



Antioxidant and anti-inflammatory activities of *Ganoderma resinaceum* (Boud) fruiting bodies extracts

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ABSTRACT

Introduction: *Ganoderma resinaceum* is used to treat oxidative and inflammatory-related diseases such as cardiovascular and liver diseases. Thus, this study aimed to evaluate the antioxidant and anti-inflammatory activities of different extracts from *G. resinaceum* fruiting bodies.

Methods: Aqueous crude (GRT), mycelial (MYC), exopolysaccharide (EPS I, EPS II) and water-soluble polysaccharide-rich (GRP I and GRP II) extracts of *G. resinaceum* were assessed for their free radical scavenging and metal chelating ions assays. The *in vitro* anti-inflammatory activity was evaluated by stabilization of erythrocytes' membranes and protein denaturation assays. For the *in vivo* study, paw oedema was induced by administration of κ -carrageenan (0.1 mL; 1%) to male Wistar rats aged 4 to 6 weeks. Animals were pre-treated with *G. resinaceum* extracts (125 mg/kg) and diclofenac sodium (20 mg/kg). Inflammatory cytokine and chemokine levels were determined, and histological analysis of paw tissue was performed.

Results: *G. resinaceum* polysaccharide-rich extracts (GRP I and GRP II) showed the best bioactivities. They scavenged DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2-azino-bis-3-ethylbenzylthiazoline-6-sulfonic acid, and NO (nitric oxide) radicals, and chelated ferrous ions, stabilized murine erythrocyte membranes, and inhibited protein denaturation. At 125 mg/kg, GRP I and GRP II restored the microarchitecture with a weak infiltration of immune cells in the subcutaneous tissues. Moreover, they decreased the overproduction of proinflammatory cytokines growth colony-stimulating factor (G-CSF), interferon gamma (IFN γ), tumour necrosis factor alpha (TNF α), chemokines (eotaxin, fractalkine) and increased the levels of anti-inflammatory cytokines (IL-10, IL-12p70).

Conclusion: *G. resinaceum* polysaccharide extracts could be potent antioxidant and anti-inflammatory agents.

Implication for health policy/practice/research/medical education:

Research on safe and novel potential antioxidant and anti-inflammatory agents from medicinal plants such as mushrooms is necessary to palliate the side effects of anti-inflammatory drugs in long-term use. This article valorises *G. resinaceum* polysaccharide-rich extracts as an alternative phytodrug for this purpose.

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Introduction

Oxidative stress is an imbalance between oxidants and antioxidants in favour of oxidants, leading to a disruption of redox signalling, molecular damage,

mutation, and cell death. Thus, reactive oxygen/nitrogen species are the end products of essential biological processes such as metabolism and respiration, and can initiate an intracellular signalling cascade that enhances

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proinflammatory gene expression (1-3). In the human body, the mechanism of defence against physical, chemical, and biological attacks is ensured by inflammation. The latter is generally characterized by redness and swelling of tissues, which is associated with hyperthermia and pain (4). During the acute inflammatory response, immune cells, including macrophages, lymphocytes, neutrophils, mast and dendritic cells, are recruited reactive oxygen species (ROS), and different inflammatory mediators are secreted to stop the spread of cellular injury (5). Exposure to external factors such as heat and chemicals modifies the tertiary structure and secondary structure of proteins (6). Moreover, lysosomal constituents of activated neutrophils, such as bactericidal enzymes and proteases, cause further tissue inflammation and damage upon extracellular release (7). Therefore, anti-denaturation of proteins and stabilization of human erythrocyte membranes are adopted as possible mechanisms of *in vitro* inflammatory activities.

Carrageenan-induced paw oedema is a well-established model for studying the *in vivo* anti-inflammatory activities of natural or synthetic products. The administration of carrageenan leads to an increase in inflammatory and oxidative stress mediators, including histamine, prostaglandins, pro-inflammatory cytokines/chemokines, neutrophil-derived free radicals, and nitric oxide (8). It is clearly known that oxidative stress and inflammation are cross linked with a number of chronic diseases, including diabetes, hypertension, cardiovascular diseases, neurodegenerative diseases, alcoholic liver disease, chronic kidney disease, cancer, and aging (9). The use of analgesic drugs, nonsteroidal anti-inflammatory drugs, steroidal anti-inflammatory drugs, and opioids is strongly recommended. However, these factors cause deleterious effects on human health in the long run (10). Despite the efficiency of conventional methods to overwhelm these side effects, research on safe and novel potential antioxidant and anti-inflammatory agents from medicinal plants, such as mushrooms, has been carried out. Bioactive compounds isolated from medicinal plants have been widely used for the last decades in South America, Asia, and Africa as part of traditional diets and medicines.

Polysaccharides have been reported to exhibit various biological activities, such as hypoglycaemic, hypolipidemic, antiviral, antitumour, immunomodulatory, antioxidant and anti-inflammatory activities (11-13). Hence, mushroom-derived polysaccharides have particularly attracted much attention in the field of biochemistry and pharmacology to treat the aforementioned diseases (14). One of the most commonly studied and exploited mushrooms for this purpose is lignicolous basidiomycetes belonging to the *Ganodermataceae* family, such as *Ganoderma lucidum* "Reishi". It has been demonstrated that alcoholic and aqueous extracts of *Ganoderma resinaceum* fruiting bodies have hypoglycaemic, hepatoprotective, antibacterial, and antioxidant activities (15-17). This

justifies their use in African and Asian mycotherapy to treat hypoglycaemia and cardiovascular and liver diseases. Due to the seasonal nature of mushroom production, the innocuity of polysaccharides and their solubility in the current extraction solvents, many techniques have been developed for their production and isolation, including liquid fermentation and fed-batch culture (18). Therefore, the present study focused on the comparative investigation of crude, mycelial, water-soluble polysaccharide and exopolysaccharide extracts for their antioxidant and anti-inflammatory activities.

Materials and Methods

Chemicals, reagents, drugs, and kits

Diclofenac sodium (product number: 15307-79-6, molecular formula: $C_{14}H_{10}Cl_2NO_2-Na$) as a white powder packaged in a one gram box, purity $\geq 98\%$, concentration 25 mg/mL) and κ -carrageenan (product number: 11114-20-8, solid-state, white colour, concentration 5 mg/mL) were purchased from Sigma-Aldrich GmbH, Sternheim, Germany. The rat cytokine/chemokine bead panel was supplied from EMD Millipore Corporation (USA). Chemical reagents for antioxidant assays were purchased from GIBCO (Grand Island, NY, USA). All of the chemicals were analytical grade.

Extracts preparation

Crude extract preparation

G. resinaceum fruiting bodies were collected locally and authenticated in comparison to voucher specimen DM 764 at the Laboratory of Mycology of University of Yaoundé 1, Cameroon. The crude extract was obtained according to the modified protocol of Liu et al (19). The mushrooms were cut into small pieces, air-dried in the shade at room temperature and ground. The powder of *G. resinaceum* (100 g) was boiled in distilled water (1:15; w/v) at 100°C for 90 minutes and the solution obtained was cooled at room temperature, then filtered using Whatman paper N° 10. The filtrate was freeze-dried to obtain an aqueous crude extract of *G. resinaceum* (GRT).

Isolation of water-soluble polysaccharides

G. resinaceum polysaccharide-rich extracts were obtained as described by Hua et al (20) with slight modifications. Briefly, the powder (650 g) was sieved and incubated in 6 L of 50% methanol at room temperature for 24 hours. Then, the mixture was filtered using Whatman paper N° 10 and dried at 40°C for 2 hours. The residue in distilled water 1:15 (w/v) was boiled at 80°C for 2 hours and filtered with Whatman paper N° 10. The polysaccharides were precipitated by stepwise addition of ethanol to concentrations of 60% and 80%. After centrifugation (5000 g, 20 minutes), the pellet was re-suspended in distilled water, dialyzed against distilled water (MWCO 14.000), and freeze-dried for further analysis. The polysaccharide-rich fractions precipitated by 60 and 80% were termed

GRP I and GRP II, respectively.

Preparation of mycelial extract

Mycelial extract of *G. resinaceum* (MYC) was obtained with respect to the modified protocol of Kim et al (21). Freshly harvested fruiting bodies were washed and sterilized by soaking basidiocarps three times in 95% ethanol, 3% hydrogen peroxide and distilled water. Then, sterile basidiocarps were inoculated on sterile PDA medium in petri dishes. Mycelia were grown after 07 days of incubation at 30°C. The 5 mm agar disks of mycelium were fermented on MCM by shaking (Excelsa E24) at 150 rpm at 30°C for 4 days. The culture broth was centrifuged (10000 g, 20 minutes), and the pellet collected was re-suspended in distilled water and freeze-dried.

Production and isolation of exopolysaccharides

G. resinaceum was initially grown on Potatoes' Dextrose Agar (PDA) medium in a Petri dish and then transferred into the seed culture medium by punching out 5 mm of the agar plate culture with a house-developed cutter. The precultures were carried out in 250 mL flasks containing 50 mL of MCM pH=6 (20 g/L glucose, 2 g peptone, 2 g yeast extract, 0.46 g KH_2PO_4 , 1 g K_2HPO_4 and 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) at 25°C for 4 days using 4% (v/v) inoculum. The mixture was further transferred into 500 mL shake flasks containing 125 mL of MCM pH=6 composed of glucose: 35 g/L; peptone: 8 g/L, yeast extract: 2 g/L; K_2HPO_4 : 1 g/L; KH_2PO_4 : 0.46 g/L, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.5 g/L. All experiments were performed at least in triplicate to ensure reproducibility. Fermentations were carried out in Mushroom Complete Medium at 150 rpm at 25°C for 14 days on a shaker (Excelsa E24). Culture broths were centrifuged at 10000 g for 20 minutes. The resulting supernatant was then filtered through Whatman N° 10, and the filtrate collected was precipitated by stepwise addition of ethanol to concentrations of 60% and 80%, stirred vigorously and kept overnight at 4°C. The mixtures were separately centrifuged at 5000 g for 15 minutes, and the collected pellets were re-suspended in distilled water. The exopolysaccharide extracts precipitated by 60 and 80% were named EPS I and EPS II, respectively.

Antioxidant assays

Antioxidant activities were determined based on the 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-bis-3-ethylbenzylthiazoline-6-sulfonic acid (ABTS) and nitric oxide radical scavenging activities and as well as the ferrous ion chelating assay.

DPPH scavenging activity

DPPH is a stable free radical characterized by the delocalization of spare electrons over the molecule, but the free radicals formed cannot dimerize like others. The DPPH quenching ability was measured as described by Katalinić et al (22). In each test tube, 0.5 mL of a different

G. resinaceum extract at various concentrations (25-200 µg/mL) was mixed with 0.5 mL of the freshly prepared solution of 400 µmol/L DPPH. After incubation at 25°C for 30 minutes in darkness, the absorbance was read at 517 nm using a UV-1605 Shimadzu spectrophotometer. Ferulic acid was used as standard. The DPPH activity of the extract was determined using the following equation: (%) = $\{[(A_0 - A_1)/A_0] \times 100\}$, where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample.

ABTS scavenging assay

The ABTS free radical scavenging activity was carried out using a previously described method (23). To this effect, 0.5 mL of different concentrations of *G. resinaceum* extract (25 - 200 µg/mL) or the reference compound ferulic acid was added to 0.5 mL of ABTS reagent, the mixture was homogenized and kept in the dark for 30 min at room temperature. The absorbance was then measured at 734 nm using a UV-VIS 1605 Shimadzu spectrophotometer. The ABTS radical scavenging effect of the fraction was calculated using the equation in the preceding section.

Nitric oxide (NO) radical scavenging activity

The Griess reagent was used to quantify nitrite, which is a stable product of NO oxidation (24). In the test tubes, 0.25 mL of different *G. resinaceum* extracts were introduced (25-200 µg/mL) and 1 mL of Sodium Nitroprusside (SNP) (10 mM) was added. The mixture was incubated at room temperature for 90 minutes. Thereafter, 1 mL of this mixture was mixed with 0.5 mL of fresh Griess reagent (1% sulfanilamide, 0.1% naphthylethylene-diamine in 5% HCl, V/V). The violet colour obtained was measured at 540 nm using a UV-VIS 1605 Shimadzu spectrophotometer. The inhibition percentage was calculated using the following formula:

% Inhibition of NO radical = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance before the reaction and A_1 is the absorbance after reaction, taken place with Griess reagent.

Ferrous ions chelating assay

The ferrous metal ion chelating activities of *G. resinaceum* extracts were investigated according to the method of Dinis et al (25) with slight modifications. Briefly, samples at different concentrations (25-200 µg/mL) were introduced in test tubes containing 0.05 mL FeCl_2 (2.0 mM), shaken well, and kept still for 1 min at room temperature. Thereafter, 0.1 mL ferrozine (5.0 mM) was added. The mixture was incubated in the dark for 30 min, and the absorbance was determined at 562 nm. EDTA was used as positive control. The ferrous ion chelating activity was given by the following equation:

Chelating ability (%) = $[(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample.

Stabilization membrane assay

Preparation of erythrocyte suspension

Fresh whole rat blood (10 mL) was collected and transferred to heparinized tubes. The tubes were centrifuged at 2500 g for 30 minutes and washed three times with an equal volume of normal saline. The volume of the blood was measured and reconstituted as a 10% v/v suspension with normal saline.

Heat induced haemolysis

The reaction mixture (2 mL) consisted of 1 mL of *G. resinaceum* extracts at concentrations ranging from 0.5 to 2.5 mg/mL, 1 mL of phosphate buffer 0.15 M (pH 7.4) and 0.5 mL of 10% RBC suspension, instead of drug, only saline was added to the control test tube. Ibuprofen was taken as a standard drug. All centrifuged tubes containing the reaction mixture were incubated at 56°C for 30 minutes. After cooling and centrifugation (2500 g; 20 minutes), the absorbance of the supernatants was measured at 560 nm. The experiment was performed in triplicates. The percentage membrane stabilization activity was calculated by the formula mentioned below:

$$\text{Inhibition of haemolysis (\%)} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the samples.

Denaturation of proteins assay

The inhibitory effects of *G. resinaceum* extracts were assayed as described previously by Mehta et al (26) with slight modifications. Briefly, 0.1 mL of albumin from fresh chicken eggs was mixed with 1.9 mL of phosphate buffered saline (PBS, pH 6.4) and 1 mL of varying concentrations (100-500 µg/mL). Diclofenac sodium at the same concentrations was used as a reference drug. The mixtures were further incubated at 37°C for 20 minutes and heated at 70°C for 5 minutes. Then, the cooling optical density (OD) was read at 660 nm on a UV-1605 Shimadzu spectrophotometer against distilled water as a blank. The percentages of inhibition were determined as follows:

$$\% \text{ Inhibition} = [(O.D._{\text{sample}} - O.D._{\text{control}})/O.D._{\text{control}}] \times 100.$$

κ-Carrageenan induced rat paw oedema

Nine groups of five animals each were constituted of male *Wistar* rats weighing between 150 and 200 g. Paw oedema was induced in the left hind paw of each rat by intraplantar injection of 0.1 mL of 1% κ-carrageenan suspended in saline. One hour prior to carrageenan injection, the rats were pre-treated with either sterile (0.9% NaCl w/v, s.c.), EPS I, EPS II, GRP I, GRP II, MYC, GRT (125 mg/kg, p.o.) or diclofenac sodium (20 mg/kg, p.o.) as a reference. The paw volume of rats was measured by a plethysmometer (Ugo Basile, Varese, Italy) at zero time and then 1, 3, and 5 hours after injection of carrageenan (27). The variations in paw volume were expressed as percentages using the

formula below:

$$\frac{V_c - V_t}{V_c} \times 100$$

where V_c and V_t represent the mean increase in paw volume of the control and treated groups, respectively. The animals were sacrificed after 5 hours of carrageenan challenge by cervical decapitation. Subplantar tissues of the hind paw were removed, rinsed in ice-cold normal saline, and immediately homogenized in NaCl (0.9%) to give a 10% homogenate suspension used for measuring oxidative stress markers. Blood was further withdrawn, and the prepared serum was kept at -80°C to determine inflammatory marker levels.

Preparation of paw tissues for histopathology

The paws collected for histopathological observations were decalcified in 5% nitric acid, processed for paraffin embedding, and sectioned at a thickness of 5 µm with a rotating microtome (Leitz 1512, Marshall Scientific, Hampton, USA). The paw slices were stained with haematoxylin & eosin (H&E) for examination under a light microscope (Axiokop 40). The micrographs were analysed histologically by an independent observer.

Determination of inflammatory mediators' levels

The measurement of cytokines growth colony-stimulating factor (G-CSF), interferon gamma (IFN-γ), tumour necrosis factor (TNF)-α, interleukins (IL)-10, 12p70, and chemokines (eotaxin, fractalkine) in serum and paw homogenates was performed using the Rat Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore Corporation, Massachusetts, USA) according to manufacturer's instructions. Briefly, 25 µL of samples/standards and 25 µL of magnetic beads in solution were added successively to appropriate wells. The plate was incubated for 2 hours at room temperature and washed twice with 1X wash buffer. A second incubation of 1 hour was performed on an orbital shaker at 1000 rpm after adding 25 µL of detection antibodies, and Streptavidin-Phycoerythrin (25 µL) was added. The plate was newly incubated for 30 minutes at room temperature, washed, resuspended in 125 µL of sheath fluid, and read on a MAGPIX® instrument (Luminex, USA).

Statistical analysis

Data are expressed as the mean ± standard error mean (SEM) for each experimental group or treatment. Statistical analysis was performed using GraphPad Prism Software version 5.03 (San Diego, CA, USA) using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test for multiple comparisons. $P < 0.05$ was considered to be significant. Data obtained from cytokines/chemokines level determination were analysed using Luminex xPONENT® multiplex assay software.

Results

Antioxidant activities

The scavenging activity of *G. resinaceum* extracts against DPPH and ABTS revealed a concentration-dependent effect from 25 to 200 µg/mL compared to ferulic acid. GRP I exhibited the highest scavenging activity with EC₅₀ values of 24.20 and 135.7 µg/mL, respectively. Meanwhile, *G. resinaceum* extracts significantly scavenged nitric oxide at the tested concentrations. GRP I showed the best NO scavenging activity, with an EC₅₀ of 97.42 µg/mL.

G. resinaceum extracts were also found to significantly chelate metal ions at all tested concentrations. However, the *G. resinaceum* polysaccharides exhibited the highest antioxidant effects (Table 1).

Erythrocytes stabilizing membranes

G. resinaceum extracts significantly inhibited lysis of rats' erythrocytes membranes within the range of 1.48 ± 1.13% to 74.07 ± 2.71% at the tested concentrations (100-500 µg/mL). GRP I and GRP II displayed the highest inhibition (74.07 ± 2.71 and 69.46 ± 5.16%, respectively) of erythrocyte membrane haemolysis compared to Ibuprofen (93.52 ± 2.97%) at 500 µg/mL. In addition, minimal inhibitory capacity was noticed with mycelial extracts (1.48 ± 1.13%) at 100 µg/mL (Figure 1). The stabilization of erythrocyte membranes suggested preservation of lysosomal membranes because of their structural similitudes.

Anti-denaturation of proteins

The effects of *G. resinaceum* extracts on albumin-induced denaturation are depicted in Figure 2. The results showed a significant inhibitory capacity of protein denaturation ranging from 13.20 ± 2.88% to 64.03 ± 1.17% depending on the concentrations of *G. resinaceum* extracts (100-500 µg/mL). The lowest effects were observed with GRT and MYC (13.20 ± 2.88% and 13.68 ± 1.17%, respectively). However, GRP II exhibited the highest inhibitory percentages of 64.03 ± 1.17 at 500 µg/mL compared to diclofenac sodium (74.17 ± 1.32%) used as a reference drug.

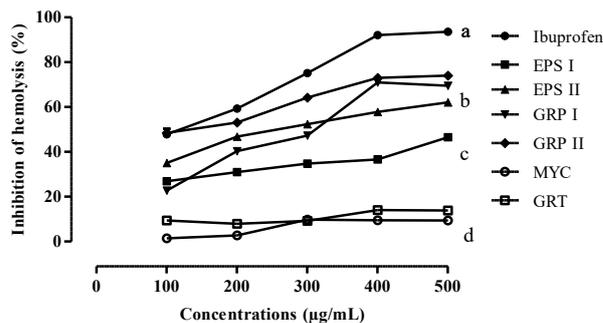


Figure 1. Inhibition of HRBCs membranes' haemolysis (%) by Ibuprofen and extracts of *G. resinaceum* fruiting bodies. Means ± SEM with different superscript letters (a, b, c, d) are significant at *P* < 0.05 using one-way ANOVA test (n= 3). EPS I and EPS II: Animals that received the polysaccharide extracts at a dose of 125 mg/kg + 0.9% NaCl p.o.; GRP I and GRP II: Animals that received the polysaccharide extracts at a dose of 125 mg/kg + 0.9% NaCl p.o.; MYC: Animals that received the mycelial extract at a dose of 125 mg/kg + 0.9% NaCl p.o.; GRT: Animals that received the crude extract of *G. resinaceum* at a dose of 125 mg/kg + 0.9% NaCl p.o.

Effects of *G. resinaceum* extracts on carrageenan-induced paw-oedema

The administration of carrageenan solution induced oedema in hind paw skin. Consequently, carrageenan enhanced the development of the vascular phase of inflammation. Both diclofenac sodium (20 mg/kg) and *G. resinaceum* extracts (125 mg/kg) pre-treatment inhibited the formation of paw edema in a time-dependent manner 1, 3, and 5 hours after carrageenan administration (Table 2). Interestingly, the most pronounced effect was noticed following GRP II administration after 3 hours (96.3 ± 2.95%) (*P* < 0.001), whereas diclofenac sodium exhibited 74.96 ± 3.92% after 5 hours when compared with the negative control group. The highest inhibitory activity of GRP II was confirmed, with the lowest diameter (4.54 ± 0.60 cm) of the hind paw noticed after 3 hours of carrageenan injection (*P* < 0.01). These results highlighted the anti-oedematous actions of *G. resinaceum* extracts at early and late phases of acute inflammation.

Table 1. Extraction yield and antioxidant activities of *G. resinaceum* extracts

	EC ₅₀ (µg/mL)				
	Yield	DPPH	ABTS	Fe ²⁺ chelating ions	NO scavenging
EPS I	0.12 g/L	58.51	153.7	153.8	319.5
EPS II	0.19 g/L	43.50	136.7	153.6	361.7
GRP I	0.2%	24.20	135.7	110.1	97.42
GRP II	0.6%	43.13	107.5	114.8	119.7
MYC	1.5 g/L	129.4	207.8	86.18	664.1
GRT	1.12%	169.1	180.8	99.20	301.0

EC₅₀: Concentration of *G. resinaceum* polysaccharide extracts leading to 50% antioxidant activity; DPPH: 1,1-diphenyl-2-picrylhydrazyl; ABTS: 2,2-azino-bis-3-ethylbenzylthiazoline-6-sulfonic acid; NO: Nitric oxide radical scavenging activities; EPS: Animals that received the polysaccharide extracts EPS I and EPS II at a dose of 125 mg/kg + 0.9% NaCl p.o.; GRP I and GRP II: Animals that received the polysaccharide extracts at a dose of 125 mg/kg + 0.9% NaCl p.o.; MYC: Animals that received the mycelial extract at a dose of 125 mg/kg + 0.9% NaCl p.o.; GRT: Animals that received the crude extract of *G. resinaceum* at a dose of 125 mg/kg + 0.9% NaCl p.o.

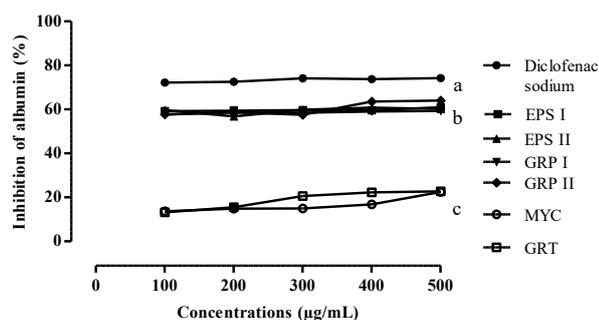


Figure 2. Effects of *G. resinaceum* extracts and diclofenac sodium against protein denaturation. Means \pm SEM with different superscript letters (a, b, c) are significant at $P < 0.05$ using one-way ANOVA test. (n= 3). EPS I and EPS II: Animals that received the polysaccharide extracts at a dose of 125 mg/kg + 0.9% NaCl p.o.; GRP I and GRP II: Animals that received the polysaccharide extracts at a dose of 125 mg/kg + 0.9% NaCl p.o.; MYC: Animals that received the mycelial extract at a dose of 125 mg/kg + 0.9% NaCl p.o.; GRT: Animals that received the crude extract of *G. resinaceum* at a dose of 125 mg/kg + 0.9% NaCl p.o.

Effects of *G. resinaceum* extracts on histopathological changes in paw tissues.

The representative histopathology of paw tissues of the experimental groups is illustrated in Figure 3. The paw tissues of carrageenan-injected rats revealed epithelial hyperplasia and infiltration of inflammatory cells, whereas the normal group showed an absence of these signs. The different treatment-based *G. resinaceum* extracts protected

paw tissues against these malformations. Likewise, the anti-inflammatory response of GRP-treated rats at 125 mg/kg was close to that exerted by diclofenac sodium at 20 mg/kg.

Effects of *G. resinaceum* extracts on cytokine/chemokines release.

To further investigate the mechanism of action of *G. resinaceum* extracts in inflammation, pro-inflammatory (G-CSF, TNF α), anti-inflammatory (IL-10, IL-12p70), and chemokines such as eotaxin and fractalkine were determined using the multiplex Luminex technique. The results are presented in Figures 4 and 5. Carrageenan injection induced a significant increase in G-CSF and TNF α levels in both serum and subcutaneous tissue compared to the normal group ($P < 0.001$). In contrast, the treatments with GRP (125 mg/kg) and diclofenac sodium (20 mg/kg) induced a significant depletion of those pro-inflammatory cytokines ($P < 0.001$; $P < 0.01$) in both serum and paw tissue in comparison with the negative control group. On the other hand, there was significant decreases in IL-10 ($P < 0.01$; $P < 0.05$) and IL-12p70 ($P < 0.05$; $P < 0.001$) in the negative control group compared with the normal control group. GRP and diclofenac sodium induced an increase in IL-10 and IL-12p70 levels compared to the negative control group. Carrageenan administration also provoked a significant increase in eotaxin ($P < 0.05$; $P < 0.001$) and fractalkine ($P < 0.01$) levels in both serum and paw tissues

Table 2. Acute anti-inflammatory effects of *G. resinaceum* extracts and diclofenac sodium on carrageenan induced paw oedema in rats

Groups	Time (h)			
	Hind paw diameter (cm)			
	0	1 h	3 h	5 h
	Oedema inhibition (%)			
NOR	4.31 \pm 0.46	4.31 \pm 0.46**	4.31 \pm 0.46**	4.31 \pm 0.46*
NC	3.97 \pm 0.16	5.28 \pm 0.28	5.63 \pm 0.32	5.14 \pm 0.17
PC	4.13 \pm 0.10	5.21 \pm 0.09	5.17 \pm 0.28	4.56 \pm 0.19
EPS I	4.59 \pm 0.44	5.76 \pm 0.52	5.88 \pm 0.26	5.19 \pm 0.37
EPS II	4.76 \pm 0.29	5.71 \pm 0.37	5.56 \pm 0.43	5.17 \pm 0.29
GRP I	4.27 \pm 0.41	5.07 \pm 0.32**	5.11 \pm 0.50**	4.54 \pm 0.45*
GRP II	4.21 \pm 0.59	5.00 \pm 0.57**	4.91 \pm 0.77**	4.54 \pm 0.6**
MYC	4.35 \pm 0.17	5.48 \pm 0.23*	5.32 \pm 0.18**	4.79 \pm 0.28*
GRT	4.03 \pm 0.25	5.19 \pm 0.34**	4.99 \pm 0.21**	4.34 \pm 0.20**

Data are presented as mean \pm standard error followed by oedema inhibition percentage. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to the Negative Control. #### $P < 0.001$; ### $P < 0.01$ and # $P < 0.05$ compared to the positive control. **NOR:** Normal control (animals that received only 0.9% NaCl p.o.); **NC:** Negative Control (animals that received 100 μ L of 1% κ -Carrageenan solution + 0.9% NaCl i.p.); **PC:** Positive Control (animals that received diclofenac sodium at 20 mg/kg + 0.9% NaCl p.o.); **EPS:** Animals that received the polysaccharide extracts EPS I and EPS II at a dose of 125 mg/kg + 0.9% NaCl p.o.; **GRP:** Animals that received the polysaccharide extracts GRP I and GRP II at a dose of 125 mg/kg + 0.9% NaCl p.o.; **MYC:** Animals that received the mycelial extract (MYC) at a dose of 125 mg/kg + 0.9% NaCl p.o.; **GRT:** Animals that received the crude extract of *G. resinaceum* (GRT) at a dose of 125 mg/kg + 0.9% NaCl p.o.

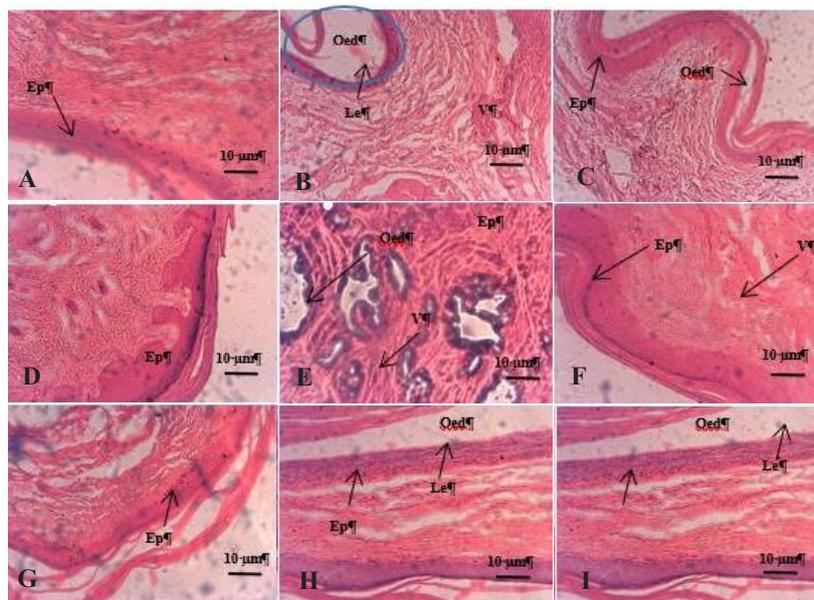


Figure 3. The representative photomicrographs of left hind paw tissue sections from the experimental groups (HE ×400).

The paw tissues of carrageenan-injected rats revealed epithelial hyperplasia and infiltration of inflammatory cells, whereas the normal group showed an absence of these signs. The different treatment-based *G. resinaceum* extracts protected paw tissues against these malformations. Likewise, the anti-inflammatory response of GRP-treated rats at 125 mg/kg is close to that exerted by diclofenac sodium at 20 mg/kg.

NOR: Normal Control (animals that received only 0.9% NaCl p.o.); **NC:** Negative Control (animals that received 100 µL of 1% κ-carrageenan solution + 0.9% NaCl i.p.); **PC:** Positive Control (animals that received diclofenac sodium at 20 mg/kg + 0.9% NaCl p.o.); **EPS:** Animals that received the polysaccharide extracts EPS I and EPS II at a dose of 125 mg/kg + 0.9% NaCl p.o.; **GRP:** Animals that received the polysaccharide extracts GRP I and GRP II at a dose of 125 mg/kg + 0.9% NaCl p.o.; **MYC:** Animals that received the mycelial extract (MYC) at a dose of 125 mg/kg + 0.9% NaCl p.o.; **GRT:** Animals that received the crude extract of *G. resinaceum* (GRT) at a dose of 125 mg/kg + 0.9% NaCl p.o.; Ep: Epidermis; Le: Leucocytes; Oe: Oedema; V: Congested vessels swollen with erythrocytes.

in comparison with the normal groups. Meanwhile, the reference drug and *G. resinaceum* extracts caused a significant increase of these chemokines compared with the negative control group.

Discussion

We herein investigated the antioxidative and anti-inflammatory activities of *G. resinaceum* extracts. To achieve this goal, 06 extracts [Crude extract (GRT), Water polysaccharide extracts (GRPI and GRPII), Mycelial extract (MYC) and exopolysaccharide extracts: (EPS I and EPS II)] were obtained with different yields. In fact, the extraction yield of polysaccharides depends on the nature of the solvent (acidic, neutral, alkaline), number of repetitions, solute-to-solvent ratio, particle size, temperature, and extraction time (28). Moreover, liquid fermentation of a substrate can be influenced by the environment, pH of the medium, and nature of the culture medium (18,20).

In most living organisms, metabolism ensures the production of energy by the oxidation of organic compounds necessary for vital functions at the cellular level. Despite the importance of oxidations, the latter can, if uncontrolled, generate ROS, which in excess levels are harmful to human health (29). Biocompounds derived from mushrooms as polysaccharides can be exploited as antioxidants to protect our bodies from oxidative damage

by diverse mechanisms, including radical scavenging, reductive capacity, prevention of chain initiation, and binding to the transition metal ion catalysts. DPPH, ABTS, NO[•] scavenging and metal chelation assays are widely used to determine the total antioxidant activities of single compounds or complex mixtures of various plants (30). In the current study, *G. resinaceum* polysaccharide extracts (GRP I and GRP II) showed higher free radical scavenging and nitric oxide quenching properties than crude, mycelial, and exopolysaccharide extracts. As a free radical, NO is implicated in the physiology and pathology of many systems (31,32). Metal ions accelerate lipid peroxidation by breaking down hydrogen and lipid peroxides formed by the Fenton free radical reaction. The *G. resinaceum* polysaccharide extracts could exert their antioxidant activities through hydrogen-donating ability to form more stable radicals, regulating NOS activity to inhibit nitrite formation and chelating metal ions (33-35). The potent *in vitro* antioxidant activities of *G. resinaceum* polysaccharide fractions could be attributed to their chemical content since phenolic compounds (data not shown) are known as antioxidant compounds (36,37). Oxidative stress can induce inflammation through diverse pathways including activation of the transcription factor NF-κB and NOD-like receptor protein 3 inflammasome leading to the maturation of pro-inflammatory cytokines and chemokines (38).

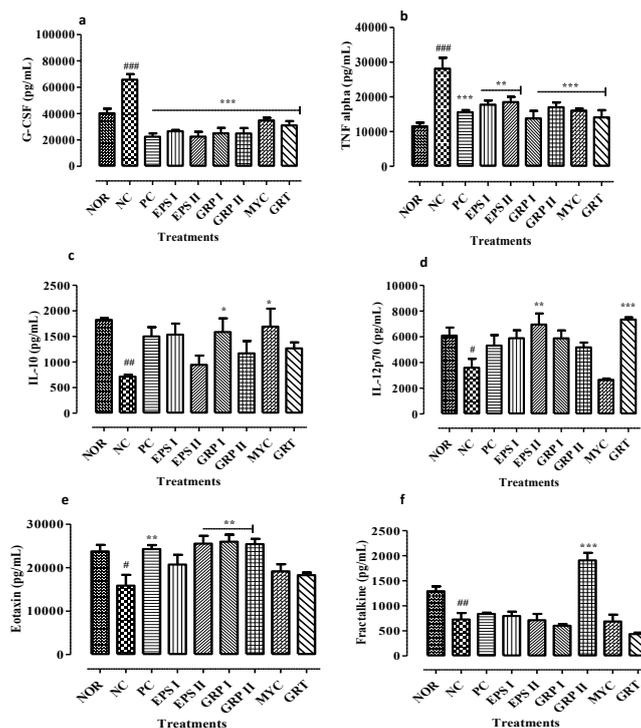


Figure 4. Effects of *G. resinaceum* extracts (125 mg/kg) or diclofenac sodium (20 mg/kg) on serum cytokines (G-CSF, TNF- α , IL-10, IL-12p70) and chemokines (Eotaxin and Fractalkine) levels in carrageenan induced paw oedema in Wistar rats.

(a) G-CSF: Growth Colony Stimulating Factor; **(b) TNF- α :** Tumour Necrosis Factor-alpha, **(c) IL-10:** Interleukin-10, **(d) IL-12p70:** Interleukin-12p70; **(e) Eotaxin;** **(f) Cytokine/chemokine levels,** which are represented as mean \pm SD ($n = 5$). ### $P < 0.001$, ## $P < 0.01$ compared to normal group, # $P < 0.05$ compared to normal group, * $p < 0.05$ compared to negative control, ** $P < 0.01$ compared to negative control, *** $P < 0.001$ compared to negative control. NOR: Normal control (Animals that received only 0.9% NaCl p.o); NC: Negative control (Animals that received 100 μ L of 1% κ -carrageenan solution + 0.9% NaCl i.p); PC: Positive control (Animals that received diclofenac sodium at 20 mg/kg + 0.9% NaCl p.o); EPS: Animals that received the polysaccharide extracts EPS I and EPS II at a dose 125 mg/kg + 0.9% NaCl p.o.; GRP: Animals that received the polysaccharide extracts GRP I and GRP II at a dose of 125 mg/kg + 0.9% NaCl p.o); MYC: Animals that received the Mycelial extract (MYC) at a dose of 125 mg/kg + 0.9% NaCl p.o.; GRT: Animals that received the crude extract of *G. resinaceum* (GRT) at a dose of 125 mg/kg + 0.9% NaCl p.o.

The *in vitro* anti-inflammatory activities of *G. resinaceum* extracts were evaluated by membrane stabilization and protein denaturation assays. Exposure to substances such as phenyl-hydrazine, methyl salicylate, hypotonic solutions, or heat leads to haemoglobin oxidation and haemolysis (39). Among *G. resinaceum* extracts, the polysaccharide extracts showed the best erythrocyte membrane protective effects against heat-induced haemolysis. This suggests their ability to preserve the integrity of the plasma membrane which is inextricably linked to cell viability. Because the membranes of erythrocytes and lysosomes are structurally similar, the ability to stabilize the erythrocyte membrane may also help protect the lysosome membrane. This mechanism prevents release into serum proteins in tissues that can activate neutrophils and lengthen the inflammatory response (40). Uncontrolled or prolonged activation of inflammation can cause dangerous alterations, such as protein denaturation that produces auto antigens. These are associated with type III hypersensitivity reaction and autoimmune diseases such as rheumatoid arthritis. The possible mechanism of denaturation is the alteration of electrostatic, hydrogen, hydrophobic, and disulfide bonds

that maintain the three-dimensional structure of proteins (41). The strong inhibitory activity of the *G. resinaceum* polysaccharide extracts found in this study could be explained by the interaction of some components as polysaccharides with two sites present in some proteins such as ovalbumin of tyrosine-, threonine- and lysine-rich bonds. The polysaccharides of *G. resinaceum* would therefore be able to control the production of auto-antigens by inhibiting protein denaturation.

Carrageenan is a phlogogenic agent whose administration leads to a biphasic inflammatory reaction. The initial or early phase lasts 90 minutes after injection of carrageenan and is characterized by the release of histamine, serotonin and bradykinin. The late phase can last up to 5 hours after the injection of carrageenan and is characterized by neutrophil infiltration and the release of prostaglandin mediated by cyclooxygenases. These mediators increase capillary permeability; thus, an exudate is formed that is responsible for the oedema that compresses the nerves and causes the sensation of pain (42,43). In this experiment, *G. resinaceum* polysaccharide extract (125 mg/kg) showed very strong anti-oedema activity 3 hours after injection of carrageenan.

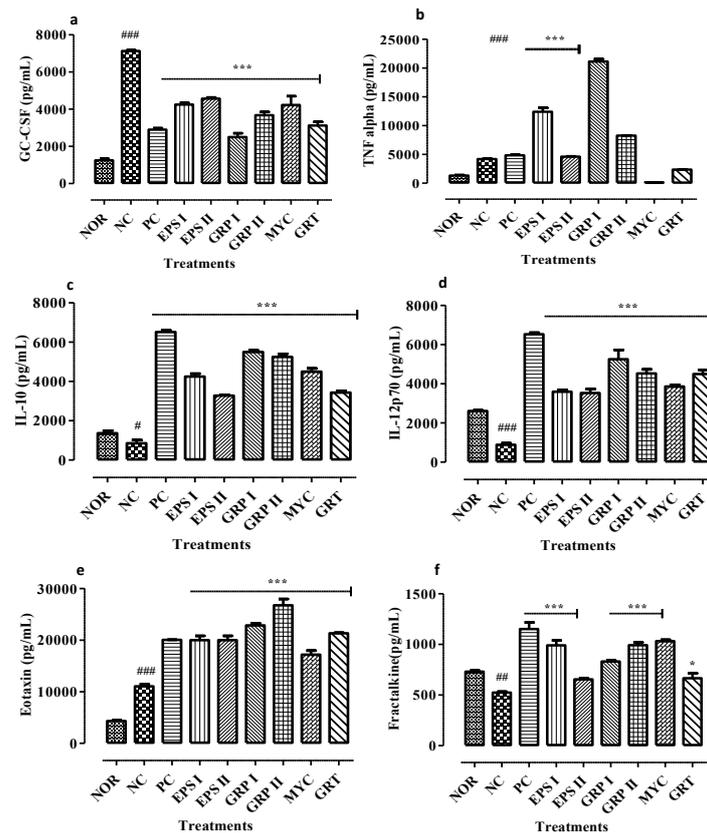


Figure 5. Effects of *G. resinaceum* extracts (125 mg/kg) or diclofenac sodium (20 mg/kg) on subcutaneous plantar tissue cytokines (G-CSF, TNF- α , IL-10, IL-12p70) and chemokines (eotaxin and fractalkine) levels in carrageenan induced paw oedema in Wistar rats.

(a) G-CSF: Growth Colony Stimulating Factor; **(b) TNF- α :** Tumour Necrosis Factor alpha, **(c) IL-10:** Interleukin-10, **(d) IL-12p70:** Interleukin-12p70; **(e) Eotaxin;** (f) Cytokine/chemokine levels, which are represented as mean \pm SD (n = 5). ### P < 0.001 ## P < 0.01 compared to Normal group, # P < 0.05 compared to Normal group, * P < 0.05 compared to Negative Control, ** P < 0.01 compared to Negative Control., *** P < 0.001 compared to Negative Control. **NOR:** Normal Control (Animals that received only 0.9% NaCl p.o); **NC:** Negative Control (Animals that received 100 μ L of 1% κ -carrageenan solution + 0.9% NaCl i.p); **PC:** Positive Control (Animals that received diclofenac sodium at 20 mg/kg + 0.9 % NaCl p.o); **EPS:** Animals that received the polysaccharide extracts EPS I and EPS II at a dose 125 mg/kg + 0.9% NaCl p.o.; **GRP:** Animals that received the polysaccharide extracts GRP I and GRP II at a dose of 125 mg/kg + 0.9% NaCl p.o); **MYC:** Animals that received the mycelial extract (MYC) at a dose of 125 mg/kg + 0.9% NaCl p.o.; **GRT:** Animals that received the crude extract of *G. resinaceum* (GRT) at a dose of 125 mg/kg + 0.9% NaCl p.o.

The histological data revealed that carrageenan induction caused acute inflammation characterized by oedema, which was reflected microscopically by pale, poor staining, and distended appearance of the connective tissue. In animals treated with *G. resinaceum* extracts, there was a decrease in the intensity of the infiltrate and a reduction in the size of the follicles corresponding to the repair phase, which results in new blood vessels on the surface of the lesions.

The late phase of acute inflammation is also marked by the production of proinflammatory cytokines and chemokines in serum and subcutaneous tissue. *G. resinaceum* extracts significantly decreased the levels of proinflammatory cytokines and chemokines and significantly increased the levels of anti-inflammatory cytokines compared to the negative control group. Several authors have highlighted the anti-inflammatory properties of bioactive compounds in medicinal plants. For example, diallyl disulfide, an organosulfur compound

isolated from garlic inhibits the development of oedema in mice by significantly reducing CRP, TNF- α , IL-1 β , and IL-2, and thus the infiltration and migration of macrophages and leukocytes to the site of injury (44). Betulinic acid significantly reduces serum levels of IL-1 α , IL-1 β , IL-5, IL-6, GM-CSF, KC, MCP-1, and PGE2 and increases those of IL 10 and IL-12 in mice (45). Vinpocetine significantly reduces inflammatory pain by regulating oxidative stress and pro-inflammatory cytokine production through the NF κ -B pathway in mice (46). Pro-inflammatory cytokines (G-CSF, TNF- α , IL-1 β , IL-2, etc) are produced by immune (macrophages, monocytes) and non-immune (fibroblasts, endothelial cells) cells in response to tissue attack (47). TNF- α stimulates the secretion of G-CSF, IL-1 β , kinins, and leukotrienes that regulate and control nociception during the late phase. TNF- α is also involved in the regulation of other pro-inflammatory cytokines, expression of adhesion molecules on the surface of neutrophils, endothelial cells, activation and chemotaxis

of leukocytes (48). Chemotaxis of immune cells at the site of inflammation is mediated through chemokines such as eotaxin and fractalkine. Thus, in the test animals, the low level of eotaxin in the serum and subcutaneous tissue reflects a low infiltration of eosinophils at the site of inflammation. These cells are capable of producing ROS, pro-inflammatory cytokines, and granular proteins with high cytotoxic potential. Similarly, fractalkine is a chemoattractant of monocytes, T cells, and natural killers under the induction of proinflammatory cytokines (49,50). These anti-inflammatory activities are greater in the groups treated with GRP I and GRP II, suggesting that the polysaccharide fractions of *G. resinaceum* would exert their anti-inflammatory activities *in vivo* by inhibiting the synthesis and production of prostaglandins and lysosomal enzymes and by regulating the levels of cytokines and chemokines, thus preventing massive invasion of immune cells at the site of inflammation.

Conclusion

The aim of this study was to evaluate the antioxidant and anti-inflammatory activities of *G. resinaceum* extracts. *G. resinaceum* polysaccharides exhibited antiradical, NO scavenging, and ferrous chelating ions activities at concentrations ranging from 25 to 200 µg/mL. It was also found that *G. resinaceum* polysaccharide extracts exhibited remarkable membrane protective effects on murine erythrocyte membranes and inhibit protein denaturation at concentrations of 100 to 500 µg/mL. Moreover, at a dose of 125 mg/kg, GRP I and GRP II inhibited the development of oedema induced by injection of carrageenan in the left hind paw of the animal. This anti-oedematous activity was confirmed by the restoration of the microarchitecture and a weak infiltration of immune cells in the subcutaneous tissues. Biochemically, GRP I and GRP II significantly inhibited the overproduction of proinflammatory cytokines (G-CSF, TNF-α), chemokines (eotaxin, fractalkine) and significantly increased the levels of anti-inflammatory cytokines (IL-10, IL-12p70). Therefore, *G. resinaceum* polysaccharide-rich fractions would be good candidates for the development of new therapies against oxidative- and inflammatory-related disorders.

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Authors' contributions

MTKS and BT were involved in the conceptualization and design of the study, MTKS and MKF carried out experiments, interpreted the data, wrote the paper and revised the manuscript, and SNY and MA carried out

experiments. All authors approved the final version for publication.

Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

The experimental protocol was approved by the Cameroon Institutional National Ethics Committee (Ref. number FWA-IRD 0001954). The handling of animals and experiments were performed with respect to the principles and procedures of the European Union Animal Care (CEE Council 86/609) guidelines adopted by the Cameroon Institutional National Ethics Committee.

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