>	AP-AU1	39.5	Positive	0.5	2.7	N.A.
<i>Alchornea cordifolia</i>	AP-AU2	5.9	Negative	8.3	15.6	N.A.
<i>Clerodendrum splendens</i>	CS-NU1	60.6	Positive	7.8	0.2	N.A.
<i>Clerodendrum splendens</i>	CS-NU2	5.5	Negative	12.8	6.5	N.A.

^aDetails regarding isolation and characterization of polysaccharide fractions from *O. polyacantha*, *A. tripartita*, *T. vulgare*, and *E. oleracea* were described previously [22, 29-31] or are unpublished (*A. cordifolia* and *C. splendens*).

^bHuman erythrocytes were from donor group O. NA, no agglutination was observed.

Table 4

Effect of enzymatic pretreatment of human erythrocytes on hemagglutinating activity of *Combretum*-derived polysaccharide fractions and reference lectins

Erythrocytes (species) and Treatment ^a	Polysaccharide Fraction		Lectin	
	CP-AU	CP-AB	ConA	WGA
	Hemagglutination Titer ^b			
Mouse erythrocytes, none-treated	16	N.A.	1024	-
Human erythrocytes, none-treated	32	N.A.	256	512
Human erythrocytes, neuraminidase (0.2 mg/ml)	64	N.A.	2048	4096
Human erythrocytes, trypsin (1 mg/ml)	4	N.A.	1024	32768
Human erythrocytes, pronase E (1 mg/ml)	4	N.A.	16384	131072
Rabbit erythrocytes, none-treated	16	N.A.	2048	8192
Rabbit erythrocytes, trypsin (1 mg/ml)	64	N.A.	65536	131072
Rabbit erythrocytes, pronase E (1 mg/ml)	32	N.A.	2048	8192

^aHuman erythrocytes were from donor of group O.

^bHemagglutination titer is reported as the inverse of the last dilution of tested sample (5 mg/ml) with agglutinating activity. N.A., no agglutination was observed.