THE PROTECTIVE EFFECTIVENESS OF *Phaleria macrocarpa* EXTRACT ON THE PERIPHERAL IMMUNE SYSTEM IN IMMUNOCOMPROMISED RABBITS

Imad Dawood Saleh*, Haniza Hanim Mohd Zain*, Husni Ibrahim*, Omar Emad Ibrahim, Noman D Salih***, Hanan Kumar Gopalan*****

*Universiti Pendidikan Sultan Idris, Faculty of Science and Mathmatics, 35900 Tanjong Malim, Perak, Malaysia.

**Centre of Preclinical Sciences Studies, Faculty of Dentistry, Universiti Teknologi MARA, 47000 Sungai Buloh, Selangor, Malaysia.

***Universiti Kuala Lumpur, Institute of Medical Science Technology, A1-1 Jalan TKS1, Taman Kajang Sentral, 43000 Kajang, Malaysia.

ABSTRACT: *Immunonutritions are effective in improving outcomes in wide range of individuals, particularly malnourished, and reported to improve the immune status of perioperative patients, thereby reducing complications and length of hospital stay. This study aimed to investigate the protective effectiveness of Phaleria macrocarpa extract on the peripheral immune system in immunocompromised rabbits and to assess whether the daily uptake of specific dosage could improve the immune cells responses in these animals. Prior to subdividing experimental groups and sampling we run several pilot studies to design our protocol and to set the accurate dosage of the immunosuppresser drug (Imuran®). Daily doses of 20 mg/kg for 15 days were adequate to produce immunocompromised status in rabbits and to have them fully immunocompromised, followed by treatment with the plant extraction (500 mg/kg for another 15 days). The mean of absolute full blood count (FBC) showed a significant improvement in the treated group (9.16±0.39 x10^9) compared to non-treated (3.20±0.59 x109) and control (9.68±0.30 x109) groups. Differential cells profiles analysis (flowcytometry technique) of lymphocytes, neutrophils, B-Cells, as well as, CD4 and CD8 T-lymphocyte showed that treatment with the extraction has maintained the populations of these cells significantly, treated group showed increment in the cell percentages of CD4 (24%), lymphocytes (23%), neutrophils (19.8%) and B-Cells (18.9%), Compared to the non-treated group CD4 (10.6%) and neutrophils (13.3%), while control group showed less than 10% of all cells populations (ANOVA analysis).This extract has significantly improved specific and nonspecific immune system cells, through the modulation of their populations and peripheral ratios, which thus, can reflect to improvement in overall immunity. The use of this herbal treatment can be developed and used by human to develop promising supplement, which could make it easier to adapt the systemic inflammation and oxidative stress, caused by immunosuppressive medications, given usually a result of disease, malnutrition, HIV infections, pregnant females, patients who are undergoing chemotherapy or radiation therapy for certain cancers and genetic disorders.*

Key word: Immunonutritions, Immune System, Immunocompromised Rabbits, *Phaleria macrocarpa*

1. INTRODUCTION

Immunodeficiency refers to the state when immune system's ability to fight infectious disease is compromised or absent. Most cases of immunodeficiency are secondary (acquired), but some individuals are born with defects in their immune systems, or primary immunodeficiency. A person who has an immunodeficiency of any kind is said to be immunocompromised. An immunocompromised person is more susceptible to additional opportunistic infections, and liable to more serious infections and/or complications than normal people [1], because of the failure of his immune system to develop normal immune responses compared to healthy individuals. There are variety of factors could enhance the body to induce inflammatory responses those could become excessive, and damaging in some patients, attenuated by administration of immunosuppressive drugs, such as chemotherapy therapy, radiation therapy for cancer, patients undergo organ or tissue transplant, malnutrition, pregnant females, or certain disease processes such as Human Immunodeficiency Virus (HIV) or Acquired Immune Deficiency Syndrome (AIDS), in addition, certain cancers and genetic disorders [2]. Health and immunity has been thought to depend, somehow, on nutrition, since long time ago, by preventing nutritional diseases, like malnutrition, rickets, and scurvy. Aside of that, the food we consume can contribute to maintain our health and to prevent broad range of diseases [3], in addition, veriety of folklore medicines and potions are claim to enhance the healing process naturally at times of stress, accelerating a return to normal [4]. The additional effects exhibited by the type of cumcumed food occur by modulation of our basic physiological activities, including immune, endocrine, nerve, circulatory, and digestive systems. Nutrient deficiencies have confirmed to impair immune responses and lead to frequent severe infections and increased mortality, especially in children [3,4]. *Phaleria macrocarpa*, is a plant from the family of Thymelaeaceae, indigenous to Indonesia and Malaysia [5]. Traditionally, it contributes to the vitality of human health, whereas, it has reported lately for numbers of valuable medicinal properties, such as anti-cancer, anti-inflammatory, anti-fungal, anti-oxidant, anti-bacterial, wound healing, and vasorelaxant activities [5,6], in addition, recent studies on animal models have confirmed the anti-diabetic efficacy of the fruits and some other parts of this plant [5-10]. Therefore, the current study has designed to investigate the possible effectiveness of daily uptake of Phaleria macrocarpa extract on the peripheral immune system and improvements of immune cells responses in immunocompromised rabbits.

2. MATERIALS AND METHODS

2.1. Plant Extract

Leaves of *P. macrocarpa* were collected from the northwestern part of Malaysia (Kedah), and taxonomically identified. They are dried and crushed in an electric grinder, and pulverized into a coarse powder form. The methanolic extraction has prepared by soaking of 100g of the coarse powder in a conical flask with the mixture solvent, which consists of 240ml distilled water and 320ml absolute methanol. The whole mixture kept for three days in an incubator at 37ºC. Then after it stirred intermittently at 4 hrs intervals. Then filtered, filtrated, and dried under low temperature and low pressure of rotary evaporator that fitted with a vacuum pump, and at the end of

the process, 24g of the powder was collected. This powder sample dissolved in normal saline and subdivided depending on the chosen dose for treatment.

2.2. Animal Preparation

The study has approved earlier by our Ethics Committee. Total of 20 New Zealand white rabbits (3 months, 2.5-3.0kg) obtained from the "Experimental Animal Breeding and Research Centre" (Selangor, Malaysia), hosted under controlled laboratory conditions, and inspected twice a week for healthy checkup purposes. The rabbits have randomly divided into four groups of five animals each: group 1 consists of normal rabbits (control), group 2 Immunocompromised rabbits (up to 15 days), group 3 Immunocompromised with normal diets (up to 30 days), and group 4 Immunocompromised with extract treatment, as immunonutrition, (up to 30 days).

2.3. Immunosuppresser Induction

Azathioprine (AZA), also known as Imuran®, is an immunosuppressive drug, used in major hospitals for admitted patients with organ transplantation and autoimmune diseases, belongs to the chemical class of purine analogues. It is well known to strongly affect proliferating cells, such as T-cells and B-cells of the immune system. The Immune suppression of our experimental rabbits has been created by an admission of daily doses (20 mg/kg) of imuran solution for 15 days. Blood samples collected every 72 hrs to monitor the blood cells counts and immune cells status of each rabbit.

2.4. Plant Extract Treatment

The treatment with the extraction has done by enteral route administration directly into the mouth by orogastric gavage. The Immunocompromised rabbits (group 4) continued with the treatment (enterally fed) of a single dose the methanolic extraction (500 mg/kg) for another 15 days.

2.5. Blood Measurements

Blood samples were collected via the marginal ear vein. Each sample is divided and kept in EDTA tube (BD Vacutainer) and plain tube (BD vacutainer) for laboratory tests. The evaluation of immune cells status in blood was measured through full blood count, standard panel of T-cells (CD3), B-cells (Mouse anti Rabbit IgM), and subset T-Lymphocyte (Mouse anti Rabbit CD4, Mouse anti Rabbit CD8) using Flow Cytometry (BD FacsCanto II) Technique, included mixture kits of antibody CD45 APC, antibody CD14 PE, antibody T-Lymphocyte FITC, antibody B-cells FITC, antibody CD4 FITC, and antibody CD8 FITC), using 4 ml of whole blood sample (EDTA tube). Full blood count (FBC) analysis (Beckman Coulter) is used, and leukocyte differential count (LDC), which is not just the count of total white blood cells (WBC) but counts also each WBC type, such as neutrophils, basophils, eosinophils, monocytes, and lymphocytes. The instrument measures the type of blood cells by analyzing data about the size and aspects of light as they pass through the cells (called front and side scatter), and measures different characteristics of all the cells to categorize them.

2.6. Statistical Analysis

Rresults are expressed by Mean±SM. The significance of differences is confirmed by one-way ANOVA and multiple Dunnett t-tests, where $P<0.05$ is considered significant, while $P<0.001$ is highly significant differences for all cases.

3. RESULTS

The mean of the total WBC count for the four groups of rabbits, showed a significant decrease in the WBC count of the Immunocompromised animals group (4.36±0.59) and Immunocompromised without treatment group (6.48±2.2) compared to control group (9.60±0.3), while, treated Immunocompromised animals where almost back to normal levels (9.24±0.39) in comparision with the controls, along the total of 30 days of the experiment (Figure 1).

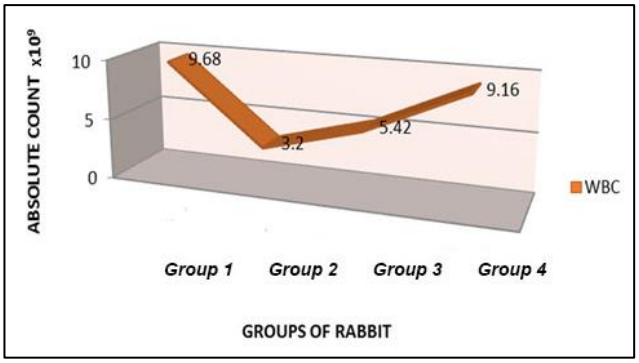

Figure 1: WBC counts for the four groups studied (x10^9)

FBC differential analysis indicated that lymphocytes level of the treated group has scored the higher percentage (32%) compared to immunocompromised group (21%) and immunocompromised without treatment at day 30 (26%). However Neutrophils have did not show any significant differences among immunocompromised groups, while monocytes were higher in treated group (22%) compared to non treated (12%), these results are shown in Figure 2.

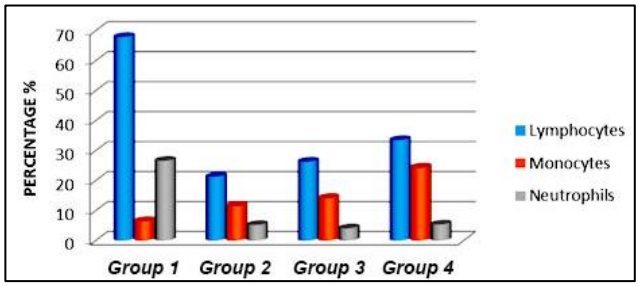

Figure 2: Comparisson of the percentages of differential blood for the four groups studied

The differentiated analysis of lymphocyte cells populations showed an increased percentage of the CD4 (24%), lymphocytes (23%), neutrophils(19.8%) and B-cells (18.9%) in the treated group compared to non treated rabbits that showed 10.6% of CD4 and 13.3% of neutrophils. While the rest of cell groups showed less than 10% of populations (e.g. monocytes and T-lymphocytes) were unreadable values (Figure 3).

4. DISCUSSION

The results indicated that treatment with this extract have given greater improvement to heal the immunocompromised state and to enhance the bone marrow to produce more leucocytes, that bring them up to the normal levels compared to non treated, as indicated by the results of of WBC count and differential full blood count. Therefore, the results demonstrates that the extract could interfere with the adaptive immunity of the immunocompromised rabbits, since they have

been induced to low levels of immunity for long period of time. Our findings would support others conclusions whom declared

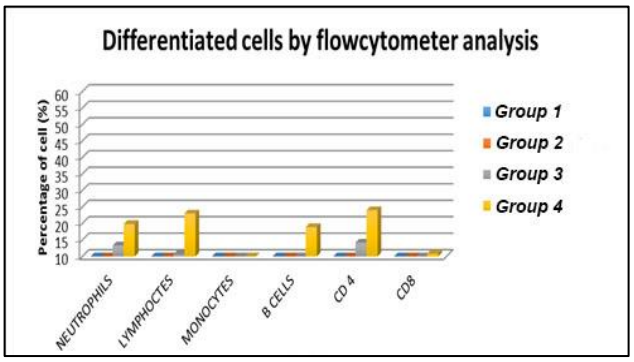

Figure 3: The differentiated lymphocuyte cells analysis, as indicated by the Flowcytometer

that certain nutrients can play roles to modulate inflammation, enhance cellular immunity, decreased hyperinflamation, improve wound healing and, in general, improve immune functions [11-14]. Improvements of immune functions by certain nutrients are usually, result in several clinical advantages, such as enhancement of cellular immunity, modulate tumour cell metabolism, cytokine production, augment lymphocyte and macrophage proliferation, improve wound healing and decrease nitrogen loss postoperatively [11]. A previous study have recorded increases in blood ratio of CD4+ to CD8+ cells in intensive care patients by giving them enteral glutamine, while increased mitogenstimulated proliferation of blood lymphocytes recorded by parenteral administration of glutamine in post-colorectal surgery patients [12]. Another study added additional benefits of enteral glutamine supplement, where they found decreased length of stay and improvement of wound healing in 41 patients with burn injury, by decreasing the rates of infections and mortality compared to other patients [13].

The enteral plant extract 'nutrient' following FBC and Standard T-Panel analysis might reduce infection and results in fast recovery. The results showed that this tested 'nutrient' reported in higher total blood lymphocyte, neutrophils and WBC after treatment, in addition, Standard T-panel analysis showed the specificity of antibodies binding for each individual differentiated cells, which are indicating a special string asscociation between these two immunological profiles.

CONCLUSION

The tested extract showed a very hoping supplement that could be improved in future to develop an effective 'immunonutrient' to help mainly groups of patients whom under surgery, trauma, burns, and injury by enhancing their immunity and maintaining their body immune system. For HIV/AIDS, cancer and chemotherapy patients, thess kind of nutrients will support their immune functions, since they are at immunodeficiency state. This plant extraction might be a promising compound to develop a natural supplement product that could enhance immune system functions and serves to avoid, treat, or lessen most of the complications of Immunodeficiency mostly accompanied with serious infectios and sever health complications.

ACKNOWLEDGMENTS

The authors would like to acknowledge the Faculty of Science and Mathematics (UPSI), Institute of Medical Science Technology (UniKL), for providing necessary facilities. Special acknowledge to Ms. Siti Hasrizan Hassan, Faculty of Medicine and Health Sciences (UPM) for her valuable assistance.

REFERENCES

1. Thomas Yoshikawa. "Infectious Disease in the Aging: A Clinical Handbook" Content Technologies Inc. & Cram 101 Publishing; ISBN 9781603275330 (2013).
2. Nevin S. Scrimshaw. Immunonutrition in health and disease" British Journal of Nutrition; **98**(Suppl.1):S3- S4 (2007).
3. Sanders M. "Overview of functional foods: Emphasis on probiotic bacteria" Int Dairy J; 8: 341-347 (1998).
4. Levy J. "Immunonutrition: The Pediatric Experience" Nutrition **14**(7/8):641-647 (1998).
5. Hendra R, Ahmad S, Sukari A, Shukor MY, Oskoueian E. "Flavonoid Analyses and Antimicrobial Activity of Various Parts of Phaleria macrocarpa (Scheff.) Boerl Fruit" Int J Mol Sci; **12**(6):3422-3431 (2011).
6. Gopalan H.K., Noman D Salih, Roslan F.H., Azmi N., Hing, H. L. "Evaluation of Antibacterial Effect of Phaleria macrocarpa Extract against five bacteria species from Human Diabetic Wound Injuries Using Scanning Electron Microscopy. Sci Int (Lahore); **27**(5):4229-4233 (2015).
7. Noman D Salih, Azmi N., Gopalan H.K. "The Protective Effects of Phaleria macrocarpa Leaves Methanol Extract on Pancreatic Islets Histology in Streptozotocin–Induced Diabetic Rats" Sci. Int. (Lahore); **27**(5): 4219-224 (2015).
8. Noman D Salih, Azmi N., Roslan F.H., Gopalan H.K. "Phytochemical Analysis of Phaleria macrocarpa Leaves Methanol Extraction and Its Medicinal Effects on Diabetic Rats. Res J Pharm Biol Chem Sci; **7**(1): 195-200 (2016).
9. Atlaf R., Mohammad Zaini A., Adiahmad D., Amirin S., Muhammad Iltizam U. "Phytochemistry and Medicinal Properties of Phaleria Macrocarpa (Scheff.) Boerl. Extracts" Art Pharmacognosy Rev; **7**(13):73-78 (2013).
10. Ali R.B., Atangwho I.J., Kuar N., Mohamed E.A.H., Mohamed A.J., Asmawi M.Z., Mahmud R. "Hypoglycemic and anti-hyperglycemic study of Phaleria macrocarpa fruits pericarp" *J Med Plants Res*; **6**(10):1982-1990 (2012).
11. Singh R., Gopalan S., Sibal A. "Immunonutrition" *Indian J Pediatr*; **69**(5):417-419 (2002).
12. Cruzat V.F., Krause M., Newsholme P. "Amino acid supplemtatation and impact on immune function in context of exercise". *Journal of the International Society of Sports Nutrition*; **11**:61 (2014).
13. Marano L., Porfidia R., Pezzella M. "Clinical and immunological impact od early postoperative enteral immunonutrition after total gastrectomy in gastric cancerpatients: a prospective randomized study". *Ann Surg Oncol*; **20**:3912–3918 (2013).
14. Oliveira1 V., Oliveira1 T., Maria V. "Immunonutrition effects in the treatment of cancer patients and its complications-a review" *Semina: Ciências Biológicas e da Saúde, Londrina*; **35**(2):107- 116 (2015).