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Modulation of Macrophage Activity by a Herbo-mineral Formulation in Murine Model

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Authors' contributions

This work was carried out in collaboration between all authors. Author FK designed and analyzed the study. Authors AE, SB and YS performed the statistical analysis and managed the literature searches and author SA wrote the first draft of the manuscript and provided overall guidance. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aim: The present paper investigates the effect on immune responses *by a* herbo-mineral formulation, Khamira Marwarid [KM], prescribed in the Indian subcontinent as a rejuvenator, especially to convalescing patients of typhoid fever, in murine model.
Study Design: KM was administered orally for short, intermediate and long duration [5, 15 and 30 days respectively] at a dose of 2g/kg body weight.
Results: Administration of KM enhanced the antigenic and mitogenic activity, induced by ovalbumin and Con A (mitogenic stimuli), of mice whole splenocytes. KM caused a marked increase of production of Th-1 cytokine (IFN-γ) and a non significant decrease of production of Th-2 cytokine (IL-4) by splenocytes when stimulated with Concanavalin A. Oral administration of KM, by itself did not induce the production of NO by macrophages, but enhanced the production of NO in response to LPS as compared to unstimulated control. However, dose duration related suppression of NO production was observed. KM also enhanced significantly the phagocytosis that was evaluated using the phagocytic rate (PR) and phagocytic index (PI).
Conclusion: The results indicate the immunomodulatory potential of KM leading to a Th1 dominant immune state and activation of macrophages and may find use in immunotherapy of tumors.

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1. INTRODUCTION

Immunomodulation, leading to enhanced protective immunity, as a low cost alternative to expensive management of diseases, is of immense economic benefit especially to developing regions of the world. Also, immunotherapy is a well documented approach for treatment of cancer [1,2]. The role of immunopotentiators, both of chemical or biological origin, in spontaneous regression of tumors has been utilized especially in patients with either inoperable tumors or operable tumors with high risk of postoperative recurrence [3,4]. Enhancing T cell mediated immunity either by prolonging T cell activation or by restoring T cell proliferation increases the patient's capacity to mount an effective anti tumor response [5]. Recently, monoclonal antibodies, ipilimumab and tremelimumab have been granted regulatory approval. Both these monoclonal antibodies are directed against CTLA-4, a molecule that down regulates T cell activation through a homeostatic feedback loop [6,7]. Similarly anti PD-1 mAb, PD-1 being an inhibitory receptor belonging to the CD28/ CTLA-4 receptor family that is expressed on activated T cells, B cells, and monocytes, are being clinically tested for their effect on cancer [8]. A number of other immune check points, like stimulation of CD40, a member of TNF superfamily, that causes a strong induction of systemic CTL [cytotoxic T lymphocyte] immunity or activation of CD137 that enhances T cell proliferation and the memory and cytotoxic activity of T cells, are being targeted using monoclonal antibody approach [9-11]. The current strategies are aimed at refining immunotherapeutic approach through more effective combinations to overcome tumor induced immunesuppression and tumor evasion. However, the economic hurdle of usage of monoclonal antibodies as therapeutic agents poses a challenge to the developing world.

In the Indian subcontinent, traditional systems of medicines rely on improvement of quality of body tissues that leads to attributes like longevity, immunity against diseases, improved mental and intellectual competence [12]. Activation of T cell mediated immune responses enhances the capacity to fight invasion and proliferation of tumor. Keeping this in mind, a traditional Unani formulation, Khamira Marwarid [KM] was chosen to investigate its properties as modulator of cell mediated immune responses. KM is a herbo-mineral formulation that containes calcined pearl, calcined jade, calcined serpentine along with extracts of *Santalum album* Linn., *Vateria indica* Linn., *Centaurea behen* Linn., *Salvia haematodus* Linn., *Punica granatum* Linn., *Pyrus melus* Linn. (Table 1) [13]. It has been prescribed since ages by Unani practitioners to strengthen body's capacity to fight infection, strengthen the vital organs of the body and as rejuvenator [14]. It is important to point out that most of the traditional Indian systems of medicines are formulations/preparations of complex integrated derivatives prepared through several specific preparatory steps. This is the reason that to point out the active component of such formulations does not give full insight into the efficacy or mode of action of these traditional formulations [12]. However, owing to their clinically proven efficacious use since centuries and the resurgent interest of the entire world on traditional knowledge of plants and minerals, it becomes imperative to investigate the efficacy of these formulations using modern scientific investigations. Such studies provide a helpful insight to modern clinical practitioners to augment and supplement modern medicine. In this study, to understand and to examine the role of KM on immune system, experiments were conducted for studying the effect of KM on T cell proliferation, cytokine analysis and production of NO by macrophages and phagocytosis.

2. MATERIALS AND METHODS

2.1 Unani Formulation Khamira Marwarid

Khamira Marwarid [KM], a product of Hamdard *Wakf* laboratories, was procured from the Unani Pharmacy at Majeedia Hospital, Jamia Hamdard, New Delhi. It is a herbo-mineral preparation and its components are mentioned in (Table1). Briefly, it is a polypharmaceutical preparation containing crude drugs of plant and mineral origin in the form of fine powder or their infusion\decoctions in sugar syrup. KM is prepared by adding microfine powder of the minerals like pearl, Jade and Serpentine to the sugar syrup of a specific consistency. Crude extracts of *Punica granatum, Pyrus malus*, *Sandal safed*, *Kehruba* and *Warq-e-Nuqurah* are then added to the cooled mixture with continuous stirring. The mixture is blended till the required consistency is achieved [13,15].

Table 1. The composition of khamira marwarid [15]

Name	Origin	Major Chemical composition	Content g/10gm
Pearl	Mineral	Calcite or Calciun carbonate	0.24
Jade	Mineral	Jadite ($AlSi_2O_6$) and Nephrite ($Ca_2(MgFe)_5(OH)_2(SiO_4)_2$	0.12
Serpentine	Mineral	Bowerite, Lizardite and Chrysotile	0.12
Argentum	Mineral	Silver	0.12
Santalum album Linn	Plant	Santalol	0.12
Punica granatum Linn.	Plant	Punicalagin	0.01L
Pyrus malus Linn	Plant	Quercetin	0.01L
Vateria indica Linn.	Plant (resin)	Fatty acids C16, C18, C18:1, C18:2, C20	0.12

2.2 Chemicals

Immunochemicals, Ovalbumin, Hank's balanced salt solution (HBSS), RPMI-1640, Fetal calf serum (FCS) and o- pheneylene diamine dihydrochloride (OPD), Concanavalin A were purchased from Sigma, St. Louis, MO. ^{3}[H] Thymidine was procured from Amersham Biosciences, UK. Goat RBC was procured from the local slaughterhouse. All other chemicals were of AR grade.

2.3 Animals

The study was approved by Institute's animal ethical committee and conformed to national guidelines on the care and use of laboratory animals. Swiss Albino mice, male, 6-8 weeks old, weighing 20-25g was used for the study. The animals were maintained at 25±2ºC, photoperiod of 12hrs, in the Institute's animal house and fed with commercial pellet diet and water *ad libitum*.

2.4 Treatment

Animals were divided into three groups comprised of a minimum of 5 mice. KM was administered orally for a short duration of 5 days, intermediate duration of 15 days and long

duration of 30 days, respectively. Animals in control group received Normal Saline for the same periods.

2.5 Dosage

The drug was suspended in normal saline and the dose to be given to mice was extrapolated from that being prescribed to humans by Unani physicians. Oral administration of *Khamira Marwarid* at a dose concentration of 2gm/Kg-body weight was given with the help of canula. Dose volume was 0.2ml/mice. Animals in the control group received the same volume of normal saline.

2.6 Lymphocyte Proliferation Assay

Animals of all the groups were injected intramuscularly and in the footpad with the antigen (ovalbumin) on day 0 (primary dose). Booster dose was given on day 8^{th} and the animals were sacrificed on day 12^{th}. Splenocytes were cultured and lymphocyte proliferation assay using ^{3}H Thymidine (Amersham, Germany) incorporation into DNA, counted using βScintillation counter (Beckman, U.S.A.) was carried out as previously described [16].

The Stimulation index was calculated as follows:

$$SI = \frac{\text{Mean cpm of cells stimulated with antigen}}{\text{Mean cpm of cells without antigen}}$$

2.7 Cytokine Analysis

The cytokines IL-4 and IFN-γ were measured in the culture supernatants collected during T cell proliferation assay. The culture supernatants were centrifuged at 5000rpm for 15min, filtered through 0.22μM membranes and assayed for cytokine levels using DuoSet® ELISA development system kits (R&D Systems, Minneapolis, USA) as per manufacturer's instructions.

2.8 Measurement of Induction of NO in Macrophages

Murine peritoneal cells were flushed from the peritoneal cavity of all the animals by administering 10ml of ice cold HBSS and NO assay was carried out as described in Ling et al. 2012 [17].

2.9 Phagocytosis by Peritoneal Macrophages

Phagocytosis by peritoneal macrophages in mice was detected using method described previously [18] with slight modification. Briefly, 0.5ml of $5X10^{6}$ goat Red blood cells (gRBC) was intraperitoneally injected into each mouse and mice were euthanized 1hr later. The fluid of abdominal cavity was collected to make a smear for each mouse. The smear were incubated at 37ºC for 30min in a wet box, fixed with 95% ethanol, and then stained by Wright-Giemsa dye. The number of macrophages ingesting gRBC out of a total of at least 100 cells was calculated by direct visual enumeration using a light microscope. The phagocytic rate (PR) and phagocytic index (PI) were calculated using the following formula:

$$PR\% = \frac{(\text{number of macrophages ingesting gRBC})X100}{\text{Total number of macrophage}}$$

$$PI = \frac{\text{Total number of ingested gRBC}}{\text{Number of macrophage ingesting gRBC}}$$

2.10 Statistical Analysis

The statistical significance of difference between groups was determined by student's t-test. Statistical significance was defined as a *P* value<0.05.

3. RESULTS

3.1 Effect on Lymphocyte Proliferation

The effect of oral administration of KM on proliferation of splenic lymphocytes was examined in ^{3}H thymidine incorporation assay. The result presented in (Table 2) demonstrates the effect of KM on antigenic (Ovalbumin) and Concanavalin A induced proliferation of splenic lymphocytes. Prior dosing of mice with KM even for a short duration of 5 days shows an enhanced antigenic and mitogenic *In vitro* lymphocyte proliferation as compared to control. Maximum antigenic and mitogenic stimulation index of 5.44 and 16.17 respectively was recorded for mice pretreated with KM for intermediate duration of 15 days. However, the antigenic and mitogenic stimulation index was observed to be slightly decreased as compared to intermediate dosing group, though was still significantly enhanced as compared to its control.

3.2 Effect on Production of Th1 and Th2 Cytokine by Splenocytes

The balance of Th1 and Th2 cells in the host is considered to be important for regulation and induction of immune functions [19,20]. We therefore investigated the effect of KM on production of Th1 and Th2 cytokines by splenocytes from control mice and KM treated mice. As shown in (Fig. 1), splenocytes from control mice and KM treated mice did not produce detectable amounts of Th1 cytokine (IFN-γ) or Th2 cytokine (IL-4) without antigenic or mitogenic stimulation. When splenocytes were incubated with antigen (ovalbumin) or mitogen (Con A) detectable amounts of these cytokines were found in cell free supernatant. The oral administration of KM resulted in a significant enhancement of IFN-γ and a non significant decrease of IL-4 production as compared with that in controls (Fig. 1). Thus oral administration of KM may lead predominantly to the production of Th1 type cytokines.

3.3 Effect on NO Production by Macrophages

To investigate the effect of oral administration of KM in mice on NO production, the accumulation of nitrite, a stable metabolite of NO, was measured in culture media of peritoneal macrophages using Greiss reagent. Cultures of peritoneal macrophage cells were stimulated with LPS (5µg/ml) to stimulate NO production. As shown in (Table 2), treatment with LPS for 24hrs induced a significant increase in nitrite production when compared with unstimulated peritoneal macrophages. Dose duration dependant suppression in NO production in LPS treated group was observed. Significant inhibition of NO production was observed in LPS stimulated macrophages isolated from mice pretreated with KM for 15 days and 30 days group as compared to control. Cell viability was not affected by exposure to LPS (data not shown).

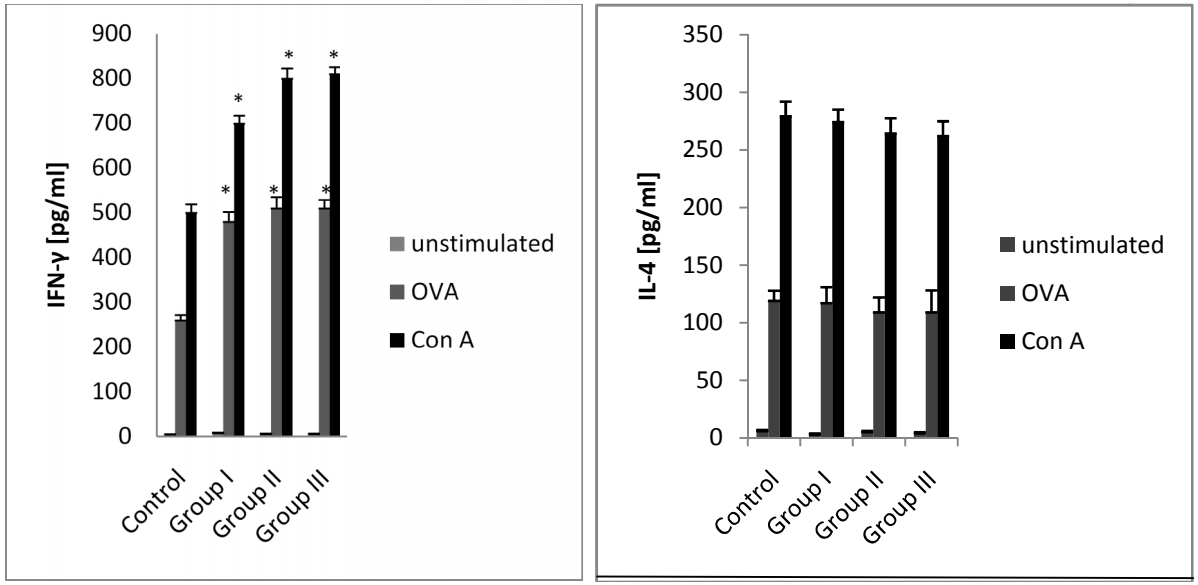

Fig. 1. Effect of KM on levels of IFN-γ anf IL-4 from splenocytes

*After the final dose of the formulation, mice were immunized with ovalbumin on day 0 and 8. Animals were sacrificed on day 12th and splenocytes were cultured for 3 days, in RPMI supplemented with 10% FCS, in presence of concanavalin a (2µg/ml) or ovalbumin (5µg/ml). The cell free supernatant was collected from each well and the amounts of IFN-γ and IL-4 were measured using ELISA. Data represents the mean cytokine level of triplicate experiments. Data represented as mean±S.E., where n=5 *P=0.05, significantly different when compared with its respective control (Dunnett's test).*

3.4 Effect on Phagocytosis

(Table 2) shows the phagocytic activity of peritoneal macrophages isolated from KM treated mice. The uptake capacity of peripheral phagocytes was increased by KM. The phagocytic rate (PR) was elevated even after 5 days of oral administration of KM, though significant increase in phagocytic index and phagocytic rate was observed in 15 and 30 days treatment groups.

Table 2. Effect of KM on activation of T cells and macrophages

Parameter analyzed		Control	Group I	Group II	Group III
Lymphocyte	Ova	1.04±0.02	*3.44±0.53	*5.44±0.42	*4.7±0.58
Proliferation assay	ConA	6.19±0.98	*10.6±0.87	*16.77±0.76	*14.62±0.89
Nitric Oxide production [μM/10 ^{6}cells]		22±1.12	21.01±0.89	*16.45±1.76	*16.23±1.06
Phagocytic rate (PR)		19.58±2.16	24.16±2.01	*43.09±2.80	*40.12±3.12
Phagocytic index (PI)		1.00 ±0.00	1.02 ±0.08	*1.13±0.093	1.11±0.042

*KM treated mice were immunized with ovalbumin on day 0 and 8. Animals were sacrificed on day 12th and splenocytes were cultured for 3 days, in RPMI supplemented with 10% FCS, in presence of concanavalin a (2μg/ml) or ovalbumin (5μg/ml). Cultures were pulsed with ^{3}H- thymidine and incorporation was detected by liquid scintillation spectroscopy. Data represents the mean stimulation indices (SI) of triplicate experiments Nitrite production by peritoneal macrophages from mice treated with KM and stimulated in-vitro with LPS (5μg/ml) was measured using Griess reagent. Peritoneal macrophages were collected from mice treated with KM and immunized i.p. with gRBC (5X10^{6}). Smear of cells were stained with Wright-Giemsa dye and enumerated using light microscope and PR and PI calculated. Data represented as mean±S.E., where n=5 C, control, Group I, II and III stands for groups treated with KM for 5,15 and 30 days; *P=0.05, significantly different when compared with its respective control (Dunnett's test).*

4. DISCUSSION

In the present study, a poly-herbo-mineral formulation, KM, was examined for its use as pharmacological immunopotentiator. Since the traditional herbal prescriptions are generally prepared from combination of many crude drugs on the basis of oriental prescriptions and herbalogy, they may have combined effects which differ from the sum of the effects of individual constituent crude drugs. Therefore the whole preparation was as such suspended in normal saline and administered to mice orally.

Lymphocyte activation and proliferation of T-cell subpopulations leads to an effective immune response. T cells in response to a foreign antigen are activated and this ensures antigen-specific clonal expansion of T cells that ultimately leads to the clearance of pathogens [21] upon stimulation by antigen or mitogen, T cells secrete IL-2, which upon binding to its receptor induce proliferation and differentiation of a number of T lymphocyte subsets. Following this stimulation, a cytokine cascade that includes IL-4 (TH2 cytokine involved in regulating antibody production), and IFN-γ (a TH1 cytokine proinflammatory cytokine) may be released [22]. In our study, it was observed that oral administration of KM to mice even for 5 days exerted an augmentative affect on lymphocyte proliferative responses to mitogen and antigen. This indicates that KM administration induces a sizeable peripheral pool of antigen and mitogen sensitive naive T lymphocytes and cytotoxic cells which ensures an improved immune response to antigenic and mitogenic challenge. Oral administration of KM caused the increased production of IFN-γ and a decreased, albeit non

significant, production of IL-4 by splenocyte stimulated with Con A when compared with untreated control. $CD4^+$ helper T cells that produce IL-2, IFN-γ and TNF-α/β, but not IL-4 are designated Th1 and are chiefly responsible for cell mediated response. They can also help B cells to produce IgG2a but not much IgG1 or IgE, respectively. CD 4^+ Th cells that produce IL-4, IL-5, IL-10 and IL-13, but not IL-2 or IFN-γ are designated Th-2 [20]. These results indicate that oral administration of KM can lead to Th-1 type dominant immune responses possibly through induction of IL-12 and IFN-γ production in mice.

Activated macrophages function effectively as antigen-presenting cells. During immune response, they also facilitate activation of T-helper cells. In addition, activated macrophages secrete various antimicrobial and cytotoxic substances that can destroy phagocytosed microorganisms. In the present study, the phagocytic rate and phagocytic index was measured as a parameter for assessing the activation of macrophages. Phagocytosis is an important step in the activation of immune responses as it is post phagocytosis that macrophages start acting like antigen presenting cells, with increased expression of CD80 and CD86 co-stimulatory molecules, and mediate activation of T cells via interaction of T cells and macrophages [23]. Oral administration of KM significantly increased phagocytic rate and phagocytic index in short term, intermediate term and long term treatment groups as compared to control indicating the enhanced activation of macrophages isolated from KM treated mice.

In the present study, macrophage function was also analyzed through NO production. Low concentrations of NO from activated macrophages are beneficial, as, along with other reactive nitrogen intermediates, they are responsible for cytostatic and cytotoxic activity against infectious organisms and tumor cells. In addition, NO plays a regulatory role in function of natural killer cells and the expression of cytokines such as IFN-γ and transforming growth factor-β [24]. However, over production of NO has been found to be associated with various diseases such as septic shock, autoimmune diseases, and chronic inflammation by increasing vascular permeability and the extravasations of fluid and proteins at the inflammatory site [25,26]. Therefore, inhibition of high-out-put NO production could be a useful strategy for treatment of various inflammatory diseases. The in vivo studies presented here demonstrated the suppressive effect of KM on NO production using freshly isolated peritoneal macrophages. The result correlates with Th-1 type of dominant immune response as observed with cytokine analysis.

5. CONCLUSION

In conclusion, the findings of the present study establish that KM has appreciable immunostimulatory activity. It modulates the cell mediated immune responses possibly via cytokine modulation. Administation of KM switched the Th1/Th2 balance towards Th1 immunity, accompanied by increased IFN-γ by Con A and OVA-stimulated mouse splenocytes. Activation of macrophages was also observed as suggested by the enhance phagocytic index. It is not possible at this junction to single out the most effective immunostimulatory constituent of KM. Further investigations on major constituents of KM are in progress.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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