A COMPARATIVE STUDY ON IMMUNOMODULATORY EFFECTS OF INDIGENOUS HERBAL DRUGS IN RABBITS

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ABSTRACT: Immunol® (syrup) (0.65ml/kg), Gen-Xing® (tablet) (7.2mg/kg) and MufarrehYakuuti® (semi-solid) (87mg/kg) were administered orally at normal, 25% (0.82ml/kg, 9mg/kg and 107.5mg/kg) and 50% (0.98ml/kg, 10.8mg/kg and 129mg/kg) increased dose to healthy male rabbits for two weeks. Non-specific and Humoral immune responses were measured through percent Neutrophil Adhesion assay and Haemagglutination Antibody assay against Sheep RBCs respectively. Immunol revealed dose dependent response with maximum percent NA (92.58 ± 2.36 & 97.87±1.31) at 50% increased dose. Effect of drugs on Humoral Immune Response was significantly higher in terms of individual antibody titer and Geometric Mean Titer (GMT) as compared to control. However, Immunol® at 50 % and MufarrehYakuuti® at 25% increased doses showed highest GMT of 1024. GMTs decreased to 50% at 45th day of experiment with highest value of 64. The herbal preparations enhanced the specific and non-specific immunity in rabbits under study. However, Immunol was found to be the best and may be potential candidate for clinical use in future.

Keywords: Herbal drugs, Immune response, Neutrophil Adhesion, Haemagglutination antibody assay and Rabbits.

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INTRODUCTION

Immunomodulators are natural or synthetic substances which can suppress or stimulate immune system of animals. The immune system is mainly responsible for protection of the individuals against potential pathogens. The search for agents to treat the residual cancer cells was the starting point in the field of immunomodulation (Yamamoto, 1996).

Medicinal Plants and their derivatives are being extensively investigated now a day for immunomodulatory properties as well as potential therapeutic agents. Minerals and Herbs have been used for the cure of disease since time immemorial. It is an established fact that immuno-stimulation can serve as an alternative to conventional antibiotics in treatment of variety of diseases whereas suppression of immunity can be induced to avoid autoimmune disorders and organ transplant rejection (Sulaiman, 2010).

Immunostimulants are used to activate immune systems both passively and actively (Rang *et al.*, 2003). However, herbal medicines are traditionally used by most of the world population and trend has increased tremendously in the last two decades (Carter, 2001; Tan and Vanitha, 2004).

Pakistan is among those developing countries where people prefer herbal remedies for their fewer side effects and reliability../AppData/Local/Zohaib Thesis/paperz for paper(WHO, 2005). Hundreds of poly herbal products are available in local markets for treatment of different ailments with claim of immunostimulatory effects. Among such products, Gen-Xing®, Immunol® and MufarrehYakuuti®are the most prominent and frequently used immune boosters. In view, present study was designed to assess the changes in non-specific and humoral immunity of rabbits, using Gen-Xing®, Immunol® and MufarrehYakuuti®.

MATERIALS AND METHODS

Animals: Male rabbits (n=60) of 1-1.6 Kg body weight were purchased from a local vendor and given two weeks of acclimatization period under standard keeping conditions. All the rabbits were housed in Laboratory Animal House of Department of Pharmacy, Government College University, Faisalabad, Pakistan at 23±2^{O}C temperature and 12 hr light/dark cycle. Adequate amount of feed and water was available at all times to the rabbits.

Herbal Preparations: Three herbal products with the brand name of Gen-Xing® (tablet), MufarrehYakuuti® (semi solid) and Immunol® (syrup) were purchased from an herbal drug store located in Chiniot Bazar, Faisalabad.

Antigen Preparation: Fresh blood (10ml) was collected by venipuncture in a sterile test tube containing anticoagulant from sheep kept at University of Agriculture Faisalabad. Packed Sheep Red Blood Cells (SRBCs) were separated by centrifugation at 3000rpm for 10 minutes aseptically. The RBCs pellet was re-suspended in 0.9% pyrogen free physiological saline and

centrifuged, the process was repeated thrice and finally 1% SRBCs suspension was prepared for inoculation (Fulzele*et al.,* 2003).

Experimental Design: All the rabbits (n=60) were placed into four groups (A, B, C and D) and each group comprised of 15 rabbits. First three groups were administered with herbal drugs whereas last group served as control. Rabbits in group A, B and C were further divided into three sub-groups (1, 2 and 3). The total duration of trial was 30 days which was further divided into Phase I: Pre-immunization & Phase II: Post-immunization each of equal duration. Dosage of herbal products was adjusted for individual rabbits based on their body weight keeping in view dosage recommended for humans. All the three herbal products i.e. Immunol®, Gen-Xing® and MufarrehYakuuti® were administered per os at normal (0.65ml/kg, 7.2mg/kg and 86mg/kg), 25% (0.82ml/kg, 9mg/kg and 107.5mg/kg) and 50% (0.98ml/kg, 10.8mg/kg and 129mg/kg) increased dose levels in sub-groups (1, 2 and 3) of group A, B and C, for 29 days respectively.

Neutrophil Adhesion Assay: Blood samples (3ml) were drawn from jugular vein using disposable sterile syringes (BD™ UK) on 14th day post administration of herbal products. Total leukocyte Count (TLC) and Differential Leukocyte Count (DLC) were determined from each sample. Afterwards all of these samples were co-incubated with 80 mg/ml of Nylon fibers at 37^{o}C for 15 min, TLC and DLC were recorded again. TLC and % neutrophil values were used to calculate the Neutrophil Index (NI) and finally Percent Neutrophil Adhesion (% NA) was determined by using the formula described bySathianarayanan and Rajasekaran (2012).

Immunization of Rabbits: 1ml of SRBCs suspension was inoculated into marginal ear vein of each rabbit in treated and control groups on 14th day of trial(Tilwari*et al*., 2011). The administration of Herbal preparations was continued for further two weeks and blood specimens were obtained again by venipuncture on 29th day of trial. All of the aforementioned parameters were recorded again on fiber treated and un-treated blood specimens for the estimation of % NA following immunization.

Haemagglutination antibody assay: Haemagglutination antibody (HA) assay was used for the evaluation of effect of herbal drugs on humoral immune response of rabbits against SRBCs (Ranjith*et al.,* 2008; Sathianarayanan and Rajasekaran, 2012). Briefly, blood samples (3ml) were collected without anticoagulant on 29th and 45th day for separation of serum. Two-fold serial dilutions of separated sera were prepared using normal saline in 96 wells, round bottom microtitration plate (Titertek™ UK). Equal volumes of SRBCs suspension (1%) added in each well as antigen along with negative control. After incubation of 30 min agglutination of SRBCs was observed. The highest dilution of serum showing agglutination in each sample was recorded as end point and its reciprocal as HA antibody titer.

Statistical Analysis: The recorded data were statistically analyzed by One Way Analysis of Variance (ANOVA) and Dennett's Multiple Comparisons test at 95% confidence level, results were presented as Mean ± SE (Steel *et al.,* 1997).

RESULTS AND DISCUSSION

Immune system plays major role in protection of the host against infections. WBCs and Neutrophils are the first lines of defense against diseases (Kuby, 1996). Certain substances form plant and animal origin possess immunomodulatory properties and stimulate the immune response against pathogens. But such substances and other herbal cocktails should be screened by systematic trials to evaluate the proclaimed effects prior to clinical use (Fulzele*et al.,* 2003)

The use of herbal medicines has increased significantly in recent years in comparison to allopathic drugs owing to the concept that natural products have least side effects. So many over the counter herbal formulations are available in herbal drug stores with the claim of immunomodulatory properties in the country. One such example is of Ginseng, a plant source preparation and contains a number of pharmacological components like; ginsenosides (tetracyclic triterpenoid saponins), polyphenolic compounds, acidic polysaccharides, and polyacetylenes. It has been reported to possess a number of therapeutic properties against insulin resistance, cancer, hypertension, and neurodegenerative disorders. Moreover, it helps in maintenance of general health, immunity and resistance to illness and infections by immune-stimulation (Min and Kang, 2012).

This study was done to assess the immunomodulation by selected indigenous herbal formulations. All of the tested products exhibited considerable increase in % NA ($P<0.05$) under various dose regimens as compared to control following two-week treatment. Immunol® and Gen-Xing® depicted a graded dose response and maximum % NA was recorded at 50% increased dose rate (92.58±5.28) and (84.60±4.96) respectively. While, MufarrehYakuuti® treated group resulted in highest % NA at normal and 25% dose levels (Table 1). All of the treated groups had significantly higher % NA ($P<0.05$) in comparison to control group, following twenty-nine-day treatment. A similar increment trend in % NA was recorded in all treatment groups following two weeks of further treatment and immunization as was recorded prior to immunization (Table 2).

The Total Leukocyte Counts, Percent Neutrophils, Neutrophil Index and Percent Neutrophil Adhesion in Phase-I (Pre-immunization) were found to be statistically significant ($P<0.05$) and promising for all the herbal preparations administered in treatment groups in comparison to untreated group (control). Non-specific Immuno-stimulation was observed for all three herbal preparations at normal, 25% and 50% dose levels. However, Immunol®(syrup) at 50% dose level was found best followed by other two formulations. A gradient dose response curve was recorded with Immunol® while, variations were recorded for the remaining two products. A significant increase in the *in-vitro* neutrophilic adhesion was recorded post treatment with Nylon Fibersand control group was exception to this, which elucidated the stimulatory effect on non-specific immunity of rabbits.

Fulzele*et al.* (2003) evaluatedHaridradiGhrita (HG) at four dose levels i.e. 50, 100, 200 and 300mg/kg/day in rats and recoded increased neutrophilic adhesion to the Nylon Fibers at dose rate of 300mg per kg per day. These findings were in line with the present study, the mere difference with our study was that we used rabbits as experimental subject in place of rats due to ease of management and convenience in multiple blood sampling.

The overall results of % neutrophils, NI, TLC and % Neutrophil Adhesion in Phase II (Post-Immunization) were highly significant ($P<0.05$) in comparison to untreated (Control). The enhanced neutrophilic adhesion to Nylon Fibers was recorded post two-week treatment only exception was the control group. The overall incremental trend in neutrophil adhesion was recorded in both phases of the trial which was a clear evidence that all the tested products were equally effective in non-specific immune stimulation for both the groups (unchallenged) and (challenged with antigen). A gradient dose response was observed for Immunol® againpost-immunization, the highest % NA was recorded at 50% dose level.On the other hand, variation in response was observedwith respect to the dose levels of other two formulations. The findings of Immunol®were in complete accord with the findings of Fulzele*et al.*(2003) with respect to % NA at 300mg per kg per day dose. The values of % NA in the current study werecomparable toBanji*et al.*, (2012), who evaluated *Moringaolifera* Lam leaves extracts for immunomodulatory properties in Wistar rats.

At 29th day, the sub-groups A1 and A2 treated with normal and 25% increased dose of Immunol showed same values (256) of GMT but sub-group B3 treated with 50% increased dose of immunol showed GMT of 1024 which was the highest value among all the treated and untreated groups. In sub-groups, B1 and B2 treated with normal and 25% increased dose of Gen-Xing, GMT was 256, whereas it was highest (512) in B3 sub-group treated with 50% increased dose of same drug. Maximum antibody titers were observed with 25% increased dose of MufarrehYakuuti with GMT of 1024 (Table 3). At 45th day of drug treatment, the values of GMTs were non-significant as compared to the control group. An overall decrease of about one half was observed in GMTs as compared to 29th day. The highest value was 64 in different treated sub-groups followed by 32 which were comparable to control group (Table 4).

The B lymphocytes responsible for humoral immunity produced immunoglobulins which recognized and eliminated extra cellular antigens. The results of Haemagglutination Antibody (HA) assay revealed a significant increase in antibody production among all the three treated groups in comparison to control group at 29th day of treatment. A gradient dose response was observed in groups A and B treated with Immunol® and Gen-Xing as the GMTs were higher with the increase in dosage. However, variable findings were observed with respect to the dosage in group C treated with MufarrehYakuuti® with the highest GMT at 25% dose level. The treatment of drugs was stopped after 29th day and rabbits were fed on normal diet up to 45th day of experiment. Serum samples tested at 45th day showed an overall decrease of 50% in GMTs of all the treated and untreated groups. The reason behind this could be either withdrawal of treatment with herbal drugs or naturally as antibodies had specific half-lives after which they were catabolized in the body and their titer gradually decreased.

The overall results of humoral immune response were in agreement with the results of Sajid*et al.* (2007), who evaluated the efficacy of Ivermectin® on the humoral and cellular immune response of rabbits. The specific antibody titer against *Pasteurellamultocida* increased with the increasing dose of Ivermectin as the highest GMT value was obtained at 600µg/kg dose level. Similarly, the results of current study were in agreement with the findings of Banji*et al.*, (2012), who used alcoholic (EEMO) and hydroalcoholic (HAMO) extracts of *Moringaolifera* for immunomodulatory effects in Wistar rats. They revealed a significantly high level of antibody titers in animals treated with higher dose of EEMO (200mg/kg) compared with the control ($P<0.05$). Whereas, Fulzele*et al.* (2003) in a study did not find any effect of HaridradiGhrita®, an Indian polyherbal formulation on humoral immune response of rats.

Table 1. Pre-immunization Neutrophil Index of Fiber untreated and Treated blood samples and % Neutrophil Adhesion in different groups at 14th day of drug treatments.

Groups/ Treatments	Subgroups	Dosage	NI (10^3) Untreated Blood	NI (10^3) Fiber Treated Blood	Percent Neutrophil Adhesion (% NA)
A Immunol	A1	Normal	623±12.74	117.68±2.38	80.968±4.21
	A2	25% increase	569.8±7.79	122.20±1.72	78.616±1.79
	A3	50% increase	728±4.68	39.40±1.32	92.578±5.28
B Gen-Xing	B1	Normal	435.4±27.21	125.24±7.70	72.524±8.42
	B2	25% increase	485.4±16.44	121.98±3.97	74.060±9.33
	B3	50% increase	402.4±9.91	58.32±1.86	84.604±4.96
C MufarrehYakuuti	C1	Normal	532±13.28	108.74±5.88	79.912±6.54
	C2	25% increase	461.6±18.33	87.60±1.99	79.782±6.35
	C3	50% increase	550±11.08	152±5.04	72.303±4.77
D	Control	---	390±15.25	165±7.50	57.69±8.22

The values are mean ± SE of 5 rabbits in each group. One-way ANOVA followed by Dennett's multiple comparisons test (P<0.05 Vs Control)

Table 2. Post-immunization Neutrophil Index (NI) of Fiber untreated and Treated blood samples and % Neutrophil Adhesion in different groups at 29th day of drug treatments.

Groups/ Treatments	Subgroups	Dosage	NI (10^3) Fiber Untreated Blood	NI (10^3) Fiber Treated Blood	Percent Neutrophil Adhesion (% NA)
A Immunol	A1	Normal	711.8±12.64	81.24±2.43	87.99±5.18
	A2	25% increase	601±7.92	43.68±1.56	92.32±0.59
	A3	50% increase	614±4.34	12.86±1.44	97.87±1.31
B Gen-Xing	B1	Normal	323±5.65	47.70±1.65	85.38±4.20
	B2	25% increase	445.8±5.33	71.68±3.63	83.65±9.00
	B3	50% increase	553.2±3.38	42.98±1.87	91.18±4.91
C MufarrehYakuuti	C1	Normal	686±16.75	96.24±3.16	85.97±3.25
	C2	25% increase	560.8±16.05	67.70±2.74	86.82±6.61
	C3	50% increase	579.6±10.12	118.64±3.44	76.98±6.74
D	Control	---	358±7.48	145.26±2.13	59.42±6.58

The values are mean ± SE of 5 rabbits in each group. One-way ANOVA followed by Dennett's multiple comparisons test (P<0.05 Vs Control)

Table 3.Hemagglutination (HA) antibody titers in serum samples of different groups at 29th day of treatment.

Groups / Treatments	Subgroups	HA Antibody Titer					GMT*
A Immunol	A1	128	1024	256	128	256	256
	A2	512	128	128	512	512	256
	A3	512	2048	512	1024	1024	1024
B Gen-Xing	B1	256	512	256	256	512	256
	B2	256	128	256	512	128	256
	B3	256	512	512	512	256	512
C MufarrehYakuuti	C1	256	512	512	512	512	512
	C2	256	2048	1024	1024	512	1024
	C3	2048	128	256	1024	1024	512
D	Control	32	512	64	128	64	128

*Geometric Mean Titers

Table 4.Hemagglutination (HA) antibody titers of serum samples of different groups at 45th day of treatment.

Groups / Treatments	Subgroups	HA Antibody Titer					GMT*
A	A1	32	32	32	32	32	32
Immunol	A2	32	32	32	64	32	32
	A3	64	128	64	64	64	64
B	B1	64	64	64	32	64	32
Gen-Xing	B2	64	32	32	32	64	32
	B 3	32	64	32	32	64	32
C	C1	128	64	64	64	32	64
MufarrehYakuuti	C2	32	64	64	64	64	64
	C3	64	32	32	64	64	64
D	Control	16	64	16	32	16	32

*Geometric Mean Titer

Conclusion: All three test herbal products (Immunol®, MufarrehYakuuti® and Gen-Xing®) had the potential to stimulate the non-specific and humoral immunity in rabbits. Immunol® syrup was found to be the best in this regard and might be a potential drug for clinical use in immuno-suppressed patients.

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