Immunostimulation by Stem Barks of *Pseudocedrela Kotschyi* (Family: *Meliaceae*): Stimulation of Phagocytosis Activities of Macrophages and Proliferative Response of Lymphocytes by Aqueous, Methanol and Hexane Extracts

OUMAR Mahamat[1*], TUME Christopher[2] and KAMANYI Albert[3]

[1]*Department of Biological Sciences, Faculty of Sciences, University of Bamenda, Cameroon*

[2]*Department of Biochemistry, Faculty of Sciences, University of Dschang, Cameroon*

[3]*Department of Animal Biology, Faculty of Sciences, University of Dschang, Cameroon*

**Corresponding Author: OUMAR Mahamat, PO. BOX 39 Bambili, Cameroon, E-mail:oumahamat@yahoo.com. Tel: 00237-696694015*

ABSTRACT: Macrophages and lymphocytes are the principal cells of defense mechanism against infection of the body. Plant extracts have been reported as the immune modulator agent owing to the presence of phytochemicals. There is small information on *Pseudocedrela kotschyi* in term of its immune modulatory effect. The present study addresses the question of whether *Pseudocedrela* kotschyi, plant of the *Meliaceae* family has ability to modulate the immune response notably the phagocytic activity of human monocytes/macrophages. The study was done by using the human cells. The response of these cells to *Mycobacterium bacillus* vaccine was observed in presence of the aqueous extract, hexanic and methanolic fractions. The effects of the extracts were evaluated by appreciating the phagocytic index in macrophages, lysosomal enzymes and myéloperoxydase activity of macrophages and production of nitric oxide (NO). We found that the three extracts stimulated the monocyte proliferation response. However only the methanol and hexane extracts caused an increase in the macrophage phagocytic activity. All the three extracts stimulated NO production by macrophages and myeloperoxidase activity, while only the aqueous and methanol extracts caused an increase in lysosomal enzyme activity in macrophages. These results show that extracts of *Pseudocedrela kotschyi* have immunostimulatory monocytes and macrophages, and thus explains the possible use of this plant extract as a putative cure for some ailments as practiced in our traditional ethnomedical domain, though pending further pharmacological investigations.

Key words: *Pseudocedrela kotschyi*, Phagocytosis, macrophages

1. INTRODUCTION

The immune system evolved to protect the host from potentially pathogenic agents including microorganisms (viruses and bacteria, parasites, and fungi), to eliminate neoplastic cells and to reject non-self components. The structural and functional alterations of the immune system may lead to immunosuppression which may modify the host defense mechanisms against infection, cancer, and induction of abnormal immune responses resulting in allergy and autoimmunity.[1] Immunostimulant drugs are required to overcome the immunosuppression induced by drugs or environmental factors, and immunosuppressants are required where there is undesired immunopotentiation. There is a strong requirement of drugs which can boost the immune system to combat the immunosuppressive consequences caused by stress, chronic diseases like tuberculosis, and conditions of impaired immune responsiveness (e.g. AIDS) etc.[2] Plants are potential means to treat diseases and to improve the immunological response against much pathology.[3] Some plant extracts was identified to boost the humoral and mediated immunity against viruses, bacteria, fungi, protozoa and cancer.[4]

Pseudocedrela kotschyi is used by traditional healers in Cameroon as an alternative to conventional chemotherapy for treatment of some diseases related to the immune system. However, no study has been done to demonstrate the evidence in support of its usage, except some study which demonstrated in mice the antiplasmodial property of stem bark of *P. kotschyi*.[5] *In-vitro*, its barks extracts also showed low cytotoxic activity against the L-6 cell line.[6]

The present study aimed at investigating the immunomodulatory activity of some extracts of this plant in modulation of immune cells activities. Given that monocytes/macrophages constitute the key components of our immune system, control in their activities can improve the immune responsiveness to infections and thus an

improvement of our health. Thus any evidence of macrophage stimulation by extracts of *P. kotschyi* can therefore be poof of the immunomolatory activity of *P. kotschyi* and therefore would justify its use by patients like these infected by the HIV.[7, 8]

2. MATERIALS AND METHODS

2.1. Plant material

The trunk bark of *P. kotschyi* (Schweinf) Harms (family: *Meliaceae*) was collected in August 2012 from Garoua, in the North region of Cameroon. The plant was authenticated in comparison with the plant sample No: 7009/SRF/Cam at the National Herbaruim, Yaounde, Cameroon. The trunk bark was chopped into small pieces and dried for one week under sunlight. After drying, it was ground using a laboratory blender and the fine powder was then extracted as described below.

2.2. Extraction procedures

The extraction was carried out with distilled water, hexane and methanol. The powder of *P. kotschyi* (500 g) was extracted as follows: For the aqueous extract, 500 g of powder was boiled in 2.0 litres of distilled water for about 20 minutes on a hot-plate. Hexane and Methanol fractions were prepared by maceration in 1.5 litres of each solvent for 72 h, with stirring for about 1 hour after every 12 hours at room temperature. The powder was first macerated as above in hexane, and then after filtration the residue was equally macerated in methanol. For each solvent, the extraction was done twice. After decantation through a perforated plastic filter to remove the coarse particles, each extract was then filtered using Whatman paper No 3. After filtration, the aqueous extract was dried in a ventilated dryer at 38°C for several days; while the methanol extract and the hexane extract were concentrated on a rotary evaporator and dried under reduced temperature (30°C) in vacuum.

The final extracts were then kept in tightly corked amber bottles at 4°C until used.

2.3. Chemicals and cell culture reagents

Eagle's Minimum Essential Medium (MEM), Foetal bovine serum (FBS), Penicillin-streptomycin solution, Neutral red, trypan blue, 3-(4,5-dimethylthiazol-2-yl)-5-(diphenyl tetrazolium) bromide reagent (MTT), Ficoll Hypaque, isopropanol, TritonX-100, *p*-nitrophenyl phosphate (N-*pp*), sulfanilamide and Napthyl ethylenediamine were purchased from Sigma Aldrich, Inc. (Germany). *Mycobacterium bacillus* vaccine commonly called Bacillus Calmette Guerin (BCG) was acquired from the Dschang District hospital pharmacy.

2.4. Isolation of monocytes and macrophages

Blood used in this study was blood collected using heparin as the anticoagulant. Blood samples were from some patients which are collected by the medical center of the University of Dschang for some analysis in respect with the principle of the minister of public health according to the world medical association declaration of Helsinki as described in the Medical ethic manual.[9] The isolation of monocytes was done by a combination of the Ficoll-hypaque gradient centrifugation and adherence on culture dishes as previously described.[10] Monocytes were then cultured for 6 days in MEM containing 10% (v/v) FBS, 2% (v/v) of a 200 mM L-glutamine solution and 1% v/v) of antibiotic solution (100 IU/ml penicillin, 100 mg/ml streptomycin) toward macrophages[11] in Falcon tissue culture bottles (Nunclon, Nalge Nunc international, U.S.A), or alternatively, using Costar cell culture dishes (medical grade polystyrene; Corning Costar Corporation GmbH, Bodenheim, Germany). For this purpose cells ($10x10^6$) were cultured in a volume of 20 ml per dish in an incubator (type BB 6220 CU; Heraeus Instruments GmbH, Hanau, Germany) at 37°C. Cells were collected by vigorous pipetting, counted, and used for the experiments described below.

2.4. Monocyte proliferation response assays

Monocytes were cultured at a density of $2.5x10^5$ per well in flat-bottomed microtiter culture plates in the presence of different extract concentrations or BCG ($3x10^6$ CFU/well) at 37°C. Cells treated with BCG alone were considered as positive controls, while cells cultured in the absence of either plant extract or BCG were the negative controls. The cultures were carried out for 48 hours and then quantified spectrophotometrically using the MTT assay method as previously described.[12, 13]. Briefly, after incubation at 37°C for 48 h, 20µl of MTT (Sigma-Aldrich, Germany) (5 mg/ml) in PBS were added. The cultures were re-incubated for 30 minutes, 100 µl of the culture medium was discarded by aspiration and added with 100 µl of 0.04M HCl in isopropanol (Sigma-Aldrich, Germany) to lyse cells. Then, the absorbance was measured at 492 nm. From the absorbance values obtained, the relative proliferation for each tested dose of the plant extract was expressed as a percentage by comparing the experimental value obtained to the baseline (OD of unstimulated cells) control value by using the following equation:

$$\% \text{ Proliferation} = \frac{OD_{sample} - OD_{control}}{OD_{control}} x100$$

2.5. Macrophage phagocytic index assays

This assay was performed using the neutral red internalization method for evaluating the phagocytic activity of macrophages in microplate wells. Briefly, macrophages ($1.5x10^5$ cells/well) were cultured at 37◦C in 5% CO2 for 48 hours either in medium plus BCG and extracts, or in medium plus BCG alone (positive control), and or medium alone (baseline control). The predetermined optimum dose of BCG at $3x10^6$ CFU/well was used. After the two days of culture, the neutral red test was carried out as described.[14] Briefly, after incubation the medium was discarded, 100 µl of 0.075% neutral red solution was added and

incubated for 1h. Then the supernatant was discarded and the cells were washed with PBS twice. Then 100 µl cell lysate solution (ethanol and 0.001% acetic acid at the ratio of 1:1) was added into 96-well plate to lyse cells at laboratory temperature for 2 h. The optical density at 492 nm was measured by a microplate reader. From optical density, the phagocytic index (PI) was calculated using the following equation:

$$\text{Phagocytic index} = \frac{OD_{sample} - OD_{control}}{OD_{control}} \text{x}100$$

2.6. Stimulation of NO production by Macrophages

Macrophages (2.5×10^5) were maintained in culture in 96-well plates for 24 hours, then pre-incubated with the different extracts at increasing concentrations (0.04 – 2.56 mg/ml) for 1 hour and then stimulated with BCG ($2.5\text{x}10^5$ CFU/ml) for 24 hours. Nitrite accumulation, an indicator of NO production, was measured in the conditioned culture medium by the Griess reaction.[10] Briefly, 100 µL of cell culture medium was mixed with 100 µl of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 2.5% (v/v) phosphoric acid and 0.1% (w/v) naphthyl ethylenediamine in 2.5% (v/v) phosphoric acid], incubated at room temperature for 30 minutes in the dark, and the absorbance at 492 nm was measured in an ELISA microplate reader (Bio-Rad Laboratories). Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was calculated from a standard curve using freshly prepared sodium nitrite in culture medium and data were expressed as the total mM nitrite produced.

2.7. Assay of lysosomal enzyme activity in Macrophages

Cellular lysosomal enzyme activity was measured according to the procedure described previously.[15] Macrophages in 96- well culture plates above were solubilized by adding 25 µl of 0.1% Triton X-100 and incubated for 30 min at room temperature. Then 150 µl of

10 mM *p*-nitrophenyl phosphate was added per well as substrate for acid phosphatase, followed by the addition of 0.1 M citrate buffer (50 µl, pH 5.0). After incubation for 1 h at 37^{o}C, 0.2 M borate buffer (50 µl, pH 9.8) was added to the mixture to stop the reaction. Optical densities were measured at 405 nm and the lysosomal enzyme activity was calculated using the following equation:

$$\text{Lysosomal Enzyme Activity} = \frac{OD_{sample} - OD_{control}}{OD_{control}} \text{x}100$$

2.8. Myeloperoxidase activity assay

Myeloperoxidase activity was evaluated on isolated macrophages as previously described.[16] Briefly, macrophages ($2.5\text{x}10^5$ cells/ml) pre-incubated for 24 hours were washed three times with fresh complete RPMI medium. Then 20 µl of a mixture 100 µl of *o*-phenylenediamine (0.4 g/ml) and 100 µl of 0.002% H_2O_2 in phosphate- citrate buffer (pH 5.0) was added to each well. The reaction was stopped after 10 min using 0.1 NH_2SO_4 and the Optical densities were measured at 490 nm. The myeloperoxidase stimulation index (SI) was expressed as follows:

$$\text{Stimulation index (SI)} = \frac{OD_{sample} - OD_{control}}{OD_{control}} \text{x}100$$

2.9. In vivo phagocytic activity by carbon clearance assay

Phagocytic activity of the plant extracts was determined as previously described.[17] Mice were used and the protocols were approved by the Laboratory committee (Laboratory of Animal Physiology and Phytopharmacology, Department of Animal Biology, University of Dschang, Cameroon) according to the standard ethical guidelines for laboratory animal use and care as described in the European Community guidelines; EEC Directive.[18] Mice were divided into five groups, of six each. The control group received vehicle (H_2O distilled). Mice in the treatment groups were administered subcutaneously with the aqueous extract (0.75, 1.25 and 2.5µ g/kg)

suspended in vehicle daily for 20 days. Colloidal carbon solution, (Rotring ink®, Hamburg, Germany) was diluted with normal saline (1:8), and injected (0.01 ml/g body weight) via the tail vein to each mouse 24 h after the last dose. Blood samples were drawn from the retro-orbital plexus under ether anesthesia at 2 and 15 minutes after injection. Blood (25µ l) was mixed with 0.1 % sodium carbonate (2 ml) for the lysis of erythrocytes, after the OD was recorded at 660 nm. The phagocytic index (K) was calculated by using following equation:

$K = \frac{\ln OD_1 - \ln OD_2}{T_1 - T_2} x100$, where OD1 and OD2 are the optical densities at times T1 and T2, respectively.[19]

2.10. Statistical analysis

Numerical data was analyzed using the Graph Path Instat (v 3.0) or Graph path prism (v 5.0) statistical package for one-way analysis of variance (ANOVA) and the Tukey-Krammer post-hoc test for comparison of means. Differences were considered statistically significant at a P value <0.05.

3. RESULTS

Proliferation assay response of plant extracts in monocyte. In preliminary studies, we determined that BCG ($3x10^6$particles/ml) induces greater proliferation of monocytes than does non stimulation (data not shown). Thus, in the present study, BCG was used for the study of mitogen-induced proliferation by monocytes. Stimulation of BCG-activated monocytes for 2 days with extracts of *P. kotschyi* resulted in significantly ($P<0.05$) greater number of cells as measured by MTT uptake than did stimulation with BCG alone. That was observed with aqueous and hexane extracts at concentrations from 640 to 2560 µg/ml and with methanol extract at concentrations from 160 to 2560 µg/ml (Table 1).

In vitro effects of extracts of *P. kotschyi* on phagocytic index of macrophages.

Phagocytosis is the first step in the response of macrophages to invading microorganisms. Phagocytic activities are crucial aspects of macrophage functional assessments.[20] Here, we examined the effects of *P. kotschyi* extracts on phagocytic index in BCG-activated macrophages. Stimulation of BCG-activated macrophages for 2 days with extracts of *P. kotschyi* resulted in significantly ($P<0.05$) greater phagocytic indices as measured by the neutral red internalization method compared to stimulation with BCG alone (Table 2).

That was observed only with the methanol extract at concentrations ranging from 320 to 2560 µg/ml. Using the NBT reduction test for measurement of the phagocytic activity of macrophages, treatment with extracts did not induce significant reduction of NBT as compared to non-treated cultures whereas reduction of NBT was significant ($P< 0.05$) in the case of macrophages treated in the presence of BCG alone (Table. 3).

Effects of Extracts of *P. kotschyi* on NO production and iNOS activity in macrophages. In order to further estimate the potential of *P. Kotschyi* to induce NO production from macrophages, an *in-vitro* concentration response experiment using the plant extracts was carried out as follows. BCG-activated macrophages were incubated with various concentrations of the plant extracts at 37^{o}C for 48 h. The culture supernatants were measured for the accumulation of nitrite, the stable end product of NO, and compared with the untreated control groups. Table 4 shows that the NO release from macrophages was significantly dependent on the dose of the extracts of *P. kotschyi*. Precisely, at the concentration range of 160-500 µg/ml, the aqueous, methanol and hexane extracts enhanced NO production in a dose-dependent manner.

Furthermore, the time dependent induction of NO production by the extracts was also examined and the results are shown in Fig. 1. It was evident that the NO production from extract-treated macrophages significantly increased from after 8 hours and peaked at 24 hours

similar to the positive control. However, it was unclear whether the stimulatory effect of the extracts on NO production was attributable to its influence on the activity of iNOS.

To elucidate the underlying mechanisms of the production of NO by extract-stimulated macrophages, the effect of L-NAME, an inhibitor of iNOS activity was evaluated after the cells were incubated with the extracts in the presence of L-NAME. Our results (Table 5) showed that, L-NAME markedly inhibited the production of NO inn then presence of *P. kotschyi* extracts. This inhibitory experiment of L-NAME showed that the effect of extracts was occurring via stimulation of the iNOS activity. This supportive evidence suggested that *P. kotschyi acted by* stimulating an inductive effect on iNOS activity in macrophages, resulting in the enhancement of NO release.

Induction of lysosomal enzyme activity in macrophages by extract of *P. kotschyi*. We also examined the effects of the extracts from *P. kotschyi* on the lysosomal enzyme activities of macrophages in vitro. The results shown in Table 6 indicate that the aqueous and methanol extracts of *P. kotschyi* at concentrations of 160 up to 2560 µg/ml significantly enhanced the relative enzyme activity in a dose-related manner ($P<0.05$ versus positive) and that the maximal effect was observed at a concentration of 2560 µg/ml (aqueous extract) and 1280 µg/ml (methanol extract). From the results it was concluded that *P. kotschyi* possesses the potential of enhancing the innate immune response.

Myeloperoxidase activity assay. The effect of *P. kotschyi* extracts on myeloperoxidase activity of macrophages is presented in Fig. 2. The extracts showed a significant ($P<0.05$) stimulation of the myeloperoxidase activity of macrophages at concentrations of 1280 and 2560 µ g/ml compared to the positive control, BCG alone.

***In vivo* phagocytic activity by the carbon clearance assay.** In order to further confirm the

results obtained with the *in vitro* essays of *P. kotschyi* activities, an *in vivo* phagocytic activity of *P. kotschyi* was determined for the aqueous extract by the carbon clearance assay in mice. The results of this assay are presented in Fig. 3. The phagocytic index (K) for the *P. kotschyi* extract was significantly higher ($P<0.05$) at 0.75, 1.25 and 2.5 µg/kg dose levels as compared to the untreated control group.

DISCUSSION

There is a growing interest in identifying herbal immunomodulators ever since their possible use in modern medicine was suggested.[21] Natural Substances and its constituents shows a pivotal role in the maintenance of immune system via the suppression or activation of various B-cells, T-cells and cytokines.[22]

In the present study, we investigated the effect of extracts from *P. kotschyi*, on the host immune defense system and identified the plant as an immunomodulator of macrophages, as reflected by the significant increase in the induction of monocyte proliferation as well the phagocytic index, NO production, cellular lysosomal enzyme activity and myeloperoxidase activity in extract-stimulated macrophages.

It is well known that the activation of monocytes results in the proliferation of these cells, which differentiate into macrophages.[23, 24] In our study, stimulation of monocytes for 2 days with BCG in the presence of the extracts from 160 to 2560 µg/ml resulted in significantly ($P<0.05$) greater monocytes proliferation as measured by MTT assay than that was with BCG alone. Thus, the extracts of *P. kotschyi* could be increase activation or differentiation of monocytes. As macrophages, they play an important role in the host protection against a wide range of tumors and microorganisms using phagocytosis process. Our results of phagocytosis assay as demonstrated by neutral red uptake or NBT dye reduction showed that the extracts of *P. kotschyi* significantly increased phagocytic index of macrophages. From

that we supposed that *P. kotschyi* has a stimulatory activity on phagocytic activity of macrophages.

It is well known that the activation of macrophages results in the expression of iNOS, which catalyzes the production of a large amount of NO from L-arginine and molecular oxygen.[25, 26][19] In our study, treatment with extracts increased NO generation in macrophages, indicating that *P. kotschyi* stimulated iNOS activity in macrophages. In addition, our study demonstrated that the production of nitrite by macrophages over a period of 3 days increased steadily with a highly significant increase within 24 hours indicating a significant duration of extract activity on NO production. Furthermore, treatment with extracts effectively suppressed L-NAME, a specific inhibitor of iNOS indicating that the extracts of *P. kotschyi* stimulated macrophages to produce NO through the induction of iNOS activity [7].

Lysosomal enzyme activity is a crucial aspect of macrophage functional assessment.[20] Myeloperoxidase also constitute a critical component of phagocytosis. Myeloperoxidase is a heme protein secreted by neutrophils and macrophages, an enzyme which uses the oxidizing potential of H_2O_2 to convert chloride ions into hypochlorous acid (HOCl), a potent bactericidal agent which is a critical component of the host defense against invading bacteria, fungi, and viruses.[22] The increase in the stimulation index of myeloperoxidase activity following exposure to the plant extract indicates an enhanced defense capability of these cells against pathogenic organisms. The release of immune mediators from murine peritoneal macrophages was significantly stimulated by exposure to the extracts. Macrophages incubated with *P. kotschyi* extract at certain concentrations for 48 hours showed a significant activation of macrophages by modulating lysosomal enzyme and myeloperoxidase activity, thus suggesting that *P. kotschyi* can effectively strengthen innate immunity against foreign particles. [8]

The process of phagocytosis involves certain body cells known as phagocytes of which

macrophages are a key component. These cells ingest and remove microorganisms, malignant cells, inorganic particles and tissue debris [27] from the body. Phagocytosis and killing of invading microorganisms by macrophages constitute the body's first line of defense against pathogens. Macrophages are an integral part of the immune system, acting as phagocytic, microbicidal and tumoricidal effector cells.[28] Through interaction with lymphocytes, macrophages play an important role in the initiation and regulation of the immune response.[29] In view of the pivotal role played by macrophages, the *P. kotschyi* extract and particularly the aqueous extract was evaluated for its *in vivo* effect on macrophage phagocytic activity. The increase in the carbon clearance i.e. phagocytic index induced by *P. kotschyi extracts* reflects the enhancement of the phagocytic function of macrophages, and thus non-specific immunity. This suggests that *P. kotschyi* is able to activate macrophages, and hence enhance their phagocytic activity *in vitro* and *in vivo.*

CONCLUSIONS

In conclusion, our results demonstrated that *P. kotschyi* can stimulate macrophage responses to foreign antigens by increasing their phagocytic activity through induction of iNOS activity resulting in NO production, and enhancement of lysosomal enzyme activities. It also stimulates monocyte proliferation. It was important to note that all extracts of this plant were not active in all the immune parameter. Methanol extract has a potential stimulatory effect in phagocytic index and intracellular mechanisms while aqueous and hexane extracts seem to stimulate intracellular mechanisms without effect on phagocytic index. Thus, methanol seems more important in stimulation of immune response than aqueous and hexane extracts. However, further investigations are being carried out to permit a better understanding of immunomodulatory effect of *Pseudecedrela kotschyi*.

Conflict of interest

We declare that we have no conflict of interest.

ACKNOWLEDGEMENTS

The authors are thankful to the “Laboratoire de Santé Animale” of University of Dschang and National Veterinary Laboratory (LANAVET) of Cameroon for collaboration.

REFERENCES

[1] Krzystyniak K, Tryphonas H, Fournier M., Approaches to the Evaluation of Chemical-induced Immunotoxicity. *Environmental Health Perspectives*. 1995, 103(9),17-22.

[2] Wagner H. (Ed). In: Immunomodulatory agents from plants, Basel: Birkhäuser, Germany. 1999, 1-35.

[3] Caroline J. R., Rama V. and Rameshkumar G. Immunomodulatory activity of aqueous extract of *Ocimum sanctum* in rat. *IJPBR*, 2011, Vol. 2(1) pp. 33-38.

[4] Noriko O. I, Nam H. Dang, Emi K., Akira K., Satoshi I., Chikao M.. Aqueous extract of *Carica papaya* leaves exhibits anti-tumor activity and immunomodulatory effects. *Ethno. Pharmacology*. 2010, 127: 760-767

[5] Dawet, A.; Yakubu, D. P.; Wannang, N. N.; Mwansat, G. S. In vivo antimalarial activity of stem bark of dry zone Cedar Pseudocedrela kotschyi (Meliaceae) in mice. *European Journal of Medicinal Plants*, 2014, 4 (3) 342-352

[6] Hay A. E., Ioset J. R., Ahua K. M., Diallo D., Brun R., Hostettmann K. Limonoid orthoacetates and antiprotozoal compounds from the roots of Pseudocedrela kotschyi. *J Nat Prod.* 2007; 70 (1):9-13.

[7] Xingbin Yang, Yan Zhao, Haifang Wang and Qibing Mei. Macrophage Activation by an Acidic Polysaccharide Isolated from *Angelica Sinensis* (Oliv.) Diels. *J. Biochem. Mol.Biol.*, 2007, Vol. 40, No. 5, pp. 636-643.

[8] Anita T., Narmada P. S., Uma D. P. Immunomodulatory Activity of the Aqueous Extract of Seeds of Abrus precatorius Linn (Jequirity) in Mice. *Iran.J.Immunol.* 2011, 8 (2), 96-102

[9] AMM : Association Médicale Mondiale. Manuel d'éthique médicale. *2ème édition*, 2009.

[10] Romani N., Gruner S., Brang D., Kampgen E., Lenz A., Trockenbacher B., Konwalinka G., Fritsch P.O., Steinmann R.M. and Schuler G. Proliferating dendritic cell progenitors in human blood. *J Exp Med.*1994, 180:83–93.

[11] Gantner F., Kupferschmidt R., Schudt C, Wendel A. and Hatzelmann A. In vitro differentiation of human monocytes to macrophages: Change of PDE profile and its relationship to suppression of tumour necrosis factor-a by PDE inhibitors. *Br J Pharmacol*. 1997, 121:221–231

[12] Oumar M., Tume C., Monique K. O. and Albert K. In vitro effect of aqueous extract, hexane and m*e*thanol fractions of *boswellia dalzeilii*, hutch (family: *burseraceae*) in immunomodulatory activities of human monocytes / macrophages. *International Journal of Biological & Pharmaceutical Research.* 2014; 5(2): 201-209.

[13] VENNILA S. BRINDHA D. In Vitro Cytotoxic AND Apoptotic Activity of Polysaccharide Rich Morinda Citrofolia Fruit on MCF-7 CELLS. *Asian Journal of Pharmaceutical and clinical research.* 2015; 8 (2) *190-193*

[14] Di G., Zhenya Z., Yingnan Y., Guoquing X. and Jiqiang L. Immunomodulatory Activity of Polysaccharide from the Roots of Actinida kolomika on Macrophages. *International of Biology*. 2011, 3 (2).

[15] Suzuki, I., Tanaka, H., Kinoshita, A., Oikawa, S., Osawa, M. and Yadomae, T. Effect of orally administered beta-glucan on macrophage function in mice. *Int. Immunopharmacol*., 1990, 12, 675-684

[16] Choi E. M.,Hwang J. K. Effects of methanolic extract and fractions from Litsea cubeb a bark on the production of inflammatory mediators in RAW264.7cells. *Fitoterapia*. 2004; 75:141-148.

[17] Yan Y., Wanshun L., Baoqin H., Changhong W., Chenwei F., Bing L., Liehuan L. The antioxidative and immunostimulating properties of D-glucosamine; International Immunopharmacology. 2007; 7: 29–35.

[18] EEC: Council directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the member states regarding the protection of animals used for experimental and other scientific purposes. *Official J Eur Comm*, 1986, L358, 1–29.

[19] Archana R. J., Amolkumar K. H., Sachin S. S., Vivek A. C. In vitro and in vivo evaluation of immunomodulatory activity of methanol extract of Momordica charantia fruits. 2009, 1(2), 89-94

[20] Jeong, S. C., Jeong, Y. T., Yang, B. K. and Song, C. H. Chemical characteristics and immunostimulating properties of biopolymers extracted from *Acanthopanax sessiliflorus*. *J. Biochem. Mol. Biol.*, 2006, 39, 84-90.

[21] Tiwari U., Rastogi B., Singh P., Saraf D. K., Vyas SP., Immunomodulatory effects of aqueous extract of *Tridax procumbens* in experimental animals. *Journal of Ethnopharm.*, 2004, 92, 113–119.

[22] ARSHAD H. and SALAH M. NIGELLA sativa and its Active Constituents Thymoquinone Shows Pivotal Role in the Diseases Prevention and Treatment. *Asian Journal of Pharmaceutical and clinical research.* 2015; 8 (1) 48-53

[23] Tacke F., and Randolph G. J. Migratory fate and differentiation of blood monocyte subsets. *Immunobiology*, 2006, 211, 609-618.

[24] Shortman K., and Naik S. H. Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol.*, 2007, 7, 19-30.

[25] Xie, Q. W., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T. and Nathan, C. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science.* 1992, 256, 225-228.

[26] Alderton, W. K., Cooper, C. E. and Knowles, R. G. Nitric oxide synthase: structure function and inhibition. *Biochem. J.* 2001, 357, 593-615.

[27] Salman H., Bergman M., Bessler H., Fenig E., Weiss J., Beilin B., Djaldetti M., Decreased phagocytic capacity of rat peritoneal macrophages following photon abdominal irradiation. *Cancer Letters*, 1999, 147 (1–2), 175–179.

[28] Son E. H., Moon E. Y., Rhee D. K., Pyo S., Stimulation of various functions in murine peritoneal macrophages by high mannuronic acid-containing alginate (HMA) exposure in vivo. *International Immunopharmacology*, 2001, 1, 147–154.

[29] Lowenstein C. J., Snyder S. H., Nitric oxide, a novel biologic messenger. *Cell.* 70, 1992, 705.

Table 1: Effects of different concentrations of the three *P. kotschyi* extracts on in vitro proliferation response of monocytes with BCG.

Concentrations	***Treatments***		
(µg/ml)	***Aqueous extract***	***Methanol extract***	***Hexane extract***
160	1,35±0,21	28,67±2,21[a]	8,18±1,40
320	15,34±4,87	32,93±1,31[b]	16,61±3,07
640	22,03±3,97[a]	56,83±2,36[c]	30,03±4,44[a]
1280	36,76±3,07[b]	87,52±2,75[c]	40,07±3,44[c]
2560	53,53±1,16[c]	85,35±3,92[c]	60,87±4,73[c]
Positive	12,97±0,73	12,97±0,73	12,97±0,73

Each value (% proliferation) represents the mean ± S.E. of triplicates comparing to the positive control (culture with BCG alone). [a]P<0.05, [b]P<0.01 and [c]P<0.001 are difference vs non-treated cultures.

Table 2: Effects of treatment with the three *P. kotschyi* extracts for 48 h on in vitro phagocytosis of macrophages as measured by neutral red uptake

Concentration (µg/ml)	*Treatments*		
	Aqueous extract	***Methanol extract***	***Hexane extract***
160	0,55±0,27^{c}	34,03±4,51	0,69±0,00
320	2,20±0,63^{c}	42,36±1,93^{b}	5,56±1,73^{c}
640	3,30±1,26^{c}	48,26±2,11^{c}	11,81±0,91^{c}
1280	13,74±2,85^{b}	65,28±1,39^{c}	17,01±0,91^{a}
2560	22,58±1,61	58,33±0,60^{c}	19,44±1,25
Positive	24,04±1,92	24,04±1,92	24,04±1,92

Each value represents the mean ± S.E. of triplicates comparing to the positive control (culture with BCG alone). $^{a}P<0.05$; $^{b}P<0.01$ and $^{c}P<0.001$ are the difference vs positive control.

Table 3: Effects of treatment with the three *P. kotschyi* extracts for 48 h on in vitro phagocytosis of macrophages as measured by NBT reduction test

Concentration (µg/ml)	*Treatments*		
	Aqueous extract	***Methanol extract***	***Hexane extract***
Control	1,00±0,00	1,00±0,00	1,00±0,00
160	0,94±0,01	0,41±0,03**	0,38±0,02***
320	1,10±0,27	0,42±0,02**	0,63±0,04*
640	1,25±0,22	0,42±0,12**	0,80±0,11
1280	1,09±0,10	0,61±0,18	1,25±0,04
2560	1,00±0,08	0,98±0,11	1,40±0,09
Positive	1,47±0,04*	1,47±0,04*	1,47±0,04*

Each value represents the mean ± S.E. of triplicates comparing to the negative control (culture in medium alone). [a]P<0.5; [b]P<0.01 and [c]P<0.001 are the difference vs negative control. Culture with BCG alone was considered as positive.

Table 4: Effects of extracts of *P. kotschyi* on *in vitro* NO production of macrophages

Concentrations (µg/ml)	***Treatments***		
	Aqueous extract	***Methanol extract***	***Hexane extract***
0	5,31±0,08	5,31±0,08	5,31±0,08
160	7,23±0,10	8,24±0,26	9,56±0,11[a]
320	8,06±0,11[a]	8,55±0,02[a]	8,88±0,30[a]
640	8,73±0,22[b]	8,96±0,28[a]	9,95±0,57[b]
1280	11,14±0,06[c]	10,70±0,00[c]	10,26±0,38[b]
2560	17,56±0,23[c]	12,03±0,62[c]	12,79±0,61[c]
Positive	6,85±0,30	6,85±0,30	6,85±0,30

Each value (Nitrite Production, mM /2.5x10^5 Cells) represents the mean ± SD. of duplicates comparing to the positive control (culture with BCG alone). [a]P<0.05, [b]P<0.01 and [c]P<0.001 are difference vs positive.

Table 5: Effects of the three *P. kotschyi* extracts on NO production of macrophages in the presence of L-NMME inhibiting the iNOS activity.

Concentrations (µg/ml)	Treatments		
	Aqueous extract	Methanol extract	Hexane extract
Negative	9,36±1,29	9,36±1,29	9,36±1,29
160	13,34±3,01	10,65±2,65	13,58±1,76
320	19,39±2,87[b]	18,19±2,34[b]	21,5±3,11[b]
640	26,72±1,49[c]	20,30±1,67[c]	21,11±2,19[b]
1280	31,90±1,80[c]	21,77±0,95[a]	23,22±0,99[c]
2560	38,33±0,38[c]	24,62±0,19[b]	26,75±1,12[c]
Positive	23,43±2,32	23,43±2,32	23,43±2,32

Each value (Nitrite Production, mM /3x10^6 Cells) represents the mean ± SD. of duplicates comparing to the negative control (culture in medium alone). [a]P<0.05, [b]P<0.01 and [c]P<0.001 are difference vs negative control.

Table 6: Effects of the three *P. kotschyi* extracts on lysosomal enzyme activity of macrophages

Concentrations	***Treatments***		
(µg/ml)	***Aqueous extract***	***Methanol extract***	***Hexane extract***
160	133,59±4,92[c]	73,74±7,00	44,53±6,67
320	177,24±1,09[c]	103,72±13,35[b]	45,30±2,84
640	237,20±6,78[c]	112,25±5,03[b]	49,89±2,41
1280	232,60±0,65[c]	120,13±1,75[b]	56,46±9,19
2560	246,17±3,94[c]	64,99±12,05	64,77±4,16
Positive	26,70±0,86	26,70±0,86	26,70±0,86

Each value (% activity) represents the mean ± SD. of duplicates comparing to the positive control (culture with BCG alone). [a]P<0.05, [b]P<0.01 and [c]P<0.001 are difference vs positive control.

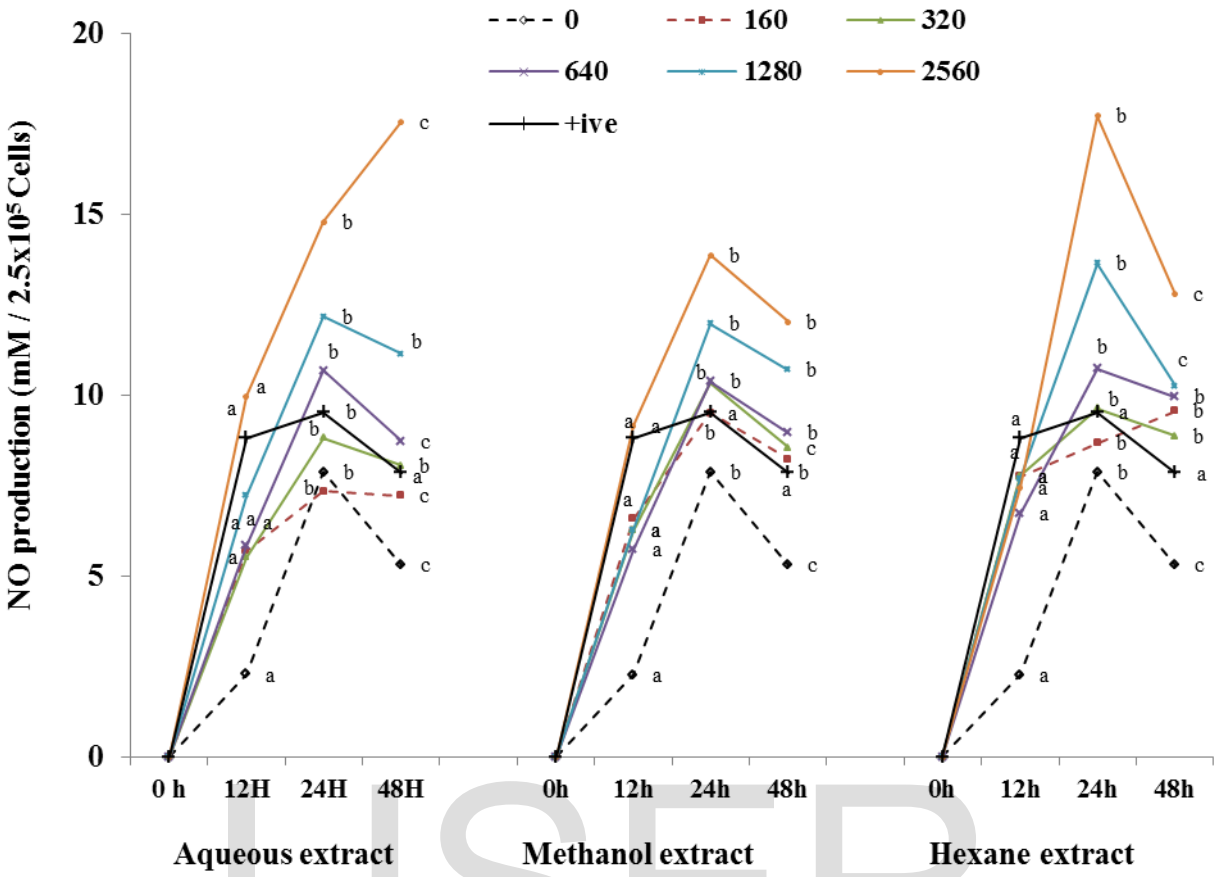

Fig. 1: Time-dependent induction of NO production by AAP in human macrophages. BCG was used as a positive reference or antigen and control indicated the untreated negative group. NO production was determined by measuring the accumulation of nitrite in the medium. Data are mean of three separate wells.

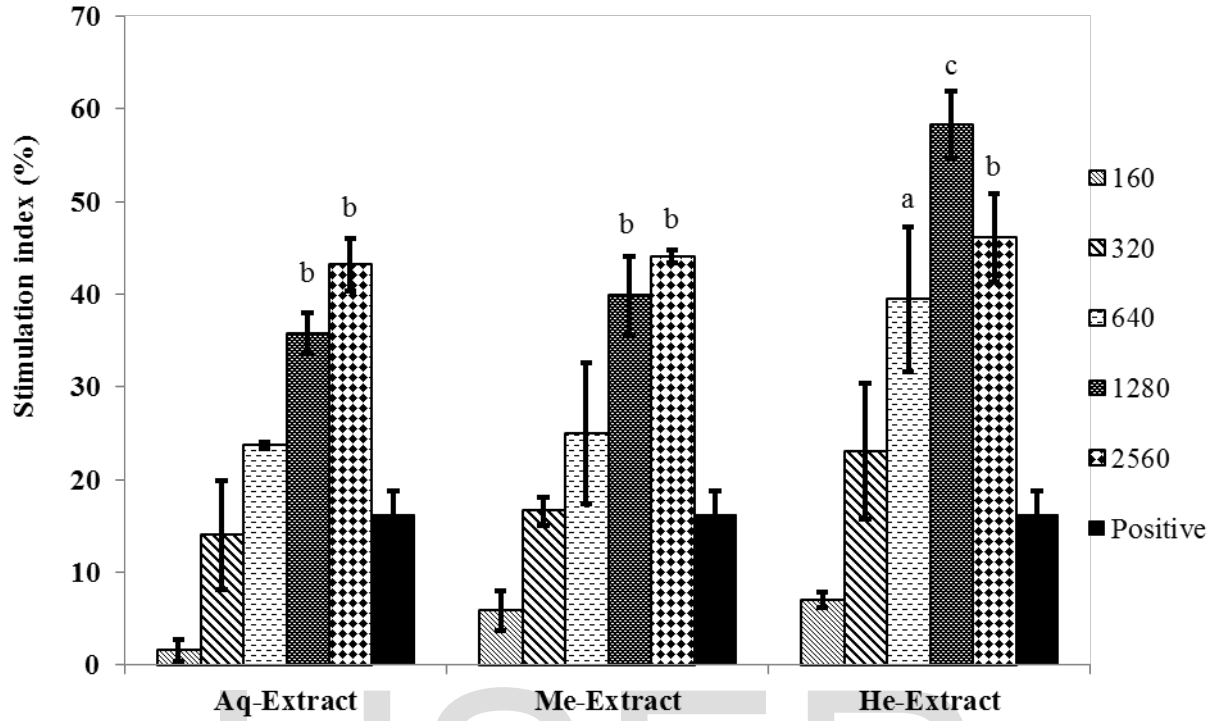

Fig. 2: *In vitro* effect of *P. kotschyi* on myeloperoxydase activity in macrophages. Histograms represents the mean ± S.E. of triplicates comparing to the positive control (culture with BCG alone). $^{a}P<0.05$; $^{b}P<0.01$ and $^{c}P<0.001$ are the difference vs positive control**.**

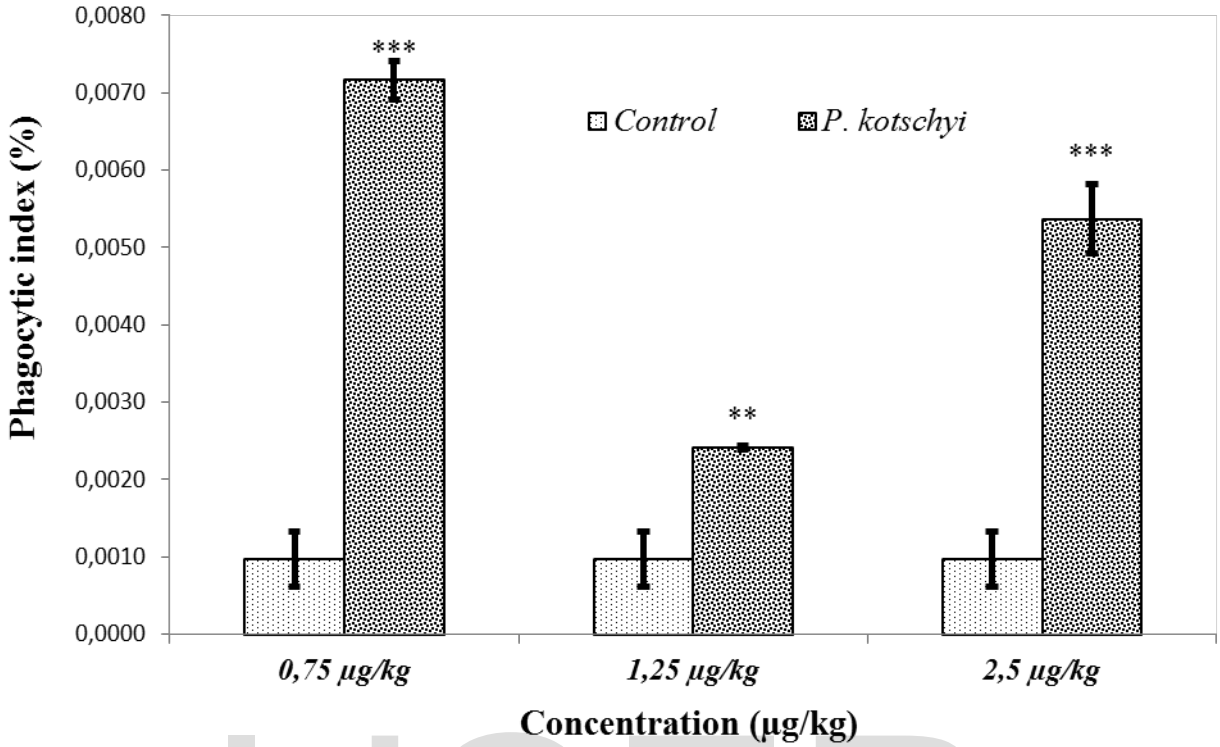

Fig. 3: *In vivo* effect of aqueous extracts on phagocytosis of macrophages. **P<0.01 and ***P<0.001 were the difference from non-treated mice (control).