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## Immunostimulant Activity of *Kalayak ghrita*

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**The focus of the present study is to investigate the immunostimulant effect of *Kalayak ghrita*, an herbal formulation. The study of this formulation in respect to humoral and cell-mediated immune response showed that oral administration of *Kalayak ghrita* enhanced the antibody titre as well as foot pad swelling response to the antigenic challenges with sheep red blood cells. The herbal formulation belongs to the *Panchgavya* class of ayurvedic formulations in which one or more of the five bovine products (milk, ghee, curd, urine and dung) are used along with herbs. The formulation was administered at doses 50, 100, 150 and 200 mg/kg/day to healthy rats. Results of the present study suggest a dose-dependent immunostimulant effect of *Kalayak ghrita* in rats.**

The field of medicine has witnessed a global resurgence of interest in traditional systems of therapy over the past few years. Apprehensions concerning the toxicity and safety of modern/synthetic drugs have played a prominent role towards promoting research in developing safer drugs for clinical therapy<sup>1</sup>. Emphasis is also laid on the integration of traditional medicine with the modern health practices<sup>2,3</sup>. With increasing understanding of disease management, it has become necessary to provide scientific basis/rationale for the clinical utility of such medicinal agents of traditional systems. A lot of focus is being given for conducting studies

that substantiate the claims of traditional formulations<sup>4</sup>. The present study attempts to investigate the claimed immunostimulant effect of an herbal formulation belonging to *Panchgavya* class of medicinal agents.

*Panchgavya* is a term used in Ayurveda to describe the five important products of bovine origin such as milk, curd, ghee, urine and dung. Several formulations based on *Panchgavya* are reported in ancient Ayurvedic texts. *Panchgavya* are used either alone or in combination with herbs for the treatment of several diseases<sup>5-7</sup>. In the present manuscript we have investigated the immunostimulant activity of *Kalayak ghrita* (KG), an herbal formulation in experimental animals. The ingredients of KG are *Berberis*

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*aristata* and cow's ghee. KG belongs to the *Panchgavya* class of Ayurvedic formulations since it contains ghee as one of its ingredient.

KG was obtained as a gift sample from Go-Vigyan Anusandhan Kendra, Nagpur and used as received. A qualified botanist authenticated the plant material and a voucher specimen of the same has been deposited in Go-Vigyan Anusandhan Kendra, Nagpur. Fresh sheep red blood cells (SRBCs) in Alsever's solution were obtained from Nagpur Veterinary College, Nagpur. SRBCs collected in Alsever's solution were washed three times in large volumes of pyrogen free 0.9% normal saline and adjusted to a concentration of  $0.5 \times 10^9$  cells/ml for immunization and challenge.

Male Wistar rats (150-200 g) were used in this investigation. Rats were housed under standard conditions of temperature, ( $23 \pm 1^\circ$ ) 12-h light/dark cycle and fed with standard pellet diet (Gold Mohur, Lipton India Ltd., Mumbai) and water *ad libitum*. The animal experimental protocols were approved by the Institutional Animal Ethics Committee. A group of six untreated rats were taken as control (Group I). KG was administered orally at doses 50 mg/kg (Group II), 100 mg/kg (Group III), 150 mg/kg (Group IV) and 200 mg/kg (Group V) to test the immunostimulant effect.

Neutrophil adhesion test<sup>9</sup> was conducted on day 14 of drug treatment. Blood samples were collected (before challenge with SRBC) by puncturing the retro-orbital plexus into heparinized vials and analysed for total leucocyte counts (TLC) and differential leucocyte counts (DLC) by fixing blood smears and staining with Field stain I and II-Leishman's stain. After initial counts, blood samples were incubated with 80 mg/ml of nylon fibres for 15 min at  $37^\circ$ . The incubated blood samples were again analysed for TLC and DLC. The product of TLC and % neutrophil gives Neutrophil Index (NI) of blood sample. Percent Neutrophil adhesion was calculated using the formula:  $NI_t - NI_u / NI_u \times 100$ , where  $NI_u$  is neutrophil index of untreated blood sample and  $NI_t$  is neutrophil index of treated blood sample.

The humoral immunity was assessed by measurement of haemagglutinating antibody (HA) titre<sup>9</sup>. Rats of Group II, III, IV and V were pretreated with KG for 14 d and each rat was immunized with  $0.5 \times 10^9$  SRBC/rat by i.p. route including control rats. The day of immunization was referred as day 0. The animals were treated for an additional period of 14 d and blood samples were collected from each rat by retro-orbital puncture on day 15 for determining the HA titre. The titre was determined by titrating two fold serum dilu-

tions with SRBC ( $0.025 \times 10^9$  cells) employing 96-well microtitreplates. After incubating the mixtures for 2 h at room temperature the haemagglutinating capacity of the sera was read. Titres of sera were determined as the highest dilution presenting positive haemagglutination.

The effect of KG on the antigen specific cellular immune response in rats was measured by determining the degree of delayed type hypersensitivity (DTH) response using the footpad swelling test<sup>9</sup>. Rats of Group II, III, IV and V were pretreated with KG for 14 d and animals of each group (control and treated) were immunized on day 0 by i.p. administration of  $0.5 \times 10^9$  SRBC/rat and challenged by a subcutaneous administration of  $25 \mu\text{l}$  of  $0.5 \times 10^9$  SRBC/ml into right hind foot pad on day +14. KG was administered orally from day -14 until day +13. The left hind foot pad was injected with  $25 \mu\text{l}$  of normal saline. Footpad swelling was measured 24 h after SRBC challenge on day +14 using a plethysmometer. The difference between the means of the right and left hind footpad volume gave the degree of footpad swelling which was used for group comparisons. Data was expressed as mean percent increase in paw volume relative to control [ $\% = (\text{mean of footpad swelling of treated animals} / \text{mean of footpad swelling of control animals}) \times 100$ ]. The data were analysed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer Multiple comparisons test. P values  $< 0.05$  were considered significant.

Control of a disease or disorder by immunomodulation has two aspects, namely the development and improvement of protective immunity and the avoidance of undesired immunological side reactions. Evaluation of immunomodulatory potential of herbal formulations is as a major thrust area of research these days. One of the explanations offered to justify the beneficial effects of herbal drugs is a non-specific enhancement of immune status of the organism<sup>10</sup>. In view of this, we have studied the immunostimulant effect of KG, an indigenous herbal formulation employed in traditional practice with a belief that it would increase longevity and promote well being. Enhancement of antigen specific responses was observed against humoral as well as cell-mediated immune response.

Oral administration of KG significantly increased the adhesion of neutrophils to nylon fibers which co-relates to the process of margination of cells in blood vessels. The increase was found to be significant at dose 100 and 200 mg/kg/day when compared to untreated control. Table 1 shows the results of neutrophil adhesion. An increase in neutrophil adhesion may represent a possible

TABLE 1: EFFECT OF KALAYAK GHRITA ON NEUTROPHIL ADHESION IN RATS.

Groups	Neutrophil index		Neutrophil adhesion (%)
	UB	FTB	
I	276±70.2	226±50.2	18.0±6.50
II	298±52.1	245±60.2	17.6±7.21
III	305±56.2	246±42.2	19.0±6.52
IV	353±66.2	275±50.1	22.0±4.21*
V	375±45.2	292±39.2	22.1±6.21*

All values are mean±SD of 6 rats in each group. Statistical significance was determined by ANOVA followed by Tukey-Kramer multiple comparisons test. \*P<0.05 Vs Group I. UB indicates untreated blood and FTB indicates fiber treated blood.

immunostimulant effect.

Significant dose-dependent increase (p<0.05) was observed in the haemagglutinating antibody titre with KG treatment as shown in Table 2. Augmentation of humoral response was significant at 150 and 200 mg/kg/day doses of KG, which resulted in titre values of 7.50±0.54 and 9.51±0.59, respectively, in comparison to that of 4.83±0.98 for untreated control group. The augmentation of humoral response is believed to be a consequence of drug treatment before and after immunization. Increase in footpad volume in immunized animals is used as a test for assessing cell-mediated immune response<sup>10</sup>. In the present study we observed that KG showed a significant increase (P<0.05) in DTH response at a dose of 200 mg/kg/day as shown in Table 3. Increase in the DTH response to SRBC is indicative of a stimulatory effect of KG on lymphocytes and accessory cell types involved in the expression of this reaction. Taken together, it seems appropriate to hypothesize that KG potentiates humoral as well as cellular immunity based on its effects on HA titre and DTH response.

KG is composed of *Berberis aristata* and cow's *ghee*. *Berberis aristata* is reported to enhance humoral as well as cellular immunity<sup>11</sup>. Traditionally cow's *ghee* is believed to increase intelligence and immunity. On the basis of the results obtained in this study, it can be concluded that KG ex-

TABLE 2: EFFECT OF KALAYAK GHRITA ON SRBC-INDUCED HUMORAL ANTIBODY TITRES IN RATS.

Groups	Dose (mg/kg, p.o.)	Haemagglutinating antibody titres Mean±S.D.
I	—	4.83±0.98
II	50	4.66±0.81
III	100	5.33±0.84
IV	150	7.50±0.54*
V	200	9.51±0.59*

n=6 per group. Results are expressed as mean±S.D. Statistical significance was determined by ANOVA followed by Tukey-Kramer multiple comparisons test. \*P<0.05 Vs Group I.

TABLE 3: EFFECT OF KALAYAK GHRITA ON SRBC-INDUCED DTH IN RATS.

Groups	Dose (mg/kg, p.o.)	DTH response (% increase in paw volume)
I	-	6.01±1.85
II	50	7.49±1.80
III	100	7.21±2.56
IV	150	8.54±1.95
V	200	11.2±2.38*

n=6 per group. Results are expressed as mean±S.D. Statistical significance was determined by ANOVA followed by Tukey-Kramer multiple comparisons test. \*P<0.05 Vs Group I.

erts immunostimulant activity and may serve as a useful adjuvant in several clinical conditions associated with depressed immune system.

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## Determination of Metoprolol Tartrate by Reverse Phase HPLC

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**A simple, precise and accurate reverse phase high performance liquid chromatographic method has been developed for the determination of metoprolol tartrate in pharmaceutical formulations. An ODS C<sub>18</sub> (25 cm X 4.6 mm) column from Shimadzu in isocratic mode, with mobile phase acetonitrile:methanol:0.5 % glacial acetic acid in triple distilled water:triethylamine (56:18:26:0.1v/v) was used. The flow rate was 1 ml/min and effluent was monitored at 280 nm. Betaxolol hydrochloride was used as the internal standard. The retention times were 4.6 min and 5.8 min for metoprolol tartrate and betaxolol hydrochloride respectively. The linearity range was found to be 0.1-40 µg/ml.**

Metoprolol tartrate<sup>1</sup> (MT) is a β-adrenoreceptor blocking agent used in the management of angina pectoris, cardiac arrhythmia and hypertension. Chemically it is known as 1-[4-(2-methoxyethyl)phenoxy]-3-(1-methylethylamino)-2-propanol and is official in USP<sup>2</sup> and IP<sup>3</sup>. Several analytical methods such as non-aqueous titration<sup>2,3</sup>, spectrophotometry<sup>3,4</sup>, potentiometry<sup>5</sup>, spectrofluorimetry<sup>6</sup>, TLC<sup>7</sup>, GC<sup>8</sup> and HPLC<sup>2,9-19</sup> for bulk and pharmaceutical dosage forms and in biological fluids have been reported in literature for its determination.

Tablets, capsules and injections, available in local market, were procured and were analysed for MT content by new RP-HPLC method which was found to be simple, precise, rapid and selective. This method obeys linearity in the concentration range of 0.1-40 µg/ml.

Shimadzu HPLC, LC-10AT solvent delivery module with UV/Vis spectrophotometric detector Shimadzu SPD-10A was used. Afcoset ER-200A electronic balance was used for weighing the samples. Reference standard of betaxolol hydrochloride and metoprolol tartrate are procured from M/s Cipla Laboratories, Mumbai. Acetonitrile HPLC grade and glacial acetic acid AR grade were procured from E. Merck (India) Ltd. Mumbai. HPLC grade methanol from Mallinckrodt Baker, Inc, Paris, Kentucky and triethylamine AR grade from Ranbaxy Ltd., S.A.S. Nagar, were procured and used.

For the preparation of stock solution of internal standard and metoprolol tartrate, 50 mg of each drug was weighed and dissolved in water separately and diluted upto 50 ml with triple distilled water. An aliquot (2.5 ml) was pipetted out from this stock solution of BX and made upto 25 ml with mobile phase. MT (1 ml) was diluted to 50 ml with mobile phase. In order to prepare test sample solutions, the

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