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Original Article

PHOENIX DACTYLIFERA SEED OIL ENHANCED CELLULAR AND HUMORAL IMMUNE RESPONSE IN CYCLOPHOSPHAMIDE-IMMUNOSUPPRESSED WISTAR RATS

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ABSTRACT

Immunosuppression is a prominent critical feature associated with certain genetic disorders, chronic infections, and conventional chemotherapeutics employed in the treatment of diverse pathological conditions. The scrutiny for bioactive principles with immunostimulatory potential from natural sources becomes crucial. This study evaluated the immunomodulatory role of *Phoenix dactylifera* seed oil (PDSO) in cyclophosphamide-immunosuppressed Wistar rats. Essential oil from the date pit was obtained via steam-distillation technique. Cellular and humoral immunity were measured respectively using delayed type hypersensitivity reaction (DTHR) and hemagglutination reaction (HR), while quantitation of serum immunoglobulin was carried out using enzyme linked immunosorbent assay. The experimental rats were randomly assigned to 7 groups each (n = 4) for DTHR, and (n = 8) for HR assays. Immunosuppression was induced in the animals using cyclophosphamide and PDSO treatment was administered. PDSO significantly ($P<0.05$) promoted delayed type hypersensitivity response (DTHR), increased antibody titres and immunoglobulin levels in the experimental rats in a dose-related manner. The magnitude of immune stimulation evoked by the essential oil at 250 µl/kg.bw compared favourably with that exhibited by the positive control. Findings from this study showcased *P. dactylifera* seed oil as a promising immunostimulatory drug candidate with potential application in immunosuppressive conditions.

KEYWORDS: *Phoenix dactylifera* seed, Essential Oil, Immunomodulation, Cyclophosphamide, Wistar rats.

1. Introduction

Deregulation of the immune response is closely linked to various inflammatory and degenerative disorders, including both chronic and acute infections [1-2]. Additionally, immunodeficiencies may arise when components of the adaptive and innate immune systems fail due to inherited genetic defects or external factors like medications or nutritional deficiencies [3]. Immunomodulation refers to processes that modify the immune system by either enhancing (immunostimulation) or suppressing its functions. Immunostimulation involves activating inactive immune components or boosting their activity, which can be beneficial in preventing infections in immunodeficient conditions. Conversely, immunosuppression indicates a decrease in immune response effectiveness, which can be advantageous in reducing inflammatory and autoimmune reactions in cases such as allergies, autoimmune diseases, and organ transplants [4-5].

The last few decades have witnessed a growing surge towards the scrutiny of natural products in the search for alternative, complimentary and novel therapeutics. Natural compounds are associated with lesser side effects, they are more cost-efficient and often show better patient compliance compared to standard conventional drugs [6-7]. Essential Oils (EOs) are highly concentrated natural compounds derived from plants that consist of aromatic, volatile, secondary plant metabolites. EOs are extracted mostly through steam distillation and exhibit a highly intense odor [8].

EOs primarily consist of mono- and sesquiterpenes, along with various oxygenated derivatives, alcohols, aldehydes, and esters [9-11]. Typically, EOs have two or three main constituents that determine their biological functions and chemical properties [12-13]. However, the overall bioactivity of EOs is influenced by their complete composition, including minor components that can have synergistic, additive, or antagonistic effects [14].

The variations in the chemical makeup of the same EO can lead to different bioactivity profiles [8,15]. Environmental factors, such as temperature, light, and location, along with the plant's genetics and physiology (including age and parts used), significantly affect the composition and biological activity of these oils.

Essential oils are reputed to possess a broad range of applications. They are commonly utilized in the food industry (as preservative and flavoring agents), aromatherapy, cosmetics, and the pharmaceutical industry (as antimicrobial and analgesic agents) [15-16]. Several plants and their essential oils have been found to remarkably affect T cells, cytokines and antibody production at the cellular and molecular levels [17-18]. The immunomodulatory activities of EOs are mediated through various mechanisms: EOs have been shown to stimulate the immune system by elevating the number of circulating lymphocytes and enhancing their phagocytic function, hence facilitating and improving bacterial clearance [19-20]. EOs are proven to suppress responses involved in inflammation and reduction of cytokine production by interfering with key mediators of inflammatory pathways [12-13, 21].

Immunosuppression is a prominent critical feature in certain genetic disorders like cancer, and during chronic infection with certain infectious agents like HIV. Furthermore, immunosuppression is a major drawback associated with conventional chemotherapeutics employed in the treatment of diverse pathological conditions including cancer [22]. Advances in the search for active principles with nontoxic immunostimulatory potential become crucial. Although *Phoenix dactylifera* is reputed to elicit diverse pharmacological activities [8,15], the immunomodulatory role of the seed oil is yet to be explored. Against this background, we evaluated the immunomodulatory role of *Phoenix dactylifera* seed essential oil in cyclophosphamide-immunosuppressed Wistar rats.

2. Materials and Methods

2.1. Materials

The materials used in this study include: dimethyl sulfoxide (DMSO), sterile distilled water, ethanol, hydrogen peroxide, test tubes, conical flask, bijou bottles, glass slides, centrifuge, membrane filter, syringe and needle, pipette, aluminium foil paper, Petri dishes, cotton wool, filter paper, Clevenger apparatus, levamisole, sheep red blood cells, microtitration plate, ELISA kit, ethylenediamine tetraacetic acid (EDTA), date (*Phoenix dactylifera*) seed, cyclophosphamide and Wistar rats.

2.2. Collection of Plant Material and Extraction of Essential oil from *Phoenix dactylifera* Seeds

The study utilized fruits from *Phoenix dactylifera* L. (dates), sourced from a local market and authenticated with the voucher specimen PSBH-240. The fruits were manually separated from their pits and milled. Essential oil was extracted from the date pits using the Clevenger steam-distillation method, as outlined by Oluyele *et al*. [8]. The milled seed powder was placed in an extraction chamber connected to a round-bottom flask with sterile distilled water, and distillation was carried out. The resulting essential oil was stored at -18°C.

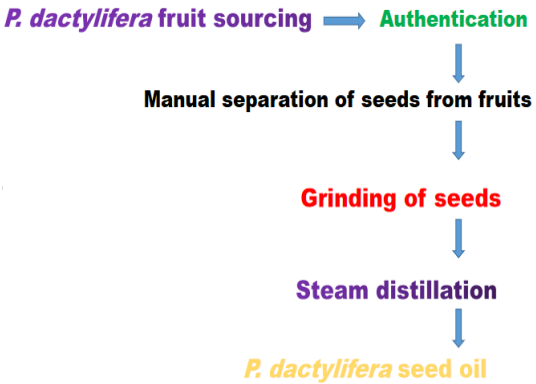

Fig. 1. Flow Chart for the Extraction of Essential oil from *P. dactylifera* Seed.

Fig. 2. Pictures of (a) *P. dactylifera* fruits (b) *P. dactylifera* seeds (c) *P. dactylifera* seed oil.

2.3. Experimental Animals

Ethical approval for the study (FUTA/ETH/22/95) was obtained from the institution's Committee for Ethical Use of Research Animals, Federal University of Technology Akure. The experiment involved Wistar rats aged 8 to 13 weeks, which were acclimatized before the study began. The rats were housed in a controlled environment with temperature of 25-27°C, humidity between 40-60%, and a 12-hour light/dark cycle. They had ad libitum access to commercial rodent feed and sterile drinking water [8].

2.4. Immunomodulatory Studies

Delayed type hypersensitivity reaction (DTH) which measures cellular immunity and hemagglutination reaction (HR) which measures the humoral immunity were used to evaluate the immunomodulatory effect of PDSO in Wistar rats. For each assay, the rats were randomly assigned to 7 groups of four rats per group for DTH; and eight rats per group for HR assays. The study was performed following the method of [23], with slight modifications. Three days prior to immunization with antigen, the rats were intraperitoneally (i.p.) injected with cyclophosphamide (100 mg/kg). All rats were immunized i.p. with 0.1 mL of 10% sheep red blood cells (SRBCs) on days 0 and 7. The treatments for both DTH and HR animal groups were administered orally for

14 days with normal saline (2 mL/kg) for the control group, levamisole (100 mg/kg) for positive control and PDSO doses ranging from 15.63 to 250 µl/kg.bw for the test group. At 24 hours after administering the last treatment, all rats were sacrificed. Whole blood samples were collected from cardiac puncture and used for total white blood cell counting and hemagglutination assay.

2.5. Antigen Preparation

Fresh blood was collected from sheep sacrificed in a local slaughter house. SRBCs were washed three times in normal saline by centrifuging at 1500 rpm for 15 minutes and adjusted to a concentration of 0.1 ml containing 1×10^6 cells for immunization and challenge [24].

2.6. Hemagglutination Assay

Blood samples were collected on the seventh day after the immunization and the challenge. Antibody levels were determined by hemagglutination technique. The antibody titer was determined by a two-fold serial dilution of one volume (50 µl) of serum and one volume (50 µl) of normal saline in a microtitration plate (U-bottom) to which there was added 50 µL volume of 2% suspension of SRBCs in normal saline. After mixing thoroughly, the plates were incubated at 37°C for 1 hour and examined for hemagglutination. The reciprocal of the highest dilution of the test serum showing agglutination was noted as the antibody titre. The data were recorded as the mean ± standard error of the mean of log titre [24].

2.7. Assay for Delayed Type Hypersensitivity Reaction

DTH response was determined by inducing immunogenic response using SRBCs in the rats' foot-pad. After pretreatment with PDSO and immunization with SRBCs, in all rats paw edema was induced by injecting 50 µL of SRBCs into the sub-plantar region of the right hind footpad. Delayed type hypersensitivity (DTH) degree was represented by difference in thickness of the right footpads before and 24hours after the challenge [24].

2.8. Immunoglobulin Assay

Blood samples were collected from the rats in a sterile plain bottle and allowed to clot. The blood samples were centrifuged at 4000 rpm for 10 minutes, the supernatant (serum) was stored at –20°C until use. Quantitation of serum immunoglobulin-G (IgG) was carried out using Enzyme Linked Immunosorbent Assay (ELISA) (Cusabio Biotech Co., Ltd., Wuhan, China) kit for rats at 450 nm wavelength following the manufacturer's protocol.

2.9. Determination of White Blood Cell (WBC) Count

As adapted from the method of [25], Ethylenediamine tetraacetic acid (EDTA)-treated whole blood at 1:20 ratio with blood cell diluting fluid (made from 3.8 grams of Sodium citrate, 0.21 grams neutral formalin and 0.5 grams brilliant cresol blue and 100 ml of distilled water) was used. The diluted blood sample was then mixed and loaded into the counting chamber. The WBC in the chamber was counted leaving out the edges of the chamber. The formula below was used to determine the Total White Blood Cell count:

$$WBC = (N \times DF \times 10^6) / (A \times D)$$

where A = area counted, N = number of cell, DF = dilution factor, D = depth of chamber.

2.10. Data Analysis

The data were analyzed using IBM SPSS Statistics version 22.0 (IBM Corp., Armonk, NY, USA). A one-way analysis of variance (ANOVA) was performed, and mean differences were assessed using Duncan's New Multiple Range Test, with statistical significance set at $p \leq 0.05$.

3. Results

3.1. Effects of Administration of *P. dactylifera* Seed Oil on Cellular and Humoral Immune Response in Wistar Rats

As shown in Table 1, PDSO significantly ($P < 0.05$) promoted delayed type hypersensitivity response (DTHR), increased antibody titres and immunoglobulin levels in the experimental rats in a dose-related manner. The magnitude of immune stimulation evoked by the essential oil at 250 µl/kg.bw, compared favourably with that exhibited by the positive control.

4. Discussion

Normally, the body's defense mechanisms maintain homeostasis; nevertheless, several endogenous and exogenous factors can alter its efficiency. A plethora of medicinal plants and their purified constituents have proven to possess valuable therapeutic properties [26]. In this study, we evaluated the immunomodulatory role of *Phoenix dactylifera* seed oil in Cyclophosphamide-immunosuppressed Wistar rats. Prolonged treatment with cyclophosphamide (CTX) in patients with health conditions such as malignancies or autoimmune diseases can lead to

Table 1. Effects of Oral Administration of *P. dactylifera* Seed Oil on Cell Mediated and Humoral Immune Response in Wistar Rats

Group	DH	HI First dose	HI Second dose	IgG level (g/L)	WBC Count ($\times10^3/mm^3$)
1	1.40 ± 0.04b	1.28 ± 0.08b	1.58 ± 0.08b	1.66 ± 0.02ab	8.37 ± 0.09a
2	2.27 ± 0.12c	1.58 ± 0.08c	1.88 ± 0.08c	1.75 ± 0.03b	8.51 ± 0.07a
3	2.62 ± 0.11d	1.81 ± 0.00d	2.26 ± 0.09d	1.88 ± 0.03c	8.83 ± 0.07b
4	3.46 ± 0.17e	1.96 ± 0.09d	2.33 ± 0.08d	2.08 ± 0.07d	9.06 ± 0.08bc
5	4.50 ± 0.11f	2.26 ± 0.09e	2.56 ± 0.09e	2.49 ± 0.02e	9.16 ± 0.09c
6	0.27 ± 0.01a	0.53 ± 0.08a	0.60 ± 0.00a	1.55 ± 0.01a	8.92 ± 0.05b
7	5.37 ± 0.05g	2.78 ± 0.08f	3.16 ± 0.09f	2.81 ± 0.04f	9.63 ± 0.07d

Legend: 1 = 15.63 µl/kg.bw PDSO, 2 = 31.25 µl/kg.bw PDSO, 3 = 62.5 µl/kg.bw PDSO, 4 = 125 µl/kg.bw PDSO, 5 = 250 µl/kg.bw PDSO, 6 = negative control (untreated), 7 = positive control (levamisole), PDSO = *P. dactylifera* essential oil, DH = delayed hypersensitivity, HI= humoral immunity. Values with same superscripts down the groups are not significantly different.

immunosuppression [27]. Both humoral and cellular immunities are suppressed by CTX use. Thus, administration of CTX is a superlative method to recognize the intricacy of the immune system.

In our study, CTX-induced immunosuppression caused a decline in WBC count in the experimental animals. This observation is supported by the study of [28] who reported that CTX triggers apoptosis and induces cytotoxic effect on murine lymphocytes. This has been proposed to occur via the alkylation of functional groups in cellular proteins and restraining of medulla hematopoietic function [28]. Conversely, the administration of varying doses of PDSO increased the total WBC count of the rats, with the most pronounced effect produced in the group given 250 µl/kg.bw of PDSO; a similar but superior response was found in levamisole treated group. Previously, levamisole was shown to be effective in promoting maturation of granulocytes and functioning of T-cells, moreover, it stimulated an increase in WBC and lymphocytes in immunosuppressed rats [23,29]. It is suggestive that PDSO might act by activating the haematopoietic system along with increasing the number of circulating leucocytes in the test animals.

In SRBC-sensitized animals, SRBC antigen primarily develops and spreads to the extravascular space through lymphatic system to the lymph nodes [30]. The administration of PDSO evoked an increase in the delayed hypersensitivity response to SRBC in a concentration related pattern. This is indicative of the stimulatory effect of PDSO on T-cells and chemotaxis dependent leucocyte migration. Delayed type hypersensitivity reaction (DTHR) is a protective localized cell-mediated immune-inflammatory response, primarily against intracellular pathogens or antigens. Upon antigen presentation, T-lymphocyte may become sensitized lymphocyte and will generate a regional abnormal reactive inflammation. This reaction is characterized by increased vascular permeability, heavy infiltration of polymorphonuclear (PMN) leucocytes and edema in response to antigenic stimulation [31].

PDSO treated animals demonstrated significantly ($P<0.05$) higher hemagglutination titre compared to the untreated control-group. The elevated values of hemagglutinating antibody titre obtained in the PDSO treated group suggest its capacity to engage B-cell towards activation and circulation of anti-SRBC antibodies. Antibody molecules which are secreted by plasma cells mediate the humoral immune response. During B-cell response, antibodies are manufactured after the recognition of the antigen by B-cells. The antibodies interact with the antigen to eliminate or neutralize the antigen [18].

These results agree with the findings of [32] who reported that *Syzygium aromaticum* essential oil stimulated cell-mediated immunity in immunocompetent mice and restored WBC count and humoral immunity in immunosuppressed mice [32]. This is further corroborated by the report of [16] who reported the stimulatory effect of *Eucalyptus globulus* essential oil on cell-mediated immune response after administration in immunosupressed rats. Furthermore, ginseng extracts and their EOs were shown to induce immunostimulatory effects by increasing the levels of tumor necrosis factor (TNFα) and IFNγ or enhancing phagocytic activity [18, 33-34].

Immunoglobulin (IgG) is the principal immunoglobulin in serum which is associated with complement activation, opsonization and neutralization of toxins [35]. Several plant based drugs promote immunoglobulin synthesis by activating B-cells or modulating the molecular processes, involved. Immunoglobulin G (1gG) levels were found to be elevated in a dose dependent fashion in the PDSO treated rats compared to the normal control rats. Though appreciable, the maximum IgG value observed in the PDSO treated group was not higher than that obtained in the positive control group. The results suggest a significant potentiating action of PDSO on humoral immune response [36], reported that the elevation of immunoglobulin level in mice treated with the crude protein extracted from *Cajanus cajan* was due to an increase in the synthesis of elevated levels of specific antibodies to SRBC. Extract of *Tridax procumbens* was also reported to potentiate the synthesis of immunoglobulin by triggering the secretion of IL-2 and IFN-ϒ [37]. Furthermore, administration of *Nyctanthes arbortristis* stem bark extracts triggered a significant boost in serum immunoglobulin levels in CTX-immunosuppressed mice [27]. The foregoing attests to the capacity of natural products to stimulate the immune system.

Interestingly, corroborating the tripartite findings on PDSO in our study, a different study reported the capacity of a plant (*Annona reticulata*) extract to promote DTHR, humoral immune response and increased antibody titres in CTX immunosuppressed rats [38].

5. Conclusions

This study underscores the potential of *Phoenix dactylifera* seed oil as an effective immunostimulatory agent, which may be useful in treating conditions associated with immune suppression. To the best of our knowledge, this is the first study to demonstrate the immune-boosting effects of *Phoenix dactylifera* seed oil in Wistar rats. More research is required before these findings can be applied to humans. Future studies should aim to clarify the underlying mechanisms, establish the ideal dosages for humans, assess potential side effects, and explore other relevant factors.

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