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Immunomodulatory Potential of Combining Some Traditional Medicinal Plants *In Vivo*

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Abstract

Background: Immunomodulatory agents initiate the activation of non-specific immune responses and work without antigenic specificity. They directly activate different cell populations of the immune system and modify the production of cytokines and initiate the production of various effect or molecules that take part in the modulation and enhancement of the immune response.

Objective: To investigate the immunomodulatory potential of combining four ethanolic extracts of *Mentha*, *Cuminumcyminum*, *Nigella sativa* L. and *Foeniculum vulgare* Mill. (MCNF) using healthy and immunocompromised animals.

Methods: Humoral and cell-mediated immunity of healthy and drug-induced myelosuppression Wistar Kyoto (WKY) rats were assessed using various standard immunological assays including T-cell population, delayed hypersensitivity, carbon clearance, haemagglutination titer, IL-4 levels and hematological parameters. These assays were used to evaluate the effect of MCNF extract at different doses of 200, 400 and 800 mg/Kg per animal. One-way ANOVA and Mann-Whitney tests were used to assess the differential statistical significance between the immunocompromised and treatment groups.

Results: The use of MCNF extract significantly increased delayed hypersensitivity responses when compared to immunocompromised rats and other control groups ($p<0.01$). The extract was found to increase the phagocytic index ($p<0.01$), White Blood Count (WBC) count ($p<0.006$), lymphocyte count ($p<0.01$) and percentage granulocytes ($p<0.006$) in parallel to a significant increase in the levels of serum immunoglobulins in a dose-dependent manner ($p<0.001$). The potential effect of MCNF extract was further confirmed by the significant increase in IL-4 levels ($p<0.01$). Moreover, the ethanolic MCNF extracts showed antagonistic capacity to zinc alpha-2-glycoprotein-1 that is usually secreted at high levels in immunocompromised animals.

Conclusion: Combination of the four ethanolic herbal extracts (MCNF) is a promising medicament with a potential therapeutic value in stimulating the suppressed or weakened immune responses in laboratory animals and may act as a potential immunomodulatory agent to enhance both cellular and humoral immunity. And for the first time we report that the antagonistic efficacy of these herbs on ZAG-1 may aid to enhance the immunity in those who are immunocompromised.

Keywords: Immunomodulation; Cellular immunity; Humoral immunity; Haemagglutination titer; Zinc alpha-2-glycoprotien, Herbal; Rats

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Introduction

Human survival is dependent upon their defensive immune mechanisms against external harms, pathogenic microbes and cancer. It is well known that the immune system helps the host to control microbes, allergic or toxic molecules and prevent cancer development [1]. Once a defect occurs within the immune system, it results in response impairment against infectious agents and cancer. The cause of impairment (immunosuppression) can be either extrinsic or intrinsic (inherited) and referred to as secondary or primary immunodeficiency, respectively [2,3].

Herbs with immunomodulatory properties are a moderately recent concept in phytomedicine. In addition to the enhancement of the humoral and cell-mediated immunity, the concept of immunomodulation initiates the activation of the "non-specific" immune responses which include

the activation of the complement system, granulocytes, macrophages and natural killer cells. Hence activation of these essential immune cells initiates the production of various effect or molecules (e.g. cytokines) that take part in the modulation and enhancement of the immune responses [4,5].

All these events produce alternatives to the current chemotherapy for immunodeficiency giving protection against infections caused by various microorganisms and prevent cancer development [6]. Immunomodulatory agents are non-specific compounds that work without antigenic specificity similar to the adjuvants that are associated with some vaccines [7,8].

Menthalongifolia L. (mint) has extensive usage in various fields such as food and pharmaceutical industries. The chemical composition of this plant contains many compounds like menthol, pulegone and piperitenone oxide that has many pharmaceutical effects such as antimicrobial and antioxidant agent with no reported side effects [9]. However, there are no reports to indicate the ability of this plant to positively boost immune responses.

N. sativa (black seed) is an herb used mainly in the food industry and traditional medicine. Its seed is well known to act against bronchial asthma, toothache, hypertension and headache [10]. Some experiments on animal model indicate that *N. sativa* can modulate the pro and anti-inflammatory cytokines profiles including interleukin-4 (IL-4) and interferon-γ [11] and acts as a balancing factor between Th1/Th2 lymphocytes [10].

C. cyminum (Cumin) was reported to have significant activation of cytotoxic ($CD8^+$) T cells and helper ($CD4^+$) T cells with an elevation of Th1 cytokines using both normal and immunosuppressed animals [12].

F. vulgare Mill. (Fennel) is used mainly in the food industry (due to its flavor) and in the medicinal field as a traditional treatment for many disorders related to the digestive, reproductive and endocrine systems [13,14].

To our knowledge no studies were performed to assess the combination effects of these four herbal extracts namely; *Mentha*, *Cuminumcyminum*, *Nigella sativa L.* and *Foeniculum vulgare* Mill (MCNF) herbs and their possible effects on alleviating the immune system. Furthermore, the study also aimed to annotate the influence of such herbs on the level of Zinc Alpha-2-Glycoprotein-1 (ZAG-1) using healthy and immunocompromised animals.

Materials and Methods

Plant materials

Mentha, *C. cyminum*, *N. sativa L.* and *F. vulgare* Mill. Herbs were collected from different localities of Oman such as Al Buraimi, Salalah, Musandam and Nizwa. Herbs were identified according to the International Ethno-medical Data in the College of Agricultural and Marine Sciences, Sultan Qaboos University (SQU). Selected leaves/seeds were washed thoroughly with water, and then air dried under shade and grounded using a pestle and mortar.

Extraction of plant materials

The ethanol extraction method was followed as described by Hasson et al. [15]. Briefly, 150 grams of ground N. sativa seeds were soaked in 200 mL of 96.9% ethanol, homogenized in an electric blender for approximately 5 minutes and incubated at room temperature for 4 days with continuous vigorous stirring. The mixture was then filtered twice using a vacuum. Solvent (ethanol) in the first filtrate was evaporated at room temperature and then exposed to a second extraction using 250 mL ethanol (96.9%) with continuous shaking in a water-bath at 70°C for 6 h. The mixture was filtered again and the ethanol was evaporated at room temperature. The residue (*N. sativa* extract) was collected and frozen at -70°C followed by lyophilization. The dried extract was stored at 4°C. The lyophilized *N. sativa* extract was weighed and re-suspended in physiological Phosphate Buffer Saline (PBS). The extraction procedure was repeated for the other herbs. The end product of each herb was mixed thoroughly in equal ratio. Finally, a combination of these herbs was obtained for further use. The prepared aliquots were kept at -20°C until utilized.

Toxicity assay

The assay was conducted to evaluate the acute toxicity and to find the effective dose of MCNF extract that boost the initial immune status as described by the OPPT (Office of Pollution Prevention and Toxics Test) guidelines [16]. Succinctly, MCNF extract was given orally to animals, on a daily basis, for a course of two weeks. Animals were distributed into two main groups. "Group I" consisted of 6 WKY rats of both sexes (weighted 300 g to 360 g) that received standard diet and tap water. "Group II" was further divided into five subgroups (each consisted of 6 WKY rats) and dosed orally (intubation) with MCNF extract with different concentrations of 100, 200, 400, 800, and 1000 mg/Kg. The animals were under the observation for any signs of acute toxicity, abnormal behavior, body weight loss, eyes, and death on a daily basis for a period of two weeks. Blood samples were collected from animals' tails at the same time during the observation period for hematological parameters analysis. The survival time, of the WKY rats, was documented and evaluated using Log-rank test. After the second week, the medication was stopped and the animals were checked for another week. At the end of the experiments, animals were sacrificed to examine them internally for any sign of toxicity and/or side effects.

Chemicals and reagents

Preparation of drugs: Azathioprine (AZP50) ≥ 98% [6-(1-Methyl-4-nitroimidazol-5-yl) thiopurine] was obtained from Sigma Aldrich (Cat#A4638). AZP50 was administered at a dose of 50 mg/Kg of body weight for induction of immunosuppression. Levamisole hydrochloride (LEV50) of 50 mg/Kg (PHR1798 Pharmaceutical Secondary Standard; Certified Reference Material (Sigma-Aldrich) was used as a positive control drug to enhance the immune function by stimulating antibody formation and T-cell activation and proliferation, strengthening monocyte and macrophage phagocytosis and increasing neutrophil chemotaxis ability.

Preparation of sheep red blood cells (SRBCs) antigen: Fresh blood of healthy sheep was collected from the slaughterhouse at Mabelah, Muscat Oman. Blood was mixed 1:1 with sterile saline solution (2.05% dextrose, 0.8% sodium citrate, 0.055% citric acid, and 0.42% sodium chloride). The blood was then centrifuged at 2000 rpm for 10 min to enable red blood cells to settle down at the bottom of the test tube. The clear supernatant was discarded and SRBCs were washed three times with pyrogen-free phosphate buffered saline (pH 7.2) and re-suspended and adjusted to $0.5 \times 10^9/mm^3$ cells concentration.

Experimental design to measure complete blood count (CBC) and ZAG-1 levels

WKY rats in the negative control group (Group I) received PBS throughout the 9 days of the study period. Group II rats were treated

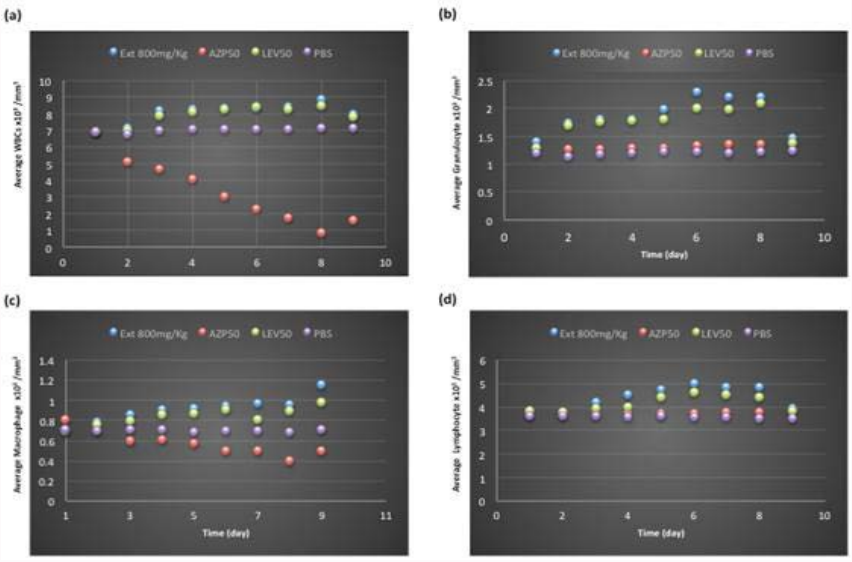

Figure 1: Influence of the MCNF extract on the WKY immune cells profile Status of different cells of: (a) white blood cells population (P ≤ 0.05), (b) granulocytes (P<0.006), (c) macrophage p ≤ 0.079 4. (d) (P ≤ 0.05), among the four groups throughout the period of the experiments.

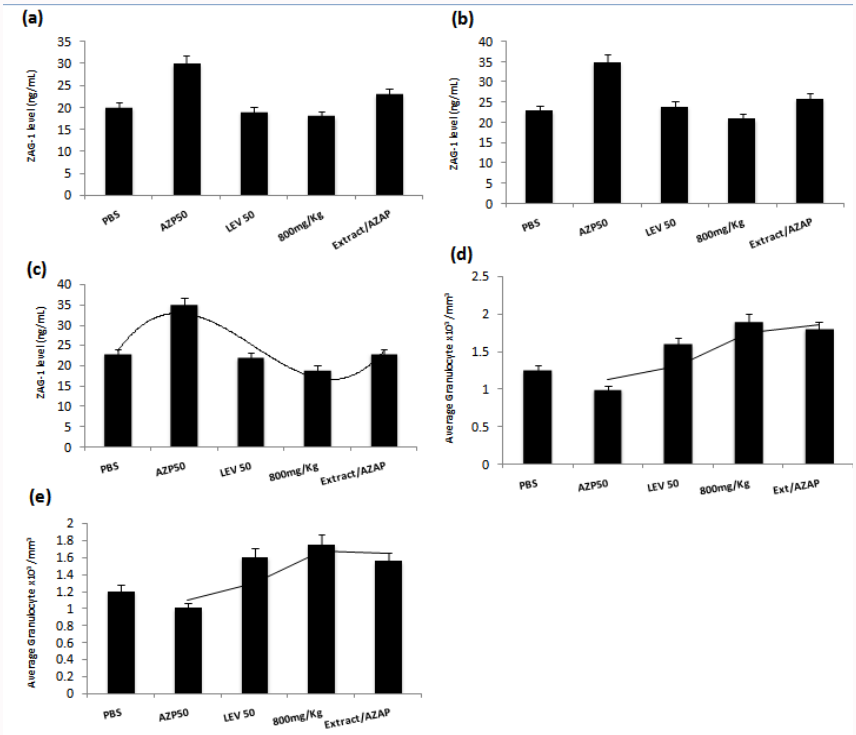

Figure 2: Effects of MCNF extract and other drugs on the ZAG-1 level. A moderate decrease in the ZAG1 level among animals treated with the extract alone or in combination of AZP50 or LEV50 in contrast with the negative control (PBS). The MNCF extracts was used at a concentration of 800 mg/Kg. Significant values were represented as *P<0.05, ns: non-significant.

with an immunosuppressant AZP50 (50 mg/Kg) on day 0 till day 9 of the study by subcutaneous injection. Animals' in group III were given a standard immunomodulatory drug-LEV50 (50 mg/Kg). Group IV animals were further subdivided into three subgroups and received different doses of MCNF extract at 200, 400 and 800 mg/Kg. Group V received both the MCNF extract and AZP50 with the same concentration as stated for group III and IV, respectively. Group VI animals were received both the MCNF extract and LEV50 with the same concentration as stated for group II and IV, respectively. However, groups V and VI were performed independently from the other groups i.e., group 1 and group IV. These two groups received the optimum concentration (800 mg/Kg) MCNF and (800 mg/Kg) MCNF with AZP50. This is to examine if such combination modeling will raise potential effect either antagonistic or synergistic.

Blood analysis: To enhance active blood circulation within the rats' tail, rats were wormed at 37°C. After that blood samples of 1.5 mL were collected weekly from the tail vein in EDTA and plain tubes for CBC and cytokine studies, respectively. CBC was done using Abx

60 machine and confirmed by manual differentiation.

Measurement of ZAG-1 levels: Determining the ZAG-1 levels in the sera samples was done utilizing a competitive enzyme-linked immunosorbent assay (ELISA) (Abnova GmbH Corp., Heidelberg, Germany). The assay was performed according to the manufacturer instructions.

IL-4 cytokine evaluation: A volume of one and a half (1.5) mL of WKY rats' blood obtained in Section 2.6.1 was centrifuged at 4000 rpm for 10 min and serum was stored at -70°C. ELISA was performed to determine the quantitative amount of IL-4 cytokine in rat's serum following manufacturer instructions (Rat's IL-4 Abcam- USA).

Evaluation of cell-mediated and humoral immunity

Delayed-type hypersensitivity (DTH) response to SRBC: DTH was measured following Doherty [17] and Puri et al. [18] with some modifications. Experiment was designed as follows: Group I received only PBS and served as a negative control, group II served as immunocompromised animals and was given 50 mg/Kg body weight of AZP50 intraperitoneally (i.p). Group III was given LEV50 of 50 mg/Kg orally. Group IV animals were subdivided into three subgroups and received different doses of MCNF extract at 200, 400 and 800 mg/Kg orally. The extract was administered orally using animal feeding needle and 1 mL syringe. Briefly, after administration of the extract and other drugs for 7 consecutive days, animals were prepared by administrating 0.5 mL containing 5×10^9 SRBC, subcutaneously into the left hind footpad. The contralateral hind paw moreover got an equal volume of 0.1% PBS. A second subcutaneous injection in another day was performed for challenging.

The degree of DTH response in the animals was assessed by measuring the footpad thickness after 4, 8, and 24 hours using Vernier calipers and expressed as mean percentage increase in thickness/edema with the help of plethysmometer.

Determination of hemagglutination antibody titer: A similar experiment to that of the DTH was performed to evaluate the efficacy of the MCNF extract in promoting the humoral immune response by determining the haemagglutinin titer of rats against SRBC as demonstrated by Fulzele et al. [19]. Administration of MCNF extract was continued further for another 7 days until day 14, and blood samples were withdrawn via cardiac puncture. Blood was centrifuged at 4000 rpm for 15 min to separate serum. Antibody titers were then assessed using the hemagglutination assay as described by Gaur et al. [20]. Serum samples in a two-fold serial dilution were made with PBS in 96-well microtiter plates and SRBC (25 µL of 1% SRBC previously prepared in normal saline) was added to each of these dilutions. The hemagglutination plates were then incubated at 37°C for one hour and then examined for hemagglutination. The highest dilution of the test serum that gave agglutination was considered as the hemagglutination antibody titer (HA units/µL).

Determination of the phagocytic index

Carbon clearance assay was used to determine the phagocytic index of macrophages in WKY rats intubated with the herbal extracts based on the method of Jayathirtha and Mishra [21]. Experiment was designed as follows: A similar experiment to that of the DTH was performed for 7 consecutive days. Blood samples (in EDTA solution, 5 µL) from retro orbital plexuses of the individual animals were drawn at the intervals of 0 min and 15 min. After collection, a 25 µL blood sample was mixed with 2 mL (0.1%) sodium carbonate

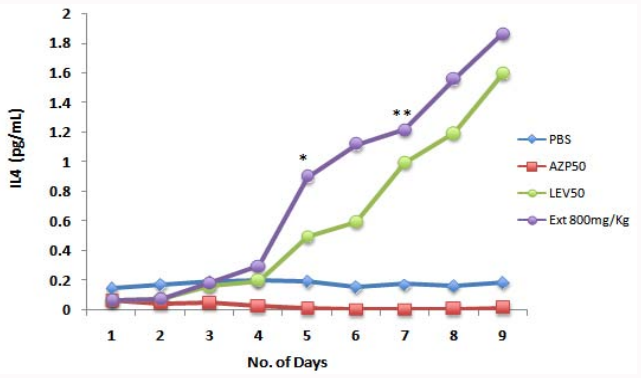

Figure 3: Effects of MCNF extract on serum levels of IL-4 in rats in contrast with immunosuppression and immunomodulation of the treated groups. IL-4 serum levels were measured on day 7 and on day 14 by ELISA. Data were represented as means ± SEM. Mann-Whitney test: *P ≤ 0.05, WKY-treated rats with AZP50 and PBS were served as immunocompromised and negative controls, respectively. **P ≤ 0.01, WKY-treated rats with LEV50.

and the absorbance, i.e., optical density was measured at 680 nm. The phagocytic index (K) was then obtained by using the equation below:

$$K = (\log OD1 - \log OD2)/15$$

Where, OD1 and OD2 were optical densities at 0 min and 15 min, respectively.

Statistical analysis

Statistical analysis was performed and all results' data obtained were expressed as mean ± SEM for six rats (unless otherwise stated) and statistical evaluation was carried out using PRISM software package (Version 5.0.4). One-way ANOVA and Mann-Whitney tests were used to assess the differential statistical significance between the immunocompromised and treatment groups. The value of probability less than 5% ($P<0.05$) was considered to be statistically significant.

Results

The MCNF extract was tested for its acute toxicity as well as to determine the optimal dose for oral administration by introducing an increasing dose of MCNF extract. It was noticed that higher dose of 1000 mg/Kg cause a rapid depolarization but no death was observed. Lower doses of 200 to 800 mg/Kg showed no toxicity to animals.

Immunological profile

In the preliminary evaluation of the herbal combination extract capacity to enhance the hematological parameters, we found a significant increase in the percentage of WBC (Figure 1a) and granulocytes counts (Figure 1b) was observed in the rats administered with extracts ($P<0.006$) in comparison with that of the control groups. A similar pattern was also observed in relation to the percentage of both lymphocytes and macrophage counts as shown in Figure 1c and Figure 1d, respectively. The results of the group IV and VI were not significant to elevate of the immune cells (data not shown).

Effects herbal extracts on ZAG-1 and IL-4 cytokine levels: The results shown in Figure 2 demonstrate that MCNF repressed the level of the ZAG-1 among animals treated with the extract alone ($P \leq 0.05$) or in combination with the addition of either AZP50 ($P<0.05$). No significant differences were observed between group IV treated with 800 mg/Kg or the combination of the MCNF extract and LEV50. We have used the results of the 800 mg/Kg as the optimum.

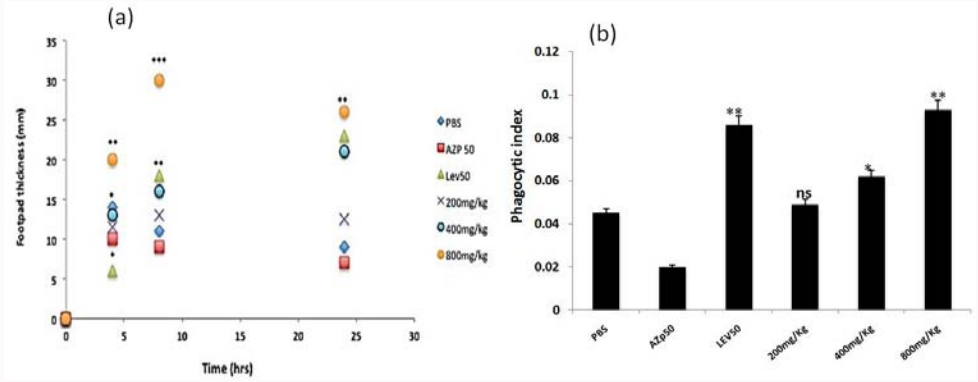

Figure 4: (a) Effect of different concentrations of MCNF extract on the delayed hypersensitivity reactions in WKY in response to SRBC as an antigen. Values were represented as mean ± with all groups. ***P<0.001, extremely significant; **P<0.01, very significant; *P<0.05, significant. (b) Determination of antibody titer in response to SRBC Values were represented as mean ± with all groups. ***P<0.001, Extremely Significant; **P<0.01 Very Significant; *P<0.05, Significant.

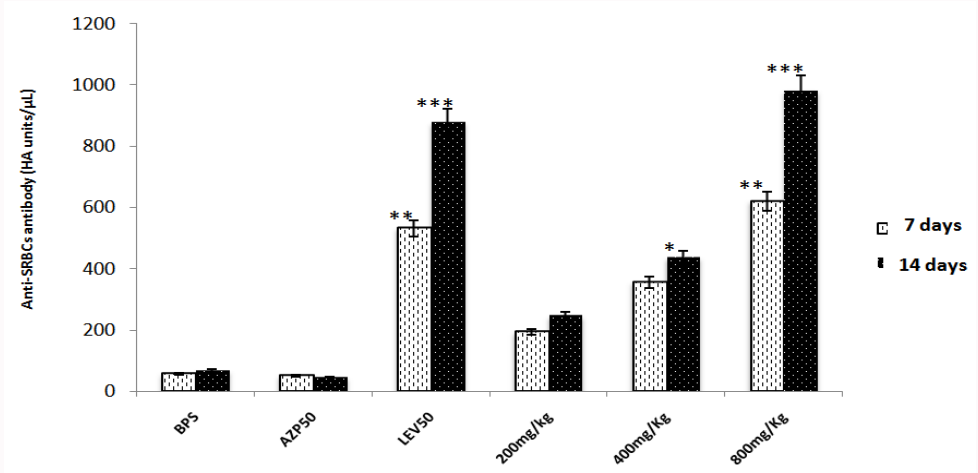

Figure 5: Effect of MNCF extract on the phagocytic index. Values were represented as mean ± SEM, (n=6). Test groups were compared with control and standard groups. Significant values were represented as **P<0.01, *P<0.05, ns - non significant.

One of the interesting findings in this study is the effect of the MCNF extract on the levels of IL-4 cytokine (Figure 3). There was a profound enhancement of IL-4 (P<0.01) among the herbal combination group especially after the fourth week of the experiments. The results of the group IV and VI were not significant as no potential elevation of the IL-4 was found (data not shown).

Analysis of NBT reduction and DTH: MCNF extract was evaluated for *in vitro* antioxidant assay using NBT inhibition assay. Having explored the NBT reduction is further confirmed the efficacy of the MCNF in enhancing the function of the granulocytes showed Figure 1b. Group treated with MCNF extract has shown significant *in vitro* antioxidant activity with NBT inhibition assay in a dose dependent manner (i.e., 1 µg/mL (54.23 ± 0.007%), 2.5 µg/mL (73.31 ± 005%) and 5 µg/mL (93.10 ± 0.003%) when results were compared with the PBS - negative control (2.5 µg/mL (4 ± 0.03%), 5 µg/mL (6 ± 0.17%), 10 µg/mL (17 ± 0.25%).

On the other hand the potential efficacy of the MCNF extract on cell-mediated immunity was evaluated (Figure 4a). Pretreatment of the MCNF has shown a significant increase in footpad thickness (P<0.01) at 24 h of a challenge at all doses in contrast with groups I and II that received PBS and AZP50, respectively. Group III that received LEV50, showed a gradual increase in footpad thickness observed within the first 24 h. However, there was a statistical potentiation of DTH elevation in percentage footpad thickness increment (P<0.01) in the group that received 800 mg/kg of the MCNF extract at 8 h of challenge. DTH response was lowered after 48 h in all MCNF extract treated groups and as compared to control animals (data not shown).

Phagocytic index activity of macrophages: Test animals treated with MCNF extract showed a significant dose-dependent increase in the phagocytic index and was found to be statistically significant (P<0.01) when compared to the phagocytic index of the negative control and group treated with AZP50, respectively. Elevation in the phagocytic index was found in all doses (200 mg/Kg, 400 mg/Kg, 800 mg/Kg) of MCNF extract, specifically at 400 and 800 mg/Kg extract (P<0.01 and P<0.05, respectively) when compared to either the negative control or the lower (200 mg/Kg) concentration (Figure 4b). This may indicate that there was an increment in the clearance of colloidal carbon from the blood after administration of the high dose extract.

Antibody titer: In this assay, serum-containing antibodies were collected from WKY rats of each group and serial dilutions (0:16:32:64:156:256:520) were done on a microtiter plate. The mean hemagglutination antibody titer to SRBC was found to be dose-dependent with significant variation in all groups and intervals for

the rats dosed with the MCNF extract as compared to the rats that received only AZP50 (Group II), with the highest mean titer for the MCNF extract concentration of 800 mg/kg at (982.0 ± 18.37; P<0.001) HA units/μL. This was followed by the results of group III that received LEV50 [(879 ± 41.21) HA units/L, P<0.003], then 400 mg/kg [(438.0 ± 3.84) HA units/L] of MCNF extract and the 200 mg/Kg of the extract [(246 ± 4.583) HA units/L] on day 14 (Figure 5). A significant decline in anti-SRBCs antibody titer was found among the immunocompromised group II animals that were treated with AZP50 [(46.23 ± 0.347) HA units/L; P<0.001] as compared with group I that received PBS (7.41 HA units/μL), though not statistically significant.

Discussion

The study was conducted to annotate the immunomodulatory potential off our herbal extracts namely; *Mentha*, *Cuminumcyminum*, *Nigella sativa L.* and *Foeniculum vulgare* Mill. (MCNF) herbs, in immunocompromised WKY rats. Also to evaluate their possible effects on either normalizes or suppressed the ZAG-1 level in such animals.

We report for the first time the potential immunomodulatory activity of these four combined herbal extracts in an *in vivo* experimental model. We have explored the immunomodulatory activity of MCNF herbs on cellular immunity by assessing their combination effects on DTH responses, hemagglutination antibody titers as well as other functional assays such as T-cell populations and NBT assays [22]. In the preliminary evaluation of these herbal medicament on the hematological parameters, a significant increase in the WBC count and granulocytes percentage in the WKY rats administered with MCNF extract (P<0.006) was observed (Figure 1a). Granulocytes are vital immune cells that play a significant role in the inflammatory process and have a profound effect on microbial infections and pathogens.

The efficacy of the MCNF extract on the IL-4 production was interesting and significant (Figure 3). There was a profound enhancement of IL-4 (P<0.01) among the MNCF extract treated animals especially after the fourth week of the experiment. This cytokine, a growth factor for B cells, normally induces differentiation of naïve Th cells (Th0) into Th2 cells that have a crucial role in the immunity against bacterial and helminthic infections along with other different functions [23]. There are many conditions that cause high-level of IL-4. The most known examples are lung cancer, breast cancer and prostate cancer, which are caused by overexpression of IL-4 receptors [24]. However, other important cytokines should also be investigated in future studies.

The DTH response is a cell-mediated immune response classified as type IV DTH [25]. This test gives a useful *in vivo* evaluation of the cell-mediated immunity [26,27]. In this study, delayed hypersensitivity was assessed, using SRBC as allergen after administration of the MCNF extract for 14 days. Results showed that treatment with MCNF extract significantly increased the DTH in contrast with the control groups. The dose-dependent increase in DTH response suggests the stimulatory effect of MCNF extract denoting an increase in the cell-mediated immunity and lymphocytes that are essential for the process of inflammation [28,18]. Increase in DTH response of WKY rats confesses the stimulatory impact of MCNF on T lymphocytes i.e. cell-mediated immunity or specific immunity.

The increase in footpad thickness of the WKY rats that were subjected to MCNF extract in this study could be ascribed to the capacity of the MCNF extract to enhance lymphocytes and other cell types leading to enhancement in the production of antibodies in the already immunosuppressed rats, consequently initiating cell-mediated immunity. This is in accordance with other findings from previous studies done utilizing some of these herbs [9]. For instance, *C. cyminum*, boosts the immune system by consolidating T-cell proliferation, increasing cytokine production and production of immunoglobulins [12], all of which are essential to initiate the inflammatory response.

The results of this study, demonstrated that the extract of MCNF could be used to boost the immune system, as there was a dose-dependent increase in paw size in response to the antigen. This was affirmed more by the carbon clearance assay performed to assess the impact of this medicament on the reticuloendothelial system. Cells of the reticuloendothelial system play important roles in the clearance of particles from the blood circulation (e.g. phagocytic cells). The rate of clearance of carbon from the blood circulation by macrophage is controlled by an exponential equation [29]. The MNCF extracts at higher dose of 800 mg/Kg demonstrated remarkable augmentation in the phagocytic index nearly comparable to that of the LEV50 drug and this may be due to the extract's ability to increase the activity of the reticuloendothelial system [30].

A hemagglutination assay was performed to ascertain the effect of MCNF extract on enhancing the humoral immune response. The results demonstrated that MCNF extract had a potential effect on stimulating the humoral immune response. This was shown by the hemagglutination antibody titer to SRBC antigens in a dose-dependent manner for the rats dosed with MCNF extract as compared to the animals that received only azathioprine [31].

Results from this study showed that the MCNF may contain potential compounds that can initiate the production of antibodies in an immunosuppressed animal. These findings were further confirmed by the results of the cytokine IL-4 as discussed earlier. This may explain the common usage of each individual herb as an immune stimulant in the traditional medicine in some countries.

In addition, intensification in rosette formation and lymphocyte formation in T-cell populations, showed that the MCNF extract increased lymphocyte formation at high dose (800 mg/Kg) followed by LEV50 treated groups while binding of lymphocytes to the SRBC also increased (P<0.01, P<0.05, respectively). Compared to the control group, lymphocyte formation was found to be elevated at both, 200 and 400 mg/Kg concentrations. However, these results were not significant.

We have also evaluated whether such herbal combination may or may not have a positive impact on inhibiting the secretion of the ZAG-1. The ZAG-1 is a 40-kilodalton (KDa) single-chain polypeptide, which has a similarity with the major histocompatibility complex - class I molecules. This adipokine is involved in several important functions such as lipid mobilization and fertilization. ZAG-1 levels have been associated with many pathological conditions such as in carcinomas, AIDS and type II diabetes clinical complications [32,33]. So proving a positive impact of these herbs in controlling ZAG-1 level would be an advantage and may enhance the findings on immunomodulation efficacy. MCNF extract alone demonstrated a significant decrease in the ZAG-1 (P<0.05) or in combination with the addition of azathioprine (P<0.05) (Figure 2). However, combination of the extract with LEV50 showed no significant effect (P>0.5).

In chemotherapy, immunomodulators are used in a similar manner to adjuvants to control and prevent infections [34]. Many researchers tried to overcome their toxicities, by using several sources of immunomodulatory materials. Compounds like phenols, alkaloids, glycosides, polysaccharides, saponins have been analyzed for their capacity as biological markers [29]. Previous reports and phytochemical screening of extracts have shown the presence of flavonoids, alkaloids, proteins, glycosides, vitamins, and traces of polyphenolic contents [30,31,35-39].

In conclusion, the data from this study suggest that the ethanolic MCNF extract enhanced both the cell-mediated and humoral immune responses as shown in the WKY rat animal model. These potential findings may be related to the different components and/or phytochemicals present in this traditional herbal medicine. Further studies may be required to identify and isolate the active components, which are responsible for rendering the immunostimulatory impacts.

Ethics Approval

An ethical approval was obtained from the Animal Ethics Committee, Sultan Qaboos University (SQU) (SQU/AEC/2017-18-11). Animals involved in all experiments were performed according to the standards accepting procedures (SQU-Animal Ethics Committee standards).

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