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Research Article

Immunomodulatory activity of methanol extract and fractions from *Borreria verticillata* on macrophages and lymphocytes

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ABSTRACT

Objectives: In recent times, focus on plant research has been intensified all over the world and large amount of evidence has been collected to show the immense potential of medicinal plant used for artisanal utilization and for most pharmaceutical giants. *Borreria verticillata* is a medicinal plant from the West region of Cameroon that is locally used for the management of epilepsy. The present study was designed to explore the effects of *B. verticillata* extract on macrophages and lymphocytes.

Key findings: *B. verticillata* extract and its fractions significantly ($p<0.05$) inhibited the production of nitric oxide from macrophages stimulated by lipopolysaccharides, no cytotoxic effects were noticeable. They decreased macrophage pinocytic activity as indicated by inhibition of neutral red dye uptake. However, the hexane fraction significantly ($p<0.05$) increased destruction of ingested particles at 512 µg/ml as revealed by nitroblue tetrazolium dye (NBT) reduction test. The hexane and ethyl acetate fractions inhibited cellular lysosomal enzyme activity of LPS-activated macrophage. Studied extracts significantly ($p<0.05$) stimulated proliferation of T and B lymphocytes.

Conclusion: The findings suggest that *B. verticillata* extract inhibited macrophages phagocytic activity and stimulate T and B lymphocytes proliferation, thereby influencing immune responsiveness to antigens.

Keywords: *Borreria verticillata*, macrophages, lymphocytes, phagocytic activity, immunomodulation

1. INTRODUCTION

Natural products have been and continue to be a rich source of leading bioactive compounds. Several communities still rely on a hugely arsenal of plant products for health-related problems and for which the active components, mechanisms of actions or even less their effects on the human body are completely unknown[1]. Despite this, it is very obvious, and recognized, that these plant products procure protective and/or curative effects[2,3]. Generally speaking, plant products may exert diverse

effects on the human body, ranging from antimicrobial, anti-inflammatory, antioxidant, immunomodulatory to toxic effects[4-6].

Vertebrates are continually exposed to pathogenic and nonpathogenic organisms and substances of exogenous or endogenous origin that are capable of invading and altering the normal physiological status of the host organism. The immune system plays a central role in the determination of the outcome via the activities of its innate and active components conjunctively[7]. Although the primary role of the immune system is protective, it may become offensive or simply fail to perform its roles as a result of infections or deficiencies as it is the case in HIV/AIDS[8]. Under these circumstances, immunomodulation is almost unavoidable. They could potentiate the activities of the immune system (immunostimulators), or inhibit its activity (immunosuppressors)[9,10]. Modulation of immune system may be an effective means of altering the immune system in favor of the host either by stimulating immune cells for better performance or by suppressing their response in case of auto-immune disorder or following tissue transplantation[11,12]. Appropriate modulation of immune system in order to boost its ability to fight infection, counteract diseases, prevent cancer, and chronic inflammation may be achieved by medicinal plants, which are considered as an alternative approach to conventional treatment[13, 14].

Borreria verticillata is a Cameroonian medicinal plant that grows in mountainous area, in the form of a shrub, and is widely used in the West Region for the management of epilepsy. It is largely accepted in several other communities as a highly active plant with several curative virtues. Despite its exploitation by local communities and other indigenous people, no scientific evaluations are conducted for confirming its role as immunomodulatory. Thus, the present study was performed to investigate the immunomodulatory properties of *B. verticillata* extract on macrophages activities and lymphocytes.

2. MATERIALS AND METHODS

2.1. Plant identification and extract preparation

The whole plant of *Borreria verticillata* was collected from the Bamboutous Mountain in the West region of Cameroon in May 2013. The plant was identified by a botanical expert at the National Herbarium in Yaounde, Cameroon by comparing to the voucher specimen registered as 22815/HNC. The stem and leaves of *B. verticillata* were dried at room temperature and then grounded to a fine powder using laboratory blender. Powdered plant material (500 g) was macerated in 3 liters methanol with frequent striking at room temperature for 48 hours. After filtration, the methanol was evaporated under reduced pressure by using rotary vacuum evaporator (Büchi R200) at 40°C resulting in the methanol extract (77.5 g). This extract was further fractionated in hexane and ethyl acetate. The hexane fraction was obtained after dissolving 40 g of the methanol extract in 1.5 liters of hexane, the mixture was then filtered and concentrated by hexane evaporation. The residue obtained was dissolved in 1.5 liters of ethyl acetate and the solution thus obtained was filtered and concentrated to yield ethyl acetate fraction. Methanol extract of *B. verticillata* and its fractions were dissolved in RPMI 5%.

2.2. Isolation of lymphocytes and generation of macrophage cells

Peripheral blood lymphocytes (PBL) were obtained by Ficoll Hypaque density gradient centrifugation of heparin-anticoagulated venous blood from healthy donors after obtaining informed consent. The blood was washed twice with RPMI supplemented with 5 % NBCS, 2 mM L-glutamine and penicillin/streptomycin 100 U/ml (RPMI 5%) and monocytes were separated from lymphocytes by plating PBL on falcon tissue plate. They were allowed to adhere for 12 hours and the non-adherent (lymphocytes) were removed. The adherent cells (macrophages) cultured for 7 days in presence of macrophage colony-stimulating factor (40 ng/ml) in RPMI 5% at 37°C in a humidified incubator at 5% CO_2. Medium was changed after 3 days culture. Macrophages viability was determined by trypan blue exclusion test (98 %) and adjusted to 2×10^5 cells/ml. In all subsequent experiments, each extract concentration was tested in triplicate.

2.3. Cytotoxicity Assay

To assess the cytotoxic of the plant extracts, macrophages and lymphocytes were treated with extract at 128 and 512 µg/ml for 24 and 72 hours respectively 37°C in a humidified incubator at 5% CO_2. Following incubation, 20 µl of MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenylterazolium bromide) 0.5 mg/ml were added into each well and incubated at 37°C for 4 hours. Formazan crystals formed from dye reduction by viable cells were dissolved using HCl-Isopropanol 0.04 N and the absorbance was recorded at 492 nm as previously described[15].

In order to verify the integrity of macrophage membranes in the presence of extract, the "dye-uptake" technique, based on the incorporation of neutral red by the living cells was used[16]. Briefly, cells were cultured at 2.5 x 10^5 cells/ml in microtitre

plate (NUNC, 96-well) in presence of extract for 24 hours at 37°C in a humidified incubator at 5% CO_2. After this incubation period, 100 µl of neutral red solution (0.075 %) was added and further incubated for 3 hours. The cells were then washed twice, the supernatant was discarded and 100 µl of 4% formaldehyde solution was added and maintained for 5 minutes to promote cell fixation. Following discarding the solution, 100 µl of cell lysis solution (acetic acid solution 1% and 50% methanol at the ratio 1:1) was added and plates were incubated for 30 minutes at room temperature on plate mixer and the optical density was read at 492 nm.

2.4. Nitric oxide (NO) quantification

The amount of nitrite in the culture medium was measured as an indicator of NO production using Griess reagent. Macrophages were cultured in 96 well plates for 24 hours as mentioned above in the presence of LPS (1 µg/ml) and plant extract. Following incubation, 100 µl of cell culture medium was incubated with equal volumes of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) for 10 min at room temperature. The absorbance was measured at 540 nm in a microplate reader. Fresh culture medium was used as blank in each experiment.

2.5. Pinocytic activity

The pinocytosis capability of lipopolysaccharides stimulated macrophages was measured by neutral red uptake[17]. Macrophages were dispensed into 96 well plates for 24 hours in the presence of plant extract and LPS 37°C in a humidified incubator at 5% CO_2. Upon incubation, culture medium was removed and 100 µl of neutral red solution added to each well followed by 3 hours additional incubation at 37°C. The dye was then discarded and macrophages were washed twice with phosphate buffer saline. They were further fixed with 100 µl of formaldehyde solution 4% for 5 minutes. Upon fixation, the formaldehyde solution was replaced by a mixture of acetic acid solution (1 %) and methanol (50 %). The plate was then kept for 30 minutes under constant agitation and the absorbance was measured at 492 nm. Phagocytic index was calculated as the ratio of the treated and control macrophages.

2.6. Macrophages oxidative metabolism

The burst of macrophages oxidative metabolism was assessed based on the reduction of Nitroblue Tetrazolium (NBT) dye reduction assay as described by Manosroi[18]. Briefly, macrophages were cultured in a humidified incubator containing 5% CO_2 for 24 hours at 37°C in microtitre plates in the presence LPS and plant extract. Upon incubation, 20 µl NBT solution 1.5 mg/ml in PBS was added in each well and the plate were incubated for additional one hour at 37°C. Upon incubation, macrophages were rinsed vigorously with RPMI-1640 medium, washed four times with 200 µl of methanol and finally air-drying. 120 µl of 2 M KOH and 140 µl of DMSO were added in each well to solubilize formazan-deposits. The absorbance was recorded at 570 nm and NBT reduction percentage was estimated.

2.7. Cellular lysosomal enzyme activity assay

Cellular lysosomal enzyme activity of macrophages was evaluated by measuring acid phosphatase activity as described earlier[19]. After 24 hours macrophages culture in the presence of plant extract and LPS in a humidified incubator containing 5% CO_2, culture medium was discarded and the cells were re-suspended by addition of 20 µl triton X-100 (0.1%) and plates were incubated once more for 30 min. 100 µl of 10 mM *p*-nitrophenyl phosphate (*p*NPP) and 50 ml of 0.1 M citrate buffer (pH 5.0) were added in each well and the mixture was incubated for 1 hour at 37°C. Thereafter, 150 µl of 0.2 M borate buffer (pH 9.8) was added to stop the reaction and the absorbance was measured at 405 nm. The percentage of lysosomal enzyme activity was calculated.

2.8. Myeloperoxidase activity assay

Macrophages were cultured as mentioned above. Upon incubation, cells were washed three times with RPMI medium and plates were incubated for 10 min with 100 µl of *o*-phenylenediamine (0.4 g/ml) and H_2O_2 (0.002%) in phosphate-citrate buffer (pH 5.0). Sulfuric acid (0.1 N) was added to stop the reaction and the absorbance was measured at 492 nm[20]. The percentage of myeloperoxidase activity was then calculated.

2.9. Mitogen-induced lymphocytes proliferation assay

The lymphocytes culture was performed in 96-well culture plates. Cell suspensions (1.5×10^5 cells/ml) were seeded for 72 hours in a humidified incubator containing 5% CO_2 at 37°C in the presence of plant extract and either Phytohemaglutinin (PHA) 4 µg/ml or LPS 1 µg/ml. PHA and LPS are T cells and B cells primer respectively. Four hours before the end of the incubation period, 20 µl of MTT solution (0.5 mg/ml) was added to each well and the plates were incubated for four more hours at 37°C. Following incubation, the medium was discarded and 100 µl of HCl-isopropanol 0.04 N was added to reduce formazan crystals formed by viable cells. After 30 min

incubation under agitation, the absorbance was read at 492 nm. Wells containing either PHA or LPS was used as control.

In each assay on macrophages, experiments were performed in triplicate and LPS was used as positive control.

2.10. Statistical analyses

The results were expressed as mean ± S.E.M. They were submitted to one way ANOVA. When the differences existed, means were separated using Waller Duncan's multiple range test at 0.05 significance level.

3. RESULTS

3.1. Cytotoxicity effect of plant extracts on macrophages

The *B. verticillata* extract and fractions was assessed on cell toxicity and membrane integrity. No cell toxicity was observed on extract treated macrophages as compared to the control (Table 1 and Figure 1). Moreover, they did not induce any alteration on membrane integrity. Cell growth was rather significantly stimulated in concentration-dependent manner in presence of plant extracts.

3.2. Effect of *B. verticillata* extracts on NO production in LPS-activated macrophages

The methanol extract, hexane and ethyl acetate fractions of *B. verticillata* were tested for their effect on nitric oxide production by LPS-activated macrophages in cultures. As shown in Figure 1, they exhibited a significant inhibition of NO production when compared to the LPS-treated macrophages ($p<0.05$). As determined by MTT assays, cell viability was not altered by the plant extracts indicating that the inhibition of NO production by these extracts was not due to cytotoxic effects.

3.3. Effect of plant extracts on macrophage phagocytosis

The pinocytic activity of macrophages was assessed by measuring the amount of neutral red uptake by LPS-activated macrophages. The extracts significantly decreased the pinocytosis of macrophages when compared to the positive control (Table 2).

The *in vitro* endocytosis effect of different extracts was also evaluated through the reduction of NBT dye. The methanol extract of *B. verticillata* didn't caused significant reduction of NBT by LPS-stimulated macrophages, however its fractions significantly decreased ($p<0.05$) this reduction, reflecting the ability of these fractions to inhibit the destruction of ingested particles (Table 3).

3.4. Effect of plant extracts on lysosomal enzyme and myeloperoxidase activity

The effect of *B. verticillata* extracts on lysosomal enzyme activity of LPS-stimulated macrophages is presented in Table 4. Ethyl acetate fraction significantly inhibited macrophages lysosomal enzyme activity when compared to the control. The methanol extract and its hexane fraction did not have any effect on this activity.

As presented in the Table 5, the myeloperoxidase activity of activated macrophages was not modified by methanol extract of *B. verticillata* but its fractions inhibited this myeloperoxydase activity at 128 µg/ml.

3.6. Effect of plant extracts on lymphocyte proliferation

Results presented in Table 6 revealed that *B. verticillata* methanol extract and its fractions didn't have any negative effect on the viability of lymphocyte cells, instead proliferation of lymphocytes were noticed. In order to appreciate the group of lymphocytes involved in proliferative activity, cultures were supplemented with PHA or LPS. Figures 2 and 3 showed that both T and B cells were involved in the proliferative activity. The stimulation was found to be more pronounced with ethyl acetate fraction in case of B cells. But as far as T cells are concerned, the crude extract and ethyl acetate fraction had best activity.

4. DISCUSSION

Macrophages constitute an important component of cell-mediated immune responses, and serve as a first line defense following infection[21]. Macrophages play a key role in the nonspecific defense mechanism during host infection and have been shown to destroy tumor cells[22]. The production of nitrogen oxide (NO), reactive oxygen species (ROS), as well as other inflammatory mediators by macrophages makes them efficient intracellular parasite killing machines as part of their role in host defense[23].

Macrophages were isolated from peripheral blood of health donor, 98% of which were viable before experimental treatment with tested extracts. Cell viability is an important parameter in cell-based assays, and can greatly influence test outcome thereby leading to false interpretation of cellular assays. Viability can be affected by several factors, including the experimental treatment being tested. The effect of *B. verticillata* extracts were investigated on lymphocytes and macrophages. Given the complexity of plant extracts, it is important to assess the effects on cell viability and integrity. The MTT assay and dye uptake assay were used to achieve this objective. Tested extracts did not show

any observable effects on cell viability and membrane integrity. This indicates that alterations in cell viability or membrane integrity can be ruled out in any further observation upon treatment.

NO is enzymatically produced by iNOS in macrophages and it is released in response to bacterial infection or inflammation[24]. Although NO is a highly reactive molecule necessary in immune defense mechanism, its overproduction can cause mitochondrial oxidative stress, adrenal insufficiency, apoptosis, arthritis neurotoxicity and cancer[25]. The study showed that *B. verticillata* extract and fractions inhibited NO production from LPS-activated macrophages.

Table 1
Effect of *B. verticillata* extract and fractions on membrane integrity of macrophages

Concentrations (µg/ml)	Membrane integrity (%)		
	Crude extract	Hexane fraction	Ethyl acetate fraction
0	100.00 ± 0.00^{b}	100.00 ± 0.00^{b}	100.00 ± 0.00^{c}
128	124.03 ± 10.42^{b}	89.53 ± 6.96^{b}	194.46 ± 2.66^{a}
512	179.06 ± 22.15^{a}	118.46 ± 8.06^{b}	167.38 ± 7.46^{b}
LPS (1µg/ml)	172.00 ± 0.00^{a}	172.00 ± 0.00^{a}	172.00 ± 0.00^{b}

Results are expressed as mean ± SD (n=3). For a given extract or fraction, values of concentration bearing different superscript letters are significantly different according to Waller-Dunkan test (at P<0.05) when compared to the non-treated cultures.

Table 2
Effect of *B. verticillata* extracts on pinocytosis of LPS-activated macrophages

Concentrations (µg/ml)	Pinocytic index		
	Crude extract	Hexane fraction	Ethyl acetate fraction
control	1.00 ± 0.08^{c}	1.00 ± 0.08^{e}	1.00 ± 0.08^{c}
128	0.93 ± 0.05^{c}	1.15 ± 0.17^{cd}	1.19 ± 0.06^{b}
512	1.10 ± 0.04^{bc}	1.26 ± 0.09^{bc}	1.59 ± 0.10^{a}
LPS (1µg/ml)	1.72 ± 0.09^{a}	1.72 ± 0.09^{a}	1.72 ± 0.09^{a}

Results are expressed as the mean ± SD (n=3). For a given extract or fraction, values of concentration bearing different superscript letters are significantly different according to Waller-Dunkan test (at P<0.05) when compared to the non-treated cultures.

Table 3
Effect of *B. verticillata* extracts on endocytosis of LPS-activated macrophages

Concentrations (µg/ml)	NBT reduction (%)		
	Crude extract	Hexane fraction	Ethyl acetate fraction
control	78.89 ± 0.00^{a}	78.89 ± 0.00^{c}	78.89 ± 0.00^{b}
128	68.33± 30.58^{a}	83.17± 3.67^{b}	77.85 ± 12.23^{b}
512	79.58± 26.91^{a}	101.91± 0.00^{a}	78.71 ± 1.22^{b}
LPS (1µg/ml)	100.00± 0.00^{a}	100.00± 0.00^{a}	100.00 ± 0.00^{a}

Results are expressed as the mean ± SD (n=3). For a given extract or fraction, values of concentration bearing different superscript letters are significantly different according to Waller-Dunkan test (at P<0.05) when compared to the non-treated cultures.

Table 4
Effect of *B. verticillata* extracts on lysosomal enzyme activity of LPS-activated macrophages

Concentrations (µg/ml)	Lysosomal enzyme activity (%)		
	Crude extract	Hexane fraction	Ethyl acetate fraction
control	68.04 ± 1.59^{b}	68.04 ± 1.59^{c}	68.04 ± 1.59^{b}
128	74.76 ± 10.47^{a}	91.36 ± 14.56^{a}	72.24 ± 4.96^{b}
512	82.68 ± 3.95^{a}	92.96 ± 2.42^{a}	76.41 ± 2.58^{b}
LPS (1µg/ml)	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00± 0.00^{a}

Results are expressed as the mean ± SD (n=3). For a given extract or fraction, values of concentration bearing different superscript letters are significantly different according to Waller-Dunkan test (at P<0.05) when compared to the non-treated cultures.

Table 5
Effect of *B. verticillata* extracts on myeloperoxidase activity of LPS-activated macrophages

Concentrations (µg/ml)	Myeloperoxidase enzyme activity (%)		
	Crude extract	Hexane fraction	Ethyl acetate fraction
control	50.19 ± 0.00^{c}	50.19 ± 0.00^{d}	50.19 ± 0.00^{c}
128	93.33± 6.28^{b}	78.72± 7.06^{bc}	87.7 ± 6.2^{b}
512	113.62± 2.20^{a}	88.23± 10.18^{ab}	97.45 ± 6.28^{a}
LPS (1µg/ml)	100.00 ± 0.00^{ab}	100.00± 0.00^{a}	100.00± 0.00^{a}

Results are expressed as the mean ± SD (n=3). For a given extract or fraction, values of concentration bearing different superscript letters are significantly different according to Waller-Dunkan test (at P<0.05) when compared to the non-treated cultures.

Table 6
Cytotoxicity effect of *B. verticillata* extracts on lymphocyte cells

Concentrations (µg/ml)	Cell viability (%)		
	Crude extract	Hexane fraction	Ethyl acetate fraction
0	100.00±0.00^{c}	100.00±0.00^{c}	100.00±0.00^{b}
128	212.85±26.49^{b}	290.87±17.10^{a}	251.26±2.96^{a}
512	228.77±26.19^{ab}	133.37±3.79^{b}	266.81±7.59^{a}

Results are expressed as the mean ± SD (n=3). For a given extract or fraction, values of concentration bearing different superscript letters are significantly different according to Waller-Dunkan test (at P<0.05) when compared to the non-treated cultures.

Incorporation of neutral red by activated macrophages was significantly decreased in presence of extracts indicating an inhibition of the pinocytic activity of macrophages. Nonspecific immunity is driven by macrophages cells through diverse functions like phagocytosis and pinocytosis[26]. These cells engulf and attack particles in the host that have been signaled for removal by various mechanisms. This process results in the activation of a membrane-bound oxidase that catalyzes the reduction of oxygen to superoxide anion, reactive oxygen intermediate that is extremely toxic to ingested microorganisms[27]. The superoxide anion also generates other powerful oxidizing agents, including hydroxyl radicals and hydrogen peroxide. Despite its host defense, exaggerated function of human phagocyte can also be involved in cell and tissue damage associated with severe inflammatory reactions and autoimmune disorders[28]. Thus, modulation of pinocytic activity and oxidative burst may play an essential role in keeping cell and tissue integrity.

All phagocytes contain membrane-bound vesicles filled with digestive enzymes that are available for the annihilation of engulfed microbes[29]. Lysosomal enzyme especially acid phosphatase can transform *p*-nitrophenyl phosphate into a colored compound which correlates with the extent of degranulation in phagocytosis[30].

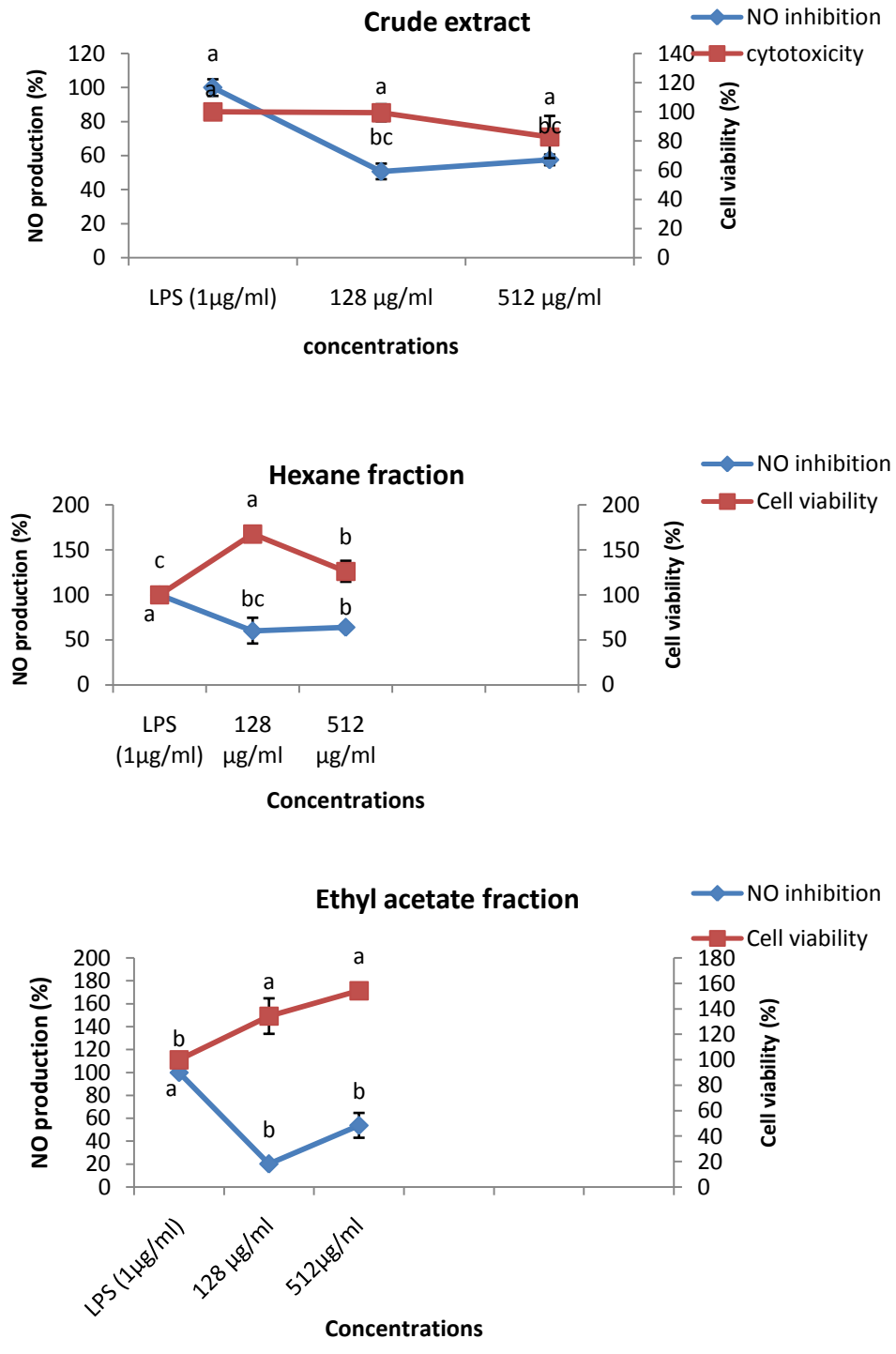

Figure 1

Effect of *B. verticillata* extract and fractions on LPS-activated macrophage NO production and cell viability

Results are expressed as the mean ± SD (n=3). For a given extract or fraction, values of concentration bearing different superscript letters are significantly different according to Waller-Dunkan test (at P<0.05) when compared to the non-treated cultures.

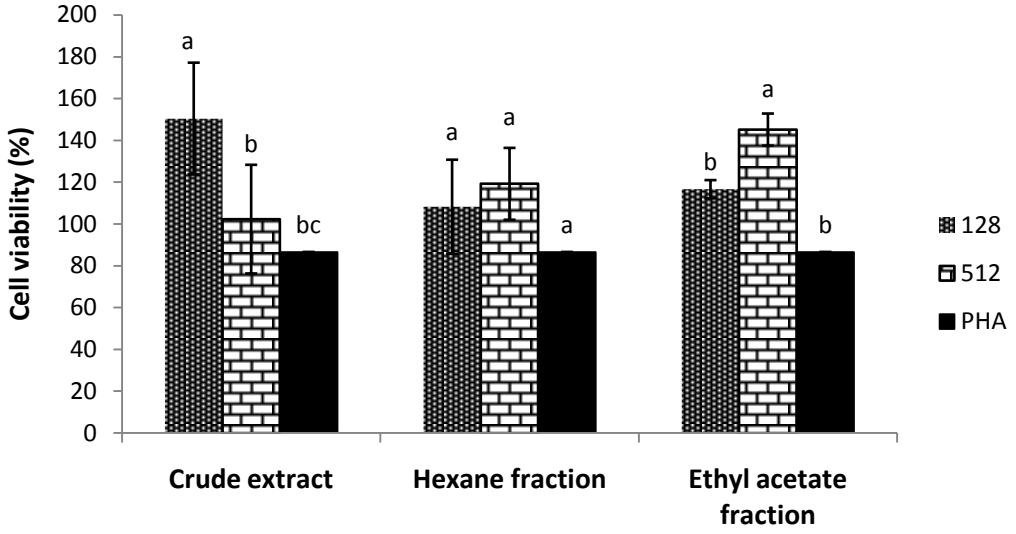

Figure 2
Effect of *B. verticillata* methanol extract and fractions on T lymphocyte proliferation
(Results are expressed as the mean ± SD (n=3). For a given extract or fraction, values of concentration bearing different superscript letters are significantly different according to Waller-Dunkan test (at P<0.05) when compared to the non-treated cultures)

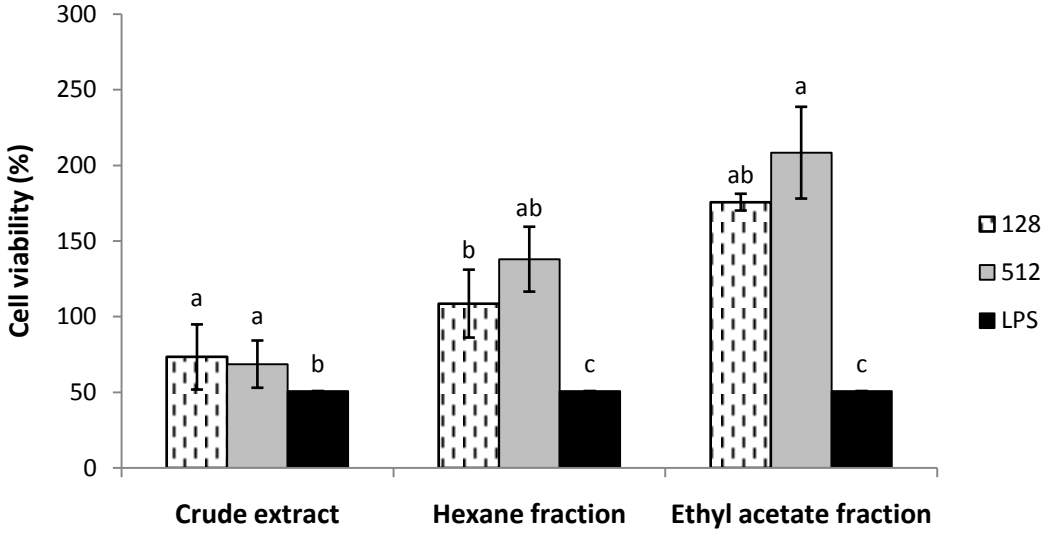

Figure 3
Effect of *B. verticillata* methanol extract and fractions on B lymphocyte proliferation
(Results are expressed as the mean ± SD (n=3). For a given extract or fraction, values of concentration bearing different superscript letters are significantly different according to Waller-Dunkan test (at P<0.05) when compared to the non-treated cultures.)

Fractions of the studied extracts inhibited the activity of lysosomal enzyme of activated macrophages. Although lysosomal enzymes are necessary for killing and engulfing pathogens, they are important mediators of acute chronic inflammatory diseases and involved in damage to connective tissue and contribute to several human diseases[31, 32]. Inhibition of the lysosomal activity of macrophages by these extract could be exploited for the management of some chronic inflammatory diseases.

Considering the overall results, it clearly appears that *B. verticillata* extract contains substances that inhibit the phagocytic activity of macrophages by altering the destruction of ingested particles as a result of

inhibition of myeloperoxidase activity and lysosomal enzyme. This property is more observed in fractions compared to the crude extract, indicating that the anti-inflammatory properties of these fractions could be expected[33].

Observing the aforementioned effects of the tested extracts on macrophages and considering the importance of macrophages and lymphocytes as the major effector cells involved in the immune responses, the effect of these extracts was explored on lymphocytes. It was found that both T and B cells proliferation is stimulated in the present of either extract or fractions. T cells are involved in cell mediated immunity, whereas B cells are primarily responsible for humoral immunity through production of antigen specific antibodies[34]. The increased T and B lymphocytes counts after treatment with extracts may be correlated with the increased humoral and cellular activities.

CONCLUSION

The effects of the methanol extract and its hexane and ethyl acetate fractions of *B. verticillata* were investigated on macrophages and lymphocytes. The extracts were capable of modulating non specific and adaptive immune responses mediators of the immune system through the inhibition of phagocyte cell activities and enhancement of lymphocyte proliferation. More so, extracts did not show any significant alteration in cell viability and integrity in vitro. Besides, the inhibitory effects of the tested extracts on macrophage activity may further be exploited for the development of disorders related to macrophage hyper activation such as macrophage activation syndrome. However, further studies are necessary to investigate their activities on other mechanisms of immunomodulatory responses.

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