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Antioxidant and immunomodulatory potential of cell wall polysaccharides from *Harungana madagascariensis* Lam

Fils Armand Ella [1], Thaddée Boudjeko [2] and Frédéric Nico Njayou [1,*]

[1] *Laboratory of Pharmacology and Toxicology, Department of Biochemistry, Faculty of Science, University of Yaounde 1, Yaounde, Cameroon.*
[2] *Laboratory of Phytoprotection and Valorisation of Plants Resources, Biotechnology Centre-Nkolbisson, Yaounde, Cameroon.*

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Abstract

Numerous plant polysaccharides have demonstrated immunomodulatory and antioxidant potentials, and can be investigated as novel molecules with biological properties that may enhance immune function. The aim of this study was to assess the antioxidant and immunomodulatory properties of cell wall polysaccharides fractions isolated from *Harungana madagascariensis* (HM). Pectin fraction (PF) and hemicellulose fraction (HCF) were extracted and tested for their antioxidant properties using reducing power, 1,1-diphenyl-2-picryl hydrazyl (DPPH) and 2,2′-azino-bis-3-éthylbenzylthiazoline6-sulphonic acid (ABTS) radical scavenging assays, chelating activity and inhibition of lipid peroxidation. Their immunomodulatory activities were carried out on peritoneal macrophages targeting NADH oxidase and lysosomal enzyme activities as well as the production of nitric oxide (NO) and hydroperoxides (H_2O_2) during phagocytosis of *Saccharomyces cereviceae*. The polysaccharides fractions showed significant antioxidant and immunostimulatory activities via stimulation of NADH oxidase and lysosomal enzyme of peritoneal macrophages and production of NO and H_2O_2. Taken together, the results of this study indicate the potential of polysaccharides from *H. madagascariensis* as antioxidant and immunomodulator agents.

Keywords: *Harungana madagascariensis*; Pectin fraction; Hemicellulose fraction; Antioxidant; Immunomodulation

1. Introduction

Polysaccharides are natural macromolecules consisting of multiple monosaccharides units. They represent a structurally diverse class of macromolecules that are widely distributed in nature and play an important role in controlling cell division, regulating cell growth and maintaining normal metabolism of living organisms. Polysaccharides of higher plants are a potential source of pharmacologically active compounds. Numerous studies have shown that polysaccharides isolated from medicinal plants could affect the immune responses both *in vivo* and *in vitro* and have the potential of being immunomodulators [1, 2].

An imbalance between oxidized cellular components and antioxidants, favoring the oxidized components, results in the phenomena of oxidative stress, which can have catastrophic repercussions including cancer and metabolic illnesses. It has been demonstrated that some plants polysaccharides may possess antioxidant properties, including those of free radical scavengers, metal chelators, lipid peroxidation inhibitors, and DNA damage reduction caused by $H_2O_2/FeCl_2$. They also have hypoglycemic, hypolipidemic, immunomodulatory effects, activate macrophages and promote lymphocyte proliferation [3, 4].

* Corresponding author: Frédéric Nico Njayou

Immunity is the ability of the body to recognize and fight off a variety of diseases as well as potentially harmful bacteria. The immune system employs a wide variety of defensive techniques to control and eliminate noxious bacteria and poisons. Immunomodulators are substances that effectively collaborate with the immune system to speed up and slow down specific aspects of the host response [5].

To find functional foods or an alternative for the treatment of many diseases, including malaria, atherosclerosis and cancer inflammation, hypertension, wounds, scabies, rashes, abscesses, diseases associated with cellular degeneration, and healing defects, the discovery and evaluation of novel and safe polysaccharides from plants has become a popular research topic. Oxidative stress and a deficient immunological response are typically used in the cell to assess these different diseases [5].

Harungana madagascariensis (Hypericaceae) is a specie widely distributed throughout inter-tropical regions of Africa. At maturity, the small, drupe-like fruits are either yellow or red. The leaves and stem barks are used as herbal remedies in traditional African medicine to cure anemia, malaria, and skin conditions [6]. There have also been reports of additional qualities, such as anti-trichomonal, anti-diarrheal, and spasmolytic capabilities. The leaves of *H. madagascariensis* are frequently employed as traditional medicines in Cameroon to treat typhoid fever [7]. Previous study done in our lab show that the plant extract displays anti-aging potential [8].

Many studies have demonstrated that polysaccharides derived from higher plants possess high antioxidant and immunomodulatory properties. These compounds have been shown to produce a range of responses by interacting with immune cells such as an increase in macrophage cytotoxic activity against tumour cells and microorganisms, activate phagocytic activity, and enhance secretion of cytokines and chemokines [4, 9].

Although, polysaccharides isolated from *H. madagascariensis* provide options for potential drug candidates, and no data currently exist on its immunomodulatory and antioxidant effects. The objective of this study was to assess the antioxidant and modulatory effects of polysaccharides fractions of *H. madagascariensis*.

2. Material and methods

2.1. Materials

Plant material: Stem barks of *Harungana madagascariensis* were collected in the Region of Centre, Yaounde, Cameroon and identified by the Cameroon National Herbier as a voucher specimen (Voucher number 4224YA).

Chemicals: TPTZ, HCl, iron II chloride, sodium acetate, DPPH, ferrozine, ABTS, potassium permanganate, hydrogene peroxide, starch, sodium chloride, Giemsa, Trypan bleu, NBT, *Saccharomyces cerevisiae*, DMEM, methanol, (KOH), DMSO, Triton X100, p-nitrophenyl phosphate, boric acid, citric acid, xylenol orange, iron chloride III, sodium nitrite, ammonium sulfate, potassium chloride, (BHT) sulfanilamide, naphtylethylene chloride, NaOH, PBS, trichloroacetic acid, thiobarbituric acid. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Methods

2.2.1. Extraction of cell wall polysaccharides

The wall was extracted according to the method of Ray et al. [10] with slight modifications. Briefly, 20 g of plant powder were mixed in 100 mL of 85% ethanol for 30 min and heated at 100 °C. The mixture was centrifuged (5000 g, 15 min, 4 °C) and the pellet was dissolved and homogenized in 100 mL of 90% dimethyl sulfoxide for 24 h at room temperature. Then, the residue was mixed with 100 mL of methanol and homogenized for 24 h at room temperature. The mixture was centrifuged (5000 g, 15 min, 4 °C), washed with acetone and the residue was dried at 45 °C.

The cell wall material was subjected to a sequential extraction of the pectin and hemicellulose fractions. Five (5) g of cell material was extracted twice with 0.5% ammonium oxalate at 100 °C for 1 h. Thereafter, the residue was incubated in 1 M KOH overnight at room temperature. The different extracts were centrifuged (5000 g, 15 min, 4 °C), dialyzed against water and lyophilized.

The polysaccharides fractions prepared were pectins fractions (PF) and hemicelluloses fractions (HCF).

2.2.2. Antioxidant activities

FRAP test

The reducing power of the plant fraction was determined according to the method of Benzie and Strain [11]. Briefly, various concentrations of polysaccharide fraction (0.01; 0.1; 1; 10; 100 µg/mL) were mixed with 2000 µL of freshly prepared FRAP reagent [1/10 volume of acetate buffer acetate buffer (pH 3.6; 300 mM), 1 of 10 mM TPTZ (in 400 mM HCl)]. The mixture was incubated for 15 min and absorbance was measured at 593 nm. Ascorbic acid (AA) was used as a standard.

DPPH radical scavenging activity

Radical scavenging activity of each fraction was estimated using a stable DPPH radical ($DPPH^{\bullet}$) assay [12]. A methanolic solution of DPPH (0.4 mM) was mixed with various concentrations (0.01; 0.1; 1; 10; 100 µg/mL) of polysaccharide fraction. After incubation in the dark at room temperature for 30 min, the absorbance was measured at 517 nm. AA was used as a standard. Results were expressed as % radical scavenging activity and calculated using the following formula.

$$\%\text{scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (1)$$

ABTS radical cation scavenging activity

Radical scavenging activity of each fraction was measured using an improved ABTS assay [13]. The ABTS radical cation ($ABTS^{\bullet+}$) stock solution was prepared by the reaction of 7 mM ABTS and 4.9 mM potassium persulfate and then incubated for 16 h in the dark at room temperature. A solution of $ABTS^{\bullet+}$ was mixed to polysaccharide fraction at varying concentrations (0.01; 0.1; 1; 10; 100 µg/mL) for 30 min and the absorbance was immediately measured at 734 nm. AA was used as a standard. Results were expressed as % radical scavenging activity and calculated using the formula (1).

Metal chelating activity

The chelation of ferrous ions by fractions was estimated as described by Kong et al. [12]. Briefly, the polysaccharide fraction (0.01; 0.1; 1; 10; 100 µg/mL) was mixed to a solution of 2 mM $FeCl_2$. The reaction was initiated by the addition of 5 mM Ferrozine, and the mixture was homogenized and left standing at room temperature for 10 min. The absorbance was measured at 562 nm. EDTA was used as a standard. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula (1).

Inhibition of lipid peroxidation

Lipid peroxidation assay was carried out by the method of Su et al. [14]. Briefly 100 µL of polysaccharide fraction (0.01; 0.1; 1; 10; 100 µg/mL) was mixed with 1ml of 10% rat's liver homogenate , followed by addition of 50 µL $FeCl_2$ (0.5 mM) and 50 µL H_2O_2 (0.5 mM). This mixture was then incubated at 37°C for 1 h and 1 mL of trichloroacetic acid 15% and 1 mL of thiobarbituric acid 0.67%. The mixture was heated for 15 min, centrifuged and the absorbance of the supernatant readed at 532 nm for inhibition percentage calculation as per the formula (1). AA was used as a standard.

2.3. Macrophage Isolation

Peritoneal macrophages were isolated from *Wistar albino* rat as described by Yang et al. [15] with slight modifications. Briefly, peritoneal macrophages were harvested from rat, which had been injected intraperitoneally with 5 mL of 2% starch solution freshly prepared, and also with 20 mL of phosphate buffered saline (PBS, pH 7.2) administered 4 days before sterile peritoneal lavage. The collected cells were incubated in DMEM. Cell viability was determined using Trypan blue dye exclusion method while Giemsa was used to characterize cells.

Thereafter, 2 × 10^6 cells/well were seeded and cultured in DMEM containing 10% FBS and 100 U/mL penicillin/streptomycin. For adherence, cells were incubated for 3 h at 37°C in 5% CO_2. Then, the cultures were washed twice with DMEM to remove non-adherent cells prior to the addition of 1 mL of fresh DMEM containing 10% FBS.

2.4. Macrophages phagocytosis assay

The adherent macrophages were pretreated with or without polysaccharide fractions (PF and HCF) (0.01; 0.1; 1; 10; 100 µg/mL) for 4h at 37 °C. Thereafter, 1 mL of cell yeast suspension of *Saccharomyces cerevisiae* (5 x 10^6 cells/mL) was added and incubated at 37 °C for 1h. At the end of the treatment, cells were then subjected to lyse in a buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTANa2, 0.2 M NaCl, 1% Triton X-100) and centrifuged (4 °C, 20 min, 10 000 g). The

supernatant was used to quantify the production of nitrites and peroxydes; and the pellet was used for the assays of enzymes activities (NADH and lysosomal enzyme). The positive control used was lipopolysaccharides.

2.4.1. Activity of NADH oxidase

After treatment, the pellet obtained was incubated with NBT (1 mg/mL) for 60 min at 37 °C in a water bath. Subsequently, the tubes were centrifuged (2000 g; 4 °C; 5 min). The pellet was washed with methanol to remove the unreduced NBT. Then, a solution of KOH and DMSO were successively added in each tube and the absorbance of the solution was measured at 570 nm with a spectrophotometer. The percentage reduction of NBT was calculated.

$$NBT\ Reduction\ (\%) = \frac{\text{Absorbance blank} - \text{Absorbance sample}}{\text{Absorbance blank}} \times 100$$

2.4.2. Activity of lysosomal cellular enzyme

After treatment, the pellet obtained was solubilized with Triton X100, then a solution of p-nitrophenyl phosphate (10 mM) and citrate buffer (0.1 M, pH 5.0) was added. The mixture was incubated for 30 min at 37 °C. Finally, the reaction was stopped by adding borate buffer (0.2 M, pH 9.8). The absorbance was measured at 405 nm and the percentage of lysosomal enzyme activity was calculated.

$$Lysosomal\ enzyme\ activity\ (\%) = \frac{\text{Absorbance sample} - \text{Absorbance blank}}{\text{Absorbance sample}} \times 100$$

2.4.3. Nitrites production

The nitric oxide secreted by macrophages is transformed into nitrite that the amount was measured according to the Griess assay. Briefly, the supernatant of the phagocytosis test was incubated with equal volumes of Griess reagent in the dark for 10 min. The absorbance was measured at 550 nm and sodium nitrate was used for calibration curve construction.

2.4.4. Peroxides production

The reaction mixture consisted of FOX reagent and supernatant of the phagocytosis test solution. After homogenization, the mixture was incubated in a water bath at 37 °C for 30 min. The absorbance was measured at 560 nm and hydrogen peroxide was used for calibration curve construction.

2.5. Statistical analysis

The results of the three replicates were pooled and expressed as Mean ± Standard Deviation. The MedCalc 8.0.0.1 software was used and One-way analysis of variance (ANOVA) with Tukey's test was performed to compare variability between the groups. Significant difference was detected at 95% confidence interval.

3. Results

3.1. Reducing and chelating powers of polysaccharide fractions of HM

The results of the Fe^{3+} reducing abilities of polysaccharides is presented in Figure 1A.The reducing ability was measured by monitoring Fe^{3+} to Fe^{2+} conversion polysaccharides fractions. The formation of Fe^{2+} is determined from the absorbance values at 700 nm at different concentrations. It can be seen that most of the polysaccharides fractions exhibited good reducing power. PF has showed highly significant reducing ability.

Also, the results for the chelating activity of polysaccharides are presented in Figure 1B. The absorbance of the red coloured complex formed in the reaction decreases as the concentration of the polysaccharide-Fe(II) complex increases. Among the polysaccharides fractions studied, PF showed significant Fe^{2+} chelating capacity. The reducing and chelating abilities of PF are less than EDTA used as standard.

3.2. Scavenging abilities of polysaccharide fractions of HM against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals

$ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals scavenging potentials of the polysaccharide fractions of HM are provided in Figures 1 C and D. The results obtained showed that PF and HCF effectively scavenged both radicals. Highly effective radical scavenging abilities were displayed by PF but the activity is less than ascorbic acid (AA).

3.3. Inhibition of lipid peroxidation by polysaccharide fractions of HM

In the lipid peroxidation inhibition assay, the activity of polysaccharide fractions of HM (PF and HCF) against the production of malondialdehyde (MDA) exhibited inhibition. The result is shown in Figure 1 E. PF showed good anti-lipid peroxidation activity but lower than AA.

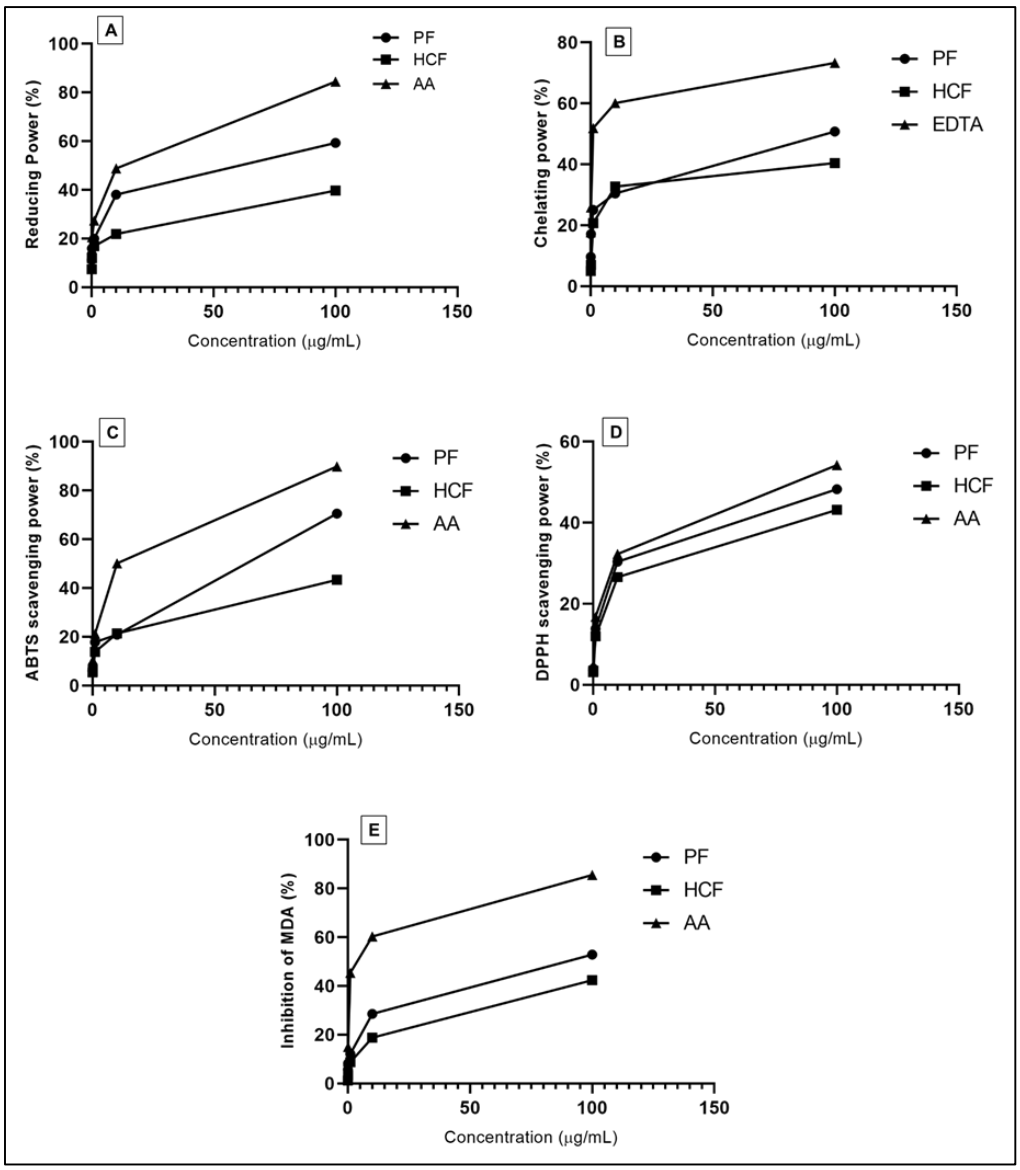

Figure 1 Antioxidant activities of polysaccharides fractions. (A): Reduction power; (B): Chelating ability; (C): Scavenging ABTS radical; (D): Scavenging DPPH radical; (E): Inhibition of lipid peroxidation. PF: pectin fraction; HCF: hemicellulose fraction; AA: ascorbic acid; EDTA: Ethylene diamine tetra acetic acid. Data were expressed as means ± SD of 3 independent experiments

3.4. Effect of polysaccharide fractions on levels of NADH oxidase and lysosomal enzyme

To investigate the effect of polysaccharide fractions on activities of NADH oxidase and lysosomal enzyme, isolated macrophages cells were pretreated with polysaccharides fractions PF and HCF for 4 h before treatment with *Saccharomyces cereviceae* for 1 h, and the activity of enzymes were determined.

The activity of NADH oxidase activity results in the reduction of NBT. PF display highest activity than HCF. This reduction increased significantly ($p<0.05$) at lowest concentrations 0.01 and 0.1 µg/mL (Figure 2A).

The lysosomal enzyme activity was estimated by targeting alkaline phosphatase activity. As the same NADH oxidase activity, PF displays significantly ($p<0.05$) activity than HCF at concentration 1 µg/mL (Figure 2B).

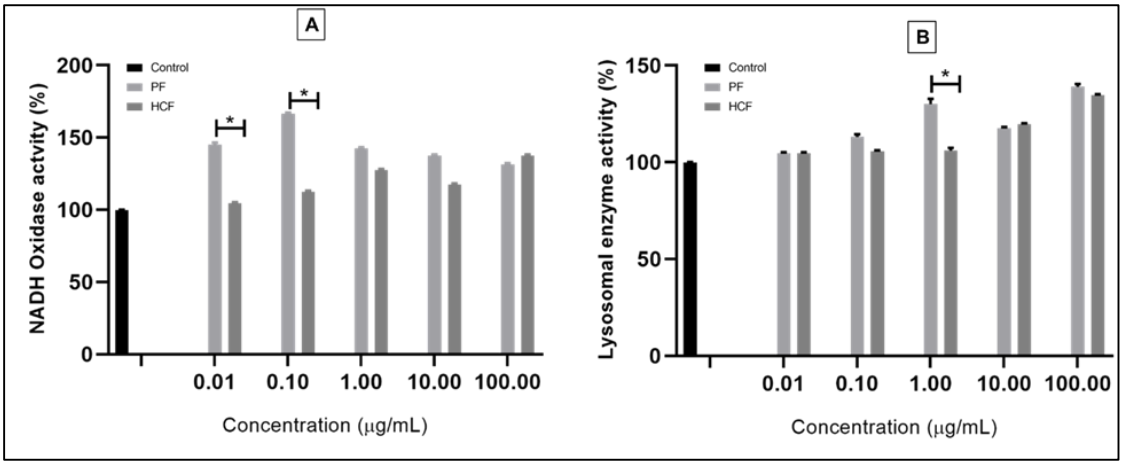

Figure 2 Enzymes Activities during phagocytosis of *Saccharomyces cereviceae* by macrophages isolated and treated with or without polysaccharides fractions. (A): activity of NADH oxydase; (B): activity of lysosomal enzyme. PF: pectin fraction; HCF: hemicellulose fraction. Data were expressed as means ± SD of 3 independent experiments. *p < 0.05

3.5. Effect of polysaccharide fractions on levels of nitrites and peroxides

To investigate whether polysaccharide fractions regulate NO and H_2O_2 production, isolated macrophages were pretreated with polysaccharides fractions PF and HCF for 4 h before treatment with *Saccharomyces cereviceae* for 1 h, and nitrite and peroxides contents were measured. Pretreatment with the polysaccharide fractions at 100 µg/mL and 10 µg/mL displayed the induction of nitrite and peroxides respectively (Figure 3 A-B). The nitrite production was ranged 0–300 mmol/mL and peroxides 0–3800 mmol/mL. The production of NO and H_2O_2 were significantly different ($p < 0.05$) between PF and HCF at 100 µg/mL and 10 µg/mL respectively.

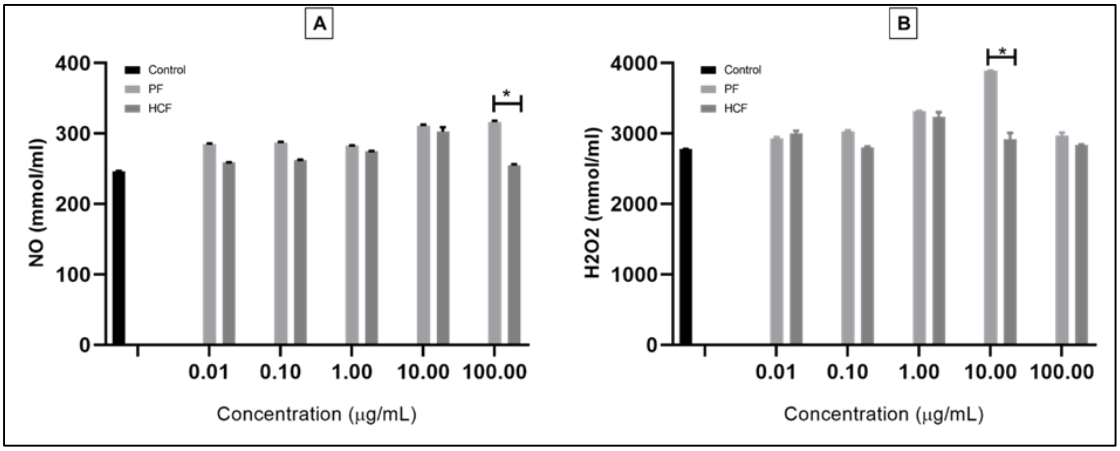

Figure 3 The concentration of mediators released during phagocytosis of *Saccharomyces cereviceae* by macrophages isolated and treated with or without polysaccharides fractions. (A): production of NO; (B): production of H_2O_2. PF: pectin fraction; HCF: hemicellulose fraction. Data are expressed Means ± SD of 3 independent experiments. *p < 0.05

4. Discussion

In this study, polysaccharide fractions were extracted from the bark of *Harungana madagascariensis*, and their antioxidant and immunomodulatory activities were assessed. In recent years, polysaccharides have attracted increasing attention because of their effective antioxidant and immune-modulatory activities. Many studies have shown that polysaccharides improved the activity of antioxidant enzymes, scavenged free radicals, and inhibited lipid oxidation [16]. One of the most important defense systems of macrophages corresponds to their ability to mediate a strong oxidative burst through the formation of reactive oxygen species (ROS). In a normal healthy cell, equilibrium between the generation of ROS and their elimination by the antioxidant system is maintained. ROS is a key regulator of homeostasis, whereas the increased generation of ROS plays an important role in inflammation, host defense response and tissue repair [17, 18]. Antioxidants have the potential to prevent oxidative stress-related illnesses: cancer,

cardiovascular disorders, and neurological disorders. In order to investigate the antioxidant capacity, five antioxidant assays were carried out: ferric reducing antioxidant potential (FRAP), DPPH free radical scavenging activity, ABTS free radical scavenging, ferrous ion chelating ability, and inhibition of lipid peroxidation. The capacity of polysaccharide fractions to supply hydrogen to a free radical was assessed using the DPPH and ABTS radical scavenging activities. The pectine and hemicellulose fractions of *H. madagascariensis* exhibited antioxidant activity but the effect of PF was more than HCF. It has been reported that polysaccharides fractions from *A. floribunda* and *C. odorata* possess highest antioxidant power [19]. Polysaccharides compounds can protect the human body from free radicals, whose formation is associated with the normal natural metabolism of aerobic cells. The antioxidant and antiradical activities of polysaccharides are principally based on the structural relationship between different parts of their chemical structure [20].

By boosting the immune system or regulating the activity of specific macrophages, polysaccharides have the potential to have immunodolatory effects, and be used as alternative medication [4]. Macrophages engulf large molecules by endocytosis while destroying microbes by phagocytosis. In phagocytosis there is generation of bactericidal enzyme like NADH oxidase with increase in the cellular oxygen consumption and glucose metabolism. Pectins showed high stimulation of NADH oxidase, with similar effect on the activity of lysosomal cellular enzyme. Potent immunomodulatory compounds isolated from medicinal plants have been demonstrated a therapeutic effect. Previous studies stated the capacity of plant polysaccharides to interfere with the function of macrophage was responsible for a number of beneficial pharmacological effects. The ethyl acetate extract of *H. madagascariensis* inhibited macrophage activation while exhibiting *in vitro* antiplasmodial activity [21].

Polysaccharides stimulate macrophages to produce pro-inflammatory cytokines and secondary mediatory, such as NO which is an important mediator of the non-specific host defense against invading microbes and tumors. Thus, NO can be used as a quantitative index of macrophage activation. Results of stimulatory effect of polysaccharides fractions of *H. madagascariensis* on macrophages NO production was dose-dependent. Pectins fractions at low concentrations were relatively more potent than hemicelluloses. Thus phagocytosis is an important indicator of macrophage effector activity and it represents the final and most indispensable step of the immunological defense system [22]. The results obtained showed that pectin fraction significantly increased the production of H_2O_2 in comparison with hemicelluloses. Previous studies also reported that polysaccharides have an immunomodulatory effect by promoting phagocytic activity of monocyte-macrophage system [1, 23].

5. Conclusion

The present study has provided evidence that polysaccharides fractions extracted from *H. madagascariensis* possess effective antioxidant activities as typified by the high reducing power, free radical scavenging ability, Fe^{2+} chelating ability, and inhibition of lipid peroxidation. The pectin fractions could be explored as a good immunomodulatory agent on macrophage activity.

Compliance with ethical standards

Acknowledgment

In the memory of the late Professor Boudjeko Thaddée.

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Author contribution statement

All authors participated in the conception and design of the study. FAE and FNJ conducted the study and prepared the first draft of the manuscript. All the authors revised, read and approved the final version of the manuscript.

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