Libyan Agriculture Research Center Journal International 1 (3): 128-133, 2010
ISSN 2219-4304

Immunologically Active Polysaccharides Isolated from *Anacyclus pyrethrum*

[1]Bendjeddou Dalila, [2]Lalaoui Korrichi and [2]Satta Dalila

[1]Department of Biology, Faculty of Sciences, 8 Mai 1945 University, Guelma (24000), Algeria
[2]Department of Animal Biology, Faculty of Natural Sciences,
Mentouri University, Constantine (25000), Algeria

Abstract: Crude polysaccharide mixture obtained from the roots of *Anacyclus pyrethrum* with hot-water extraction followed by ethanol precipitation and deproteinization by Trichloro acetic acid (TCA) and Sodium acetate (NaOAc) treatment, was divided in two main fractions by precipitation with increasing concentrations of ethanol (1:1, 1:2 v/v), respectively. Both fractions were separated by gel filtration chromatography into PSI, PSII, PSIII. The effect of those different fractions on lymphocyte activity was studied. The polysaccharides markedly stimulated the proliferation of murine spleen cells *in vitro* measured by MTT colorimetric assay. Fractionation analysis and alkaline phosphatase inducing activity method, the results showed that the mitogenic effect of PSI, PSII, PSIII was predominantly, on T cell population but they activated B cells indirectly via T cells. Furthermore, the three polysaccharides enhanced the reticulo-endothelial system potentialing activity by increasing the clearance rate of carbon particles.These results suggested that the three polysaccharides extracted from *A. pyrethrum* have immunostimulating activity which could be used clinically for the modulation of immune systems.

Key words: Plant polysaccharides % *Anacyclus pyrethrum* % Mitogenic activity alkaline phosphatase-inducing activity % Reticulo-endothelial system potentialing activity

INTRODUCTION

Polysaccharides isolated from many medicinal plants represent a large class of biopolymers with a structure variability which is the basis for their biological activities. This enormous potential of proprieties can play a role in the various applications of the polysaccharides in the broad field of pharmacy and medicine Franz [1]. The main purpose of this study is to prepare polysaccharides from the roots of *Anacyclus pyrethrum* (L.) Link Fam. Compositae (traditional medicinal plant used in Algeria for treatment of several diseases as infection, chronic bronchitis and cough) and to investigate their effect on immune system in order to find new immunostimulants for the prevention and supporting treatment of cancer and immunodeficiency related diseases.

MATERIALS AND METHODS

Plant Material: The roots *of Anacyclus pyrethrum* were harvested from the region of Constantine (North-east of Algeria). The samples were thoroughly washed with tap water and ethanol, air dried and finely powdered.

Extraction: The polysaccharide extract was prepared as described by Bendjeddou *et al.* [2]. The root powder (300 g) was suspended in distilled water at the rate of 1g powder per 20 ml of water. The suspension stirred on the water bath at 100°C for 3 hours, cooled, kept over night at 4°C and then centrifuged for 30 minutes at 6000 rpm to remove residues. The supernatant was reduced to half volume by evaporation under reduced pressure at 60°C, filtered to remove any residues and the polysaccharides were precipitated by addition of 4 volumes ethanol 95%. The precipitate was collected by centrifugation for 30 minutes at 10000 rpm, dried, dissolved in distilled water, dialysed and then treated with (TCA) and (NaOAc) to remove proteins Wagner *et al.* [3]. The preparation was precipitated with 4 volumes ethanol to obtain hot water soluble polysaccharide fraction (HWSPF).

Fractionation: The (HWSPF) was dissolved in distilled water, separated into two fractions by reprecipitation with increasing concentration of ethanol (1:1, 1:2 v/v) respectively. Both fractions were applied to a column (60 x 2cm) of Sephadex G200. The column was eluted with distilled water, the eluate was collected in 5ml portions.

Corresponding Author: Bendjeddou Dalila, Laboratory of Molecular and Cellular Biology Department of Animal Biology, Faculty of Natural Sciences, University Mentouri Constantine, Algeria Tel: ++(213) 0774288184

The eluate from the same peak was concentrated and lyophylised to obtain PSI from the fraction (1:1) and PSII, PSIII from the fraction (1:2).

Biological Activity: Each polysaccharide fraction (PSI, PSII, PSIII) was examined for its effect on the lymphocyte activity.

Preparation of Cell Suspensions: Lymphocyte suspensions were harvested from spleens of normal mice ddY (4 weeks-old) and red blood cells were removed with cell lysing buffer 0.83% ammonium chloride [4, 5]. The lymphocyte rich fluid was applied to sephadex G10 column for depletion of macrophages according to Severinson & Larsson [6]. The lymphocyte suspension was divided in two parts. One part was designated unfractionated lymphocyte suspension, the other part was placed through nylon wool columns to obtain enriched-T-cell suspension. Enriched-B lymphocytes were obtained by washing off nylon wool adherent cells into cold culture medium RPMI 1640 and recovered cells were then further depleted of T cells by a second sedimentation through a nylon wool column Julius *et al.* [7]. The number of the viable cells of each suspension was determined microscopically by trypan blue exclusion test.

Clearance Rate of Carbon Particules: The clearance rate of carbon according to the technique given by Biozzi *et al.* [8] was taken as measure of phagocytic activity of the reticulo-endothelial system of mice. The test samples (PSI, PSII, PSIII) and the positive control (Zymosan) were dissolved in physiological saline and injected intra-peritoneal (i.p) into groups of 4 weeks old ddYmice (five mice per group and each mouse on average weighed between 20 and 25g). Each mouse received 0.5 ml of the saline containing 0 (control), 50 mg of polysaccharide material or Zymosan per Kg body weight. After a stabilization period of 48 h, the fine carbon suspension prepared beforehand from black "Pelikan" Ink as described by Biozzi *et al.* [8], was injected at a rate of 0.1 ml per 10 g body weight into the tail vein of each mouse. Samples of blood (0.02 ml per mouse) were drawn with a capillary from the retro-orbital venous plexus at intervals of 5 and 15 min after the carbon injection. Each blood sample was hemolyzed immediately by the addition of 3 ml of 0.1% Na_2CO_3 solution The optical density of each sample at a wavelength of 675 nm for the two time periods was used to calculate the phogocytic index *K*, stimulation index and half-life period of carbon in the blood ($t_{1/2}$) by the following equations according to [9, 10].

$K = \text{Ln } OD_1 - \text{Ln } OD_2 / t_2 - t_1$ were OD_1 and OD_2 are the optical densities at time t_1 and t_2 respectively.

Stimulation index = K of the treated animal / K of the untreated animal $t_{1/2} = 0.693 / K$.

Mitogenic Activity: It was measured using MTT [3-(4,5-Dimethylthiazol-2yL) (2.5 Diphenyltetrazolium bromide; Sigma] colorimetric assay according to [11, 12]. Each cell suspensions obtained as described before, was adjusted to a value of ($2x10^6$ cell/ml) in complete RPMI 1640 medium containing 10% fetal calf serum, penicillin (100U/ml), streptomycin (50 µg/ml) and 2 mM glutamine. 100 µl of the lymphocyte suspension were pippetted into each well before adding in triplicate one of the polysaccharide extracted material (100 µl per well) at a concentration of 25µg/ml. The cell suspension without sample was taken as control and Pokeweed mitogen (PWM) from *Phytolacca americana* was used at a concentration of 5µg/ml as positive control culture. The cell culture was incubated at 37°C in humidified atmosphere. MTT solution was added 4 hours prior the culture termination (Bendjeddou et a.l., 2003) and the optical density was measured at 570 nm. The results were expressed as stimulation Index (MIT SI) = mean OD in treated group/mean OD in control group.

Alkaline Phosphatase Assay: This assay was measured as described by Ohno *et al.* [13]. The cell culture was performed similarly to the mitogenic assay. After 72 hours of culture, cells were collected, washed with 10 mM of phosphate buffer (pH 7.2). 50 mM of carbonate buffer (pH 9.8) containing P. nitrophenyl - phosphate (0.1 mg/ml and $MgCl_2$ (1mM) was added to the cell suspension. The reaction mixture was incubated for 1 hour and the absorbance was measured at 405 nm. The results were expressed as the stimulation Index (AL.P SI) = mean OD in treated group/mean OD in control group.

Statistical Analysis: All the data obtained during the investigation are expressed as mean ± S.E. Student's *t*-test was used to compare the difference between treated and control groups.

RESULTS

Extraction and Fractionation: The crude extract was obtained from the root material by hot water extraction, followed by precipitation with ethanol. The dried precipitate (31.98 g = 10.66%) was dialysis and deproteinized by TCA treatment to obtain the hot water

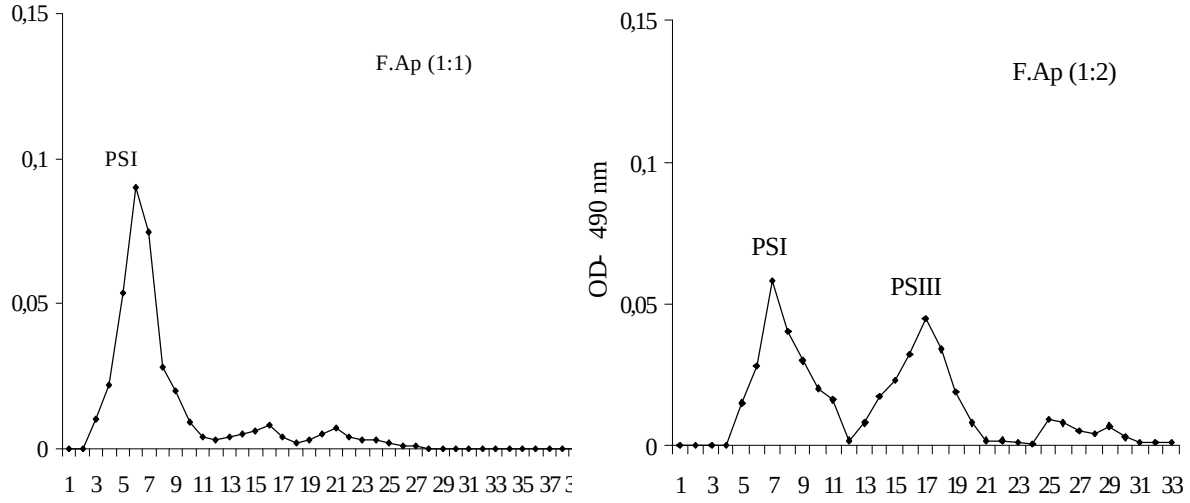

Fig. 1: Hot water polysaccharides fractions (HWPFs) from *Anacyclus pyrethrum* were applied to a column (60 x 2cm) of Sephadex G200. The column was eluted with distilled water, the eluate was collected in 5ml portions

Table 1: Effect of PSI from *A. pyrethrum* on the clearance of carbon from the circulating blood of mice

Treated groups [a]	Dose (mg/Kg) [b]	Phagocytic index K_t (mean ± S.E.)	Stimulation index K_t / K_c [c]	Half time of carbon $t_{1/2}$ (min)
PSI 50	0.0828 ± 0.0028*	1.66	8.37 ± 0.18*	
PSII 50	0.1406 ± 0.0034**	2.82	4.92 ± 0.28 **	
PSIII	50	0.1068 ± 0.0084***	2.14	6.49 + 1.24***
Zymosan	50	0.1296 ± 0.0063**	2.40	5.35 ± 0. 12**
Control		0.0498 ± 0.0077	1	13.91± 0.51

The significance was evaluated by means of Student's *t*-test

[a] In each group five mice were used. Test substances were injected *i.p.* at 48 h before the *i.v.* injection of carbon solution. The mice of control were received saline alone.

[b] 50 mg/Kg is the best efficiency dose obtained from a series of experimental tests (results not shown)

[c] $K_t / K_c > 1$ (immunostimulation), $K_t / K_c < 1$ (immunosuppression)

*$P < 0.05$, ** $p < 0.01$, *** $p < 0.001$

soluble polysaccharide extract. The yieled relative to dry sample weight was 4.92%. The extract was dissolved in water, separated by reprecipitation with increasing concentrations of ethanol into two fractions F.Ap (1:1) and F.Ap (1:2) (Yieled: 94.67 and 217.2 mg respectively). Each fraction was subjected to gel filtration through a column of Sephadex G200. The F.Ap (1:1) gave a major peak at (15-55 ml) which was designated PSI. The fraction F.Ap (1:2) was separated into two major peaks at (25-55 ml) and (65-100 ml). The fractions constituting the first and the second peaks were termed PSII and PSIII, respectively (Fig. 1). The tubes containing each of these polysaccharides were combined separately, dialysed against distilled water, concentrated, precipitated with ethanol and air dried (Yieled: 55.84, 30.93 and 24.09 mg) for PSI, PSII and PSIII respectively.

Biological Activity: Because of the importance of macrophages and lymphocytes, which are among the major effector cells involved in the induction of immune responses, our study described the immunolological effects of PSI on the reticulo-endothelal system (RES) potentialing and mitogenic activities on murine spleen cells. The effect of PSI, PSII and PSIII on the RES was demonstrated by a modification of the in vivo carbon clearance, using Zymosan as positive control. As shown in Table 1, a marked increase in the phagocytic response was obtained following single *i.p.* administration of PSI, PSII and PSIII at the dose of 50 mg/Kg. The *K*-value and the stimulation index were increased and the half-life time $t_{1/2}$ of carbon in the blood was decreased to (8.37, 4.92, 6.49 min) after PSI, PSII and PSIII treatment respectively. These values differed significantly from those of the control group. It was remarked also that PSII

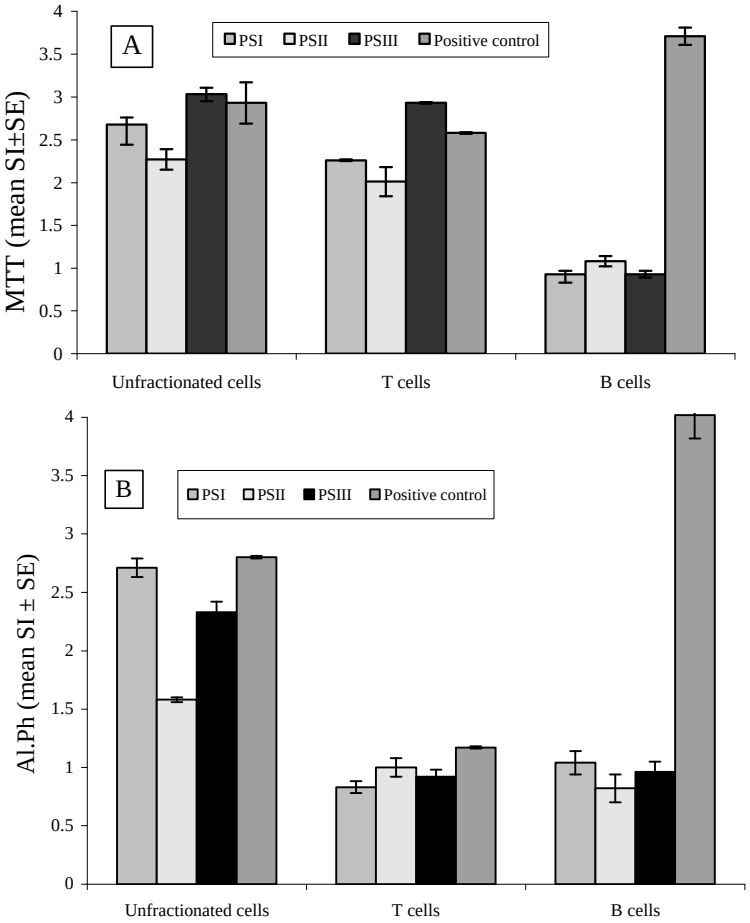

Fig. 2: Mitogenic activity of the three polysaccharides PSI, PSII, PSIII and the positive control was measured by A: MTT colorimetric assay, B: alkaline phosphatase inducing activity method. Values represent the mean SI ± SE. of triplicate cultures. Lymphocytes were collected from spleen of normal mice, separated into three populations (unfractionated cells, T cells and B cells), cultured 3 days in vitro with samples or positive control (Pokeweed for the unfractionated and T cells and LPS for B cells) and the mitogenic activity was measured.

treatment was exerted a phagocytic activity better than observed with 50 mg/Kg of Zymosan ($t_{1/2}$ = 5.35 min).

In Addition to the distinguished RES activity, we have found a remarkable mitogenic activity of the murine spleen lymphocytes. The unfractionated spleen lymphocytes B and T purified cells were treated in vitro with PSI, PSII, PSIII (50 µg/ml) or one of the specific mitogens (Pokeweed or LPS). After incubation of 72 h, the lymphocyte proliferation was measured by MTT colorimetric assay or by alkaline phosphatase activity. As shown in Fig. (2a), the three polysaccharide tested markedly enhanced the unfractionated lymphocyte transformation. The stimulating index increased to a value of 2.68, 2.27 after PSI and PSII treatment respectively this was comparable to that given by Pokeweed (SI= 2.93), while PSIII showed an activity higher than the positive control (Pokeweed) with a SI = 3.03. A similar pattern was seen using purified T cells. The stimulation increased to 2.26, 2.01, 2.93 and 2.58 after PSI, PSII, PSIII and Pokeweed treatment respectively.

In contrast to that, the purified B cells did not respond to the all polysaccharide treatments, while the LPS (B cell mitogen) exhibited a strong enhancement of the B cell proliferation with a high stimulation index (3.71). From the results of Fig. (1.B), it was remarked that an increase effect of PSI on the alkaline phosphatase activity, was observed with the unfractionated lymphocytes only followed by PSIII and PSII respectively. In this case, PSI had a stimulation index comparable to that given by the positive control (2.71 and 2.80 respectively).

DISCUSSION

From the results obtained from the carbon clearance test, it was confirmed that that PSI, PSII and PSIII enhanced the activity of macrophage in vivo, an integral component of the RES Van Furth *et al*. [14]. This effect, which was obtained with various polysaccharides especially some glucans, is of a great significance in view of the fact, that macrophages form the first line of host defense against foreign invasion and neoplastic diseases. In the other hand, the MTT assay results indicated that the polysaccharides PSI, PSII and PSIII had a mitogenic effect on T and unfractionated lymphocytes, but the stimulation in the case of unfractionated cells was higher than that observed with T cells alone. This, indicates that T cells might not to be the only class responding to the mitogenic material and that B cells were contributing to the increase proliferation of unfractionated lymphocytes of murine spleen.

This latter suggestion was confirmed by the measurement of the alkaline phosphatase activity, which was used as a marker for blastoid B cell assay Garcia *et al*. [15]. Since alkaline phosphatase activity was induced by the three polysaccharides in unfractionated spleen lymphocytes, but not with purified B cells, it was concluded that this stimulation would result from the activation of B lymphocytes stimulated indirectly by soluble factors produced by T cells after their treatment with the test samples. This effect was observed by earlier workers, with the other plant-originated polysaccharides [16, 17].

CONCLUSION

From these series of experiments we confirmed that all of PSI obtained by hot water extraction from the roots of *A. pyrethrum* enhanced the non specific immune response as provided by the data of macrophage activity. It also induced T lymphocyte stimulation not only to proliferate, but to produce and release soluble factors which play a major role in the humoral immunity. These, suggest that PSI, PSII and PSIII could potentially be immunostimulating compounds with a large therapeutic implication in the host defense.

Further work on the chemical structure determination of PSI, PSII and PSIII is progressing in order to find the structure-activity relationship of these immunostimulatory polysaccharides.

ACKNOWLEDGEMENTS

We are grateful to Dr Mazurier, J. (University of Lille-1. France), for his help to complete some parts of this work.

REFERENCES

1. Franz, G., 1989. Polysaccharides in pharmacy: Current application and future concepts. Planta Medica, 55: 493-497.
2. Bendjeddou, D., K. Lalaoui and D. Satta, 2003. Immunostimulating activity of the hot water-soluble polysaccharide extracts of Anacyclus pyrethrum, Alpinia galanga and *Citrullus colocynthis.* J. Etnopharmacol., 88: 155-160.
3. Wagner, H., H. Stuppner, W. Schafer and M. Zenk, 1988. Immunologically active polysaccharides of *Echinacea purpurea* cell cultures. Phytochemistry, 27(1): 119-126.
4. Daun, J.R., D.M. Shepherd and J.N. Randolph, 1995. Physical interactions and early signaling between Helper T lymphocytes and B lymphocytes In: G.R. Burleson, J.H. Dean, A.E. Munson, (eds.), Vol. 1. Wileys-liss-Inc., New York, Chichester, Toronto, Singapore, pp: 469-481.
5. Ducan, D.D. and D.A. Lawrence, 1995. T cells and cloned and transformed T-cell lines to assess in immune function In: G.R. Burleson, J.H. Dean, A.E. Munson, (eds.), Vol. 1. Wileys liss Inc., New York, Chichester, Toronto, Singapore, pp: 483-505.
6. Severinson, E.and E.L. Larsson, 1978. Lymphocyte responses to polyclonal B and T cell activators. In: D.M. Weir, (eds.), Vol. 2. Blackwell, Oxford, pp: 63.1-63.15.
7. Julius, M.H., E. Simpson and L.A. Herzenberg, 1973. A rapid method for isolation of functional Thymus-derived murine lymphocytes. European J. Immunol., 3: 645-649.
8. Biozzi, G., B. Bernacrraf and B.N. Halpern, 1953. Quantative study of granulopectic endothelial system. II. A study of the kinetics of the granulopectic acticity of the RES in relation to the dose of carbon injected. Relationship between the weight of the organs and their activity. British J. Experimental Pathol., 34: 441- 456.

9. Wagner, H., A. Proksch, I. Reiss-Maurer, A. Vollmar, S. Odenthal, H. Stuppner, K. Jurcic, M. LeTurdu and Y.H. Heur, 1984. Immunstimulierend Wirkend Polysaccharide (Heteroglykane) us Noheren pflanzen. Arzneimittelforschung Drug Res., 34: 659-661.
10. Kiho, T., M. Ito, K. Nagai, C. Hara and S. Ukai, 1987. Polysaccharides in fungi. XX. Structure and antitumor activity of branched (1-3)-B-D-Glucan from alkaline extract of Yu er. Chemical and Pharmaceutical Bulletin, 35: 286-293.
11. Mosmann, T., 1983. Rapid Colorimetric Assay for cellular growth and survival. Application to proliferation and cytotoxicity Assays. J. Immunological Method, 65: 55-63.
12. Smialowicz, R.J., 1995. In vitro lymphocyte proliferation assays. The mitogen-stimulated response and the mixed lymphocyte reaction in immunotoxicity testing. In: G.R. Burleson, J.H. Dean and A.E. Munson, (eds.), Vol. 1. Wileys liss Inc., New York, Chichester, Toronto, Singapore, pp: 197-210.
13. Ohno, N., Y. Aral, I. Suzuki and T. Yadomae, 1986. Induction of alkaline phosphatase activity in murine spleen cells treated with various mitogens. J. Pharmacobio Dyn, 9: 593-599.
14. Van Furth, R., Z.A. Cohn, I. Reiss-Maurer, J.G. Hirsch, J.H. Humphery, W.G. Spector and H.L. Landevooth, 1972. The mononuclear phagocytic system: A new classification of macrophages, monocytes and their precursor cells. Bulletin of WHO 46: 845-852.
15. Garcia Rozas, C., A. Plaza, F. Diaz Espada, M. Kreisler and C. Martinez-Alonso, 1982. Alkaline phosphatase activity as membrane marker for activated B cells. J. Immunol., 129: 52-55.
16. Lee, Y.S., I.S. Chung, I,R, Lee, K,H, Kim, W.S. Hong, and Y.S. Yun, 1997. Activation of multiple effector pathways of immune system by the antineoplastic immunostimulator acidic polysaccharide ginsan isolated from *Panax ginseng*. Anticancer Res., 17(1A): 323-331.
17. Shan, B.E., Y. Yoshida, T. Sugiura and U. Yamqshita, 1999. Stimulating activity of Chinese medicinal herbs on human lymphocytes in vitro. International J. Immunopharmacol., 21(3): 149-159.