Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2016; 8(11); 1788-1794

ISSN: 0975-4873

Research Article

Pharmacological Evaluation of *Roscea procera* (Kakoli) and *Lilium polyphyllum* (Kshirkakoli) Extracts for Immunomodulatory Activity

Sahu R A[1*], Itankar P R[1], Mishra R R[2], Maliye A N[2]

[1]*Department of Pharmaceutical Sciences, Pharmacognosy and Phytochemistry Division, Rashtrasant Tukadoji Maharaj Nagpur University, Amravati Road, Nagpur 440033, Maharashtra, India*
[2]*Sonekar College of Pharmacy, Mahadula, Koradi, Nagpur 441111, Maharashtra, India*

Available Online: 15th November, 2016

ABSTRACT
Astavarga group have a long history of use in traditional system of indigenous medicine. It is used in chyawanprash as a rejuvenator and health motivator. However, no systematic & scientific data is available to validate the claim. Hence, immunomodulatory activities of *Roscea procera* (kakoli) and *Lilium polyphyllum* (kshirkakoli) plants were studied on mice for haematological parameters, delayed type hypersensitive reaction and lymphoid organ weighing. There was significant modulation of immune reactivity in all the three animal models. Treatment with ethanol extract of kakoli and ethyl acetate extract of kshirkakoli (200 mg/kg of b.w.) was found to enhance WBC count ($p<0.001$ & $p<0.01$ respectively) and weight of thymus and spleen. Ethanolic extract of kakoli was accompanied by significant increase in delayed type hypersensitive reaction. In conclusion increase in immunomodulaory potential of EEK and EAEKS over the other extracts could be due to partial purification and separation achieved by successive extraction procedure which might result in increase in degree of polymerization and segregation of secondary metabolites such as alkaloids, flavonoids, steroids etc. in extracts showing highest activity.

Keywords: Kakoli, Kshirkakoli, Immunomodulatory, Haematological, Polymerization, Lymphoid organ weight.

INTRODUCTION

Immunomodulation is a process which can alter the immune system of an organism by interfering with its functions. Immunomodulators are the agents which have an activity to normalize or modulate pathophysiological process[1]. They have biphasic effects viz; If it results in an enhancement of immune reaction it is termed as an immunostimulative agent which preliminary implies the stimulation of nonspecific immune system i.e. granulocytes, macrophages, certain T-lymphocytes and different effector substances, while Immunosupression implies mainly to reduce resistance against infections, stress and may occur on account of environmental or chemotherapeutic factors[2]. Use of plants as a source of immunomodulators is still in its infancy in modern medicine[3]. In present study *Roscea procera* (Kakoli) and *Lilium polyphyllum* (Kshirkakoli) plants which have been described under "Astavarga" group is selected. Astavarga drugs suffers a lot of confusion in Ayurvedic literature in accordance to the identification and authentification. According to the various Ayurvedic Nighantu books composed or commented by different Vaidyas, astavarga drugs forms an important constituent of number of Ayurvedic preparations. These are known as Jivaka, Rishibhak, Mahameda, Meda, Kakoli, Kshirkakoli, Riddhi and Vriddhi. Kakoli and kshirkakoli comes under "Brhneeya" (the drug which promote the formation of mansadhatu that is flesh formation) in Dhanvantri Nighantu, Charaka Samhita, Sushruta Samhita and Astanghridaya[4-7]. They are employed as a 'Rasayana' and also are the constituent of 'Chyawanprash' an outstanding rejuvenator[8]. Kakoli (*Roscea procera* wall.) is an ancient Indian medicinal plant belonging to family Zingiberaceae. Tubers of kakoli are found to contain alkaloid, glycoside, flavonoid, tannin, saponins and active phenolic compounds. The modern screening methods revealed its principal pharmacological activities like antidiabetic, immunomodulator, spermopiotic, fever, burning and phthisis[6,9-12]. *Lilium polyphyllum* (D. Don.) commonly known as kshirkakoli or white lilly (family Liliaceae) is extensively used in many indigenous preparations. Kshirkakoli is reported to contain sugar, Alkaloid, flavanoids, steroids[11].Medicinally, bulb of species has been used for diuretic, antipyretic, tonic[13-16]. In the literature survey, scientific and systematic studies on haematological parameter and lymphoid organ weight which are the important parameter for immunomodulatory activity have not been reported for kakoli and kshirkakoli plants, so it became a need to validate and produce the affirmative claims.

**Author for Correspondence: rajeshashoksahu@gmail.com*

MATERIALS AND METHODS

Plant materials

Marketed rhizomes of *Roscea procera* (Kakoli) and bulb of *Lilium polyphyllum* (Kshirkakoli) plants were collected locally, authenticated by Dr. Dongarwar Department of Botany R.T.M. Nagpur University, Campus, Nagpur. A voucher specimen has been deposited in the Herbarium of Department of Botany, with collection no 9480 and 9481.

Extraction

The dried, coarsely powdered rhizomes of Kakoli and Kshirkakoli plants (500 g each) were extracted with petroleum ether (55-60˚), ethyl acetate (50-55˚), chloroform (50-55˚), acetone (55-60˚) and ethanol (60-65˚) successively by soxhlation. The dried marc after ethanol extraction was cold macerated to obtain hydroalcoholic extract. The extracts were evaporated to dryness in oven (45˚C.). The yield of ethyl acetate extract of kakoli (EAEK), chloroform extract of kakoli (CHEK), acetone extract of kakoli (AEK), ethanol extract of kakoli (EEK) and hydroalcoholic extract of Kakoli (HAEK) was obtained as 2.84 w/w, 3.17 w/w, 1.79 w/w, 2.64 w/w and 2.11 w/w respectively, while that of ethyl acetate extract of kshirkakoli (EAEKS), chloroform extract of kshirkakoli (CHEKS), acetone extract of kshirkakoli (AEKS), ethanol extract of kshirkakoli (EEKS) and hydroalcoholic extract of Kshirkakoli (HAEKS) was 3.06 w/w, 1.13 w/w, 1.74 w/w, 4.14 w/w, 5.71 w/w respectively. Chemical constituents of each extracts of both the plants was evaluated and estimated by qualitative and quantitative method of analysis[17].

Chemicals and standard drugs

Cyclophosphamide and Levamisole were purchased from Endoxan-N Cadila Healthcare Ltd. Sheep red blood cells (SRBC's) were obtained from Nagpur Veterinary College and were washed thrice with large volumes of pyrogen-free sterile saline and adjusted to concentration of 1 X 10^9 cells per ml for immunization and challenge. All other solvents used for experimental work were of analytical grade.

Animals

Swiss albino mice (25-30 g) of either sex kept at Central Animal House facility of University were used. The animal was fed a standard pallet diet and water ad libitum. They were maintained in controlled environment and temperature condition (22 ± 5°C with 12 h of light/ dark/ cycle). All experimental protocols were approved by Institutional Animal Ethical Committee (12/2009/CPCSEA).

Acute toxicity study[18]

Mice of either sex of 3 per group were administered dose ranging between 5, 50, 300, 2000, 3000 and 5000 mg/kg of each extracts of Kakoli and Kshirkakoli. the mice were observed individually after at least once during the first 30 minutes, periodically during first 24 hrs and were observed for additional 7 days. Data for mortality at different dose levels were recorded.

Dosage

Extracts of Kakoli and Kshirkakoli was suspended in 1% tween 80 and was administered orally for 14 days at doses of 200 mg/kg body weight i.e. one tenth of previously tested of 2000 mg/kg in acute toxicity study. The dose volume was 0.2 mL. Control animals received the same volume of normal saline. Levamisole was administered at dose of 50 mg/ kg while cyclophosphamide was given at a dose of 30 mg/ kg of body weight.

Statistical analysis

Data were expressed as the mean ± S.D. comparison between the groups were made by analysis of variance ANOVA) followed by Newmann. Keuls test. $P<0.05$ was considered significant.

Investigations into the immunomodulatory activity of extracts of Kakoli and Kshirkakoli

Delayed-type hypersensitive (DTH) reaction[8,19]

Animals were divided into groups (6 Nos / group). Treatments with extracts began 14 days before challenge. All the groups were immunized by injecting 20 µl of 5 X 10^9 SRBC per ml subcutaneously into the right foot pad (0 day). After 14 days of treatment, the thickness of left foot pad was measured using vernier callipers. The mice were then challenged by injecting 20 µl of 5 X 10^9 SRBC per ml intradermally on the left hind foot pad (time 0). Foot thickness was measured after + 24 h of challenge. The difference between the thickness of left foot just before and after challenge in mm was taken as a measure of DTH.

Haematological parameters[3,20,21]

Animals (6 Nos./group) were treated with the sublethal doses of drugs as per body weight. Preliminary studies indicated that this dosage was found to maximally stimulate haemopoetic system and did not produce any toxicity. Blood was collected from retro orbital plexus of animals of each group and subjected to haematological studies such as total white blood count (WBC), differential count of leukocytes such as lymphocyte, monocytes and granulocytes, total red blood count (RBC), haemoglobin content, total platelet count, mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH).

Lymphoid organ weight[3,22]

Animals were divided into groups (6 Nos /group). Body weight of animals was recorded after the extracts administration and animals were weighed and sacrificed after the last dose of treatment. The weight of vital organs such as liver, thymus and spleen were recorded and expressed in relative organ weights.

RESULTS

Quantitative and Qualitative results

The present study carried out on each extracts of both plants revealed the presence of medicinally active constituents. Results indicated that Kakoli contains Carbohydrate, Flavonoids, Alkaloids, Phenolics, Protein and glycosides, while Kshirkakoli give positive results for Saponins, Phytosterols and carbohydrate compounds. Quantitative estimation of phytochemicals presents in different solvent extracts

Table 1: Quantification of crude phenolic compound, flavanoids, alkaloids and Steroids of Kakoli and Kshirkakoli

S. No.	Plant A					Plant B	
	Extracts	Phenolics (mg/g)	Flavonoid (mg/g)	Alkaloids (mg/g)	Steroids (mg/g)	Extracts	Steroids (mg/g)
1	EAEK	7.33±0.35	6.68±0.11	5.76±0.11	--	EAEKS	11.77±0.199
2	CHEK	14.80±0.59	9.8±0.2	11.66±0.25	0.19±0.001	CHEKS	9.34±0.18
3	AEK	9.04±0.46	4.03±0.25	3.46±0.35	0.16±0.0001	AEKS	4.01±0.168
4	EEK	19.42±0.24	13.06±0.208	12.43±0.35	0.221±0.002	EEKS	--
5	HAEK	11.52±0.50	7.05±0.086	8.76±0.83	--	HAEKS	2.65±0.072

Plant A: Kakoli; Plant B: Kshirkakoli; EAEK: ethyl acetate extract of Kakoli; CHEK: Chloroform extract of Kakoli; AEK: Acetone extract of Kakoli; EEK: Ethanol extract of Kakoli; HAEK: Hydroalcohol extract of Kakoli; EAEKS: ethyl acetate extract of Kshirkakoli; CHEKS: Chloroform extract of Kshirkakoli; AEKS: Acetone extract of Kshirkakoli; EEKS: Ethanol extract of Kshirkakoli; HAEKS: Hydroalcohol extract of Kshirkakoli.

Table 2: Effect of kakoli and kshirkakoli extracts on delayed type hypersensitivity reaction

Groups		Initial paw thickness (mm)	Final paw thickness (mm)	Difference in paw thickness (mm)
Control		1.45±0.007	1.98±0.16	0.53±0.15
Plant A	Test Group I	1.52±0.16	2.12±0.02	0.60±0.14
	Test Group II	1.5±0.07	2.105±0.04	0.605±0.02
	Test group III	1.53±0.14	1.92±0.09	0.39±0.04
	Test group IV	1.72±0.20	2.89±0.16	1.16±0.03**
	Test group V	1.83±0.15	2.295±0.21	0.465±0.6
Plant B	Test Group I	1.36±0.09	1.9±0.02	0.56±0.07
	Test Group II	1.5±0.14	2.05±0.07	0.48±0.070
	Test group III	1.5±0.014	1.89±0.03	0.39±0.02
	Test group IV	1.48±0.09	1.97±0.21	0.49±0.31
	Test group V	1.915±0.007	2.71±0.014	0.795±0.0

Control (no treatment); Plant A: Kakoli; Plant B: Kshirkakoli; Test group I: ethyl acetate extract 200 mg per kg of b.w.; Test group II: chloroform extract 200 mg per kg of b.w.; Test group III: acetone extract 200 mg per kg of b.w.; Test group IV: ethanol extract 200 mg per kg of b.w.; Test group V: hydroalcoholic extract 200 mg per kg of b.w. Values are expressed in mean ± SD of six mice in a group. ** $p<0.01$, experimental group compared with control

Table 3: Effect of kakoli and kshirkakoli extracts on haematological parameters

Groups		WBC Count ($10^3/mm^3$)	RBC count ($10^6/mm^3$)	Haemoglobin count (g/dl)	Platelet count ($10^5/mm^3$)	Mean corpuscular volume (µm3)	Mean corpuscular Haemoglobin (pg)
Control		7.8±1.13	7.885±1.37	14.6±2.82	10.22±1.64	104±0.0	41.05±2.47
Group A		46.75±3.6	8.35±0.24	12.4±2.82	18.60±1.46	101±1.41	32.78±1.72
Group B		1.4±0.28	6.6±1.75	12.1±2.40	7.345±1.95	180±1.41	33.38±2.57
Plant A	Test Group I	5.95±0.21	9.04±0.08	15.5±0.42	11.93±0.10	100.5±0.70	34.5±0.70*
	Test Group II	11.39±0.15	8.74±1.21	15.405±2.0	12.51±0.17	109±1.5	37.5±0.5
	Test Group III	10.2±0.0	9.25±1.79	17.1±3.25	12.43±0.35	104±2.82	36.8±0.0
	Test Group IV	17.9±0.42***	9.76±2.0	17±3.67	14.53±0.67*	99±4.24	34.8±0.56*
	Test Group V	6.4±0.3	9±0.17	14.5±0.3	9.41±0.22	102.5±1.0	32.9±0.4*
Plant B	Test Group I	17.5±0.19**	9.51±0.58	14±1.18	5.27±0.35*	51±1.93***	31.05±0.04**
	Test Group II	15.55±0.66**	9.68±0.10	17.5±0.01	8.9±0.22	106±1.12	36.1±0.01
	Test Group III	11.8±0.10	8.56±0.73	16.3±1.20	12.77±0.03	108±0.35	38.1±0.07
	Test Group IV	10.5±0.07	9.33±0.29	16.5±0.62	12.4±0.08	98±0.66	35.5±0.25
	Test Group V	4±0.02	8.55±0.3	14±0.40	9.23±0.03	94±0.21**	32.6±0.12*

Control (no treatment); Group A: levamisol 50 mg per kg; Group B: cyclophosphamide 30 mg per kg; Plant A: Kakoli; Plant B: Kshirkakoli; Test group I: ethyl acetate extract 200 mg per kg; Test group II: chloroform extract 200 mg per kg; Test group III: acetone extract 200 mg per kg; Test group IV: ethanol extract 200 mg per kg; Test group V: hydroalcohol extract 200 mg per kg. Values are expressed in mean ± SD of six mice in a group.*$p<0.05$; ** $p<0.01$;***$p<0.001$, experimental group compared with control.

of Kakoli and Kshirkakoli indicated high amount of phytochemicals in it (Table 1). In Kakoli, phenolic (19.42±0.24 mg/g), flavanoid (13.06±0.208 mg/g) and alkaloids (12.43±0.35 mg/g) were found to be very high in ethanol extract. While quantitative study of Kshirkakoli extracts confirms that steroid (11.77±0.199 mg/g) was

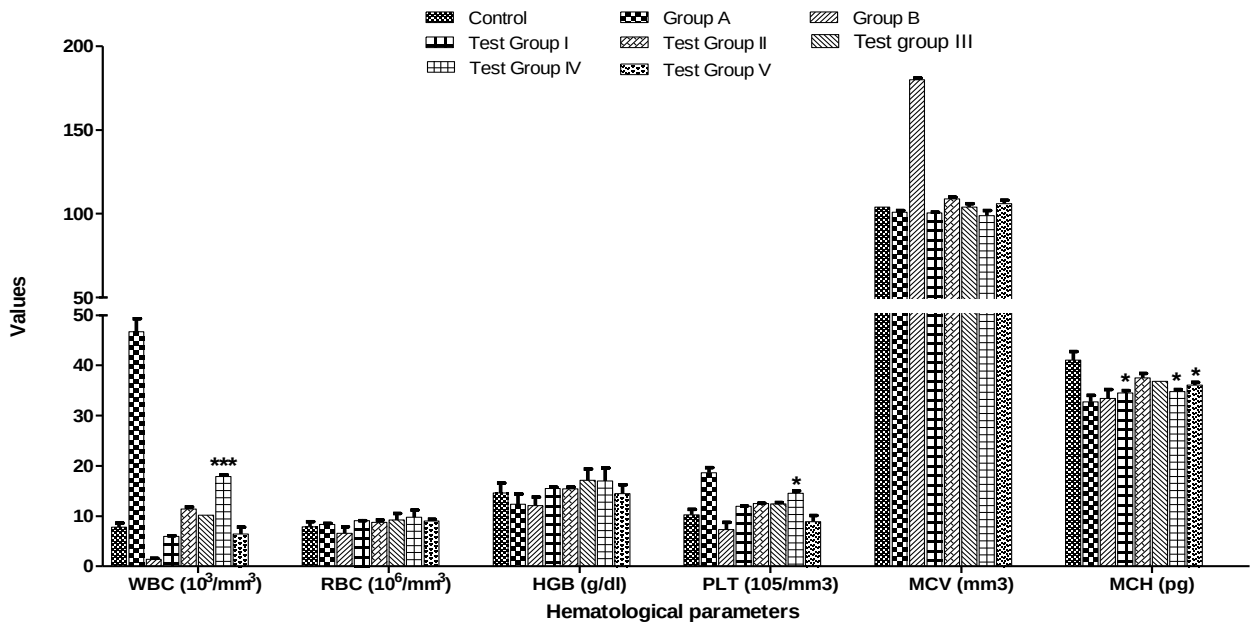

Figure 1: Effect of kakoli extracts on haematological parameters. Control (no treatment); Group A: levamisol 50 mg per kg; Group B: cyclophosphamide 30 mg per kg; Test group I: EAEK 200 mg per kg; Test group II: CHEK 200 mg per kg; Test group III: AEK 200 mg per kg; Test group IV: EEK 200 mg per kg; Test group V: HAEK 200 mg per kg. All values are expressed in Mean ± SD with six mice in a group. *p<0.05, ***p<0.001, experimental group compared with control.

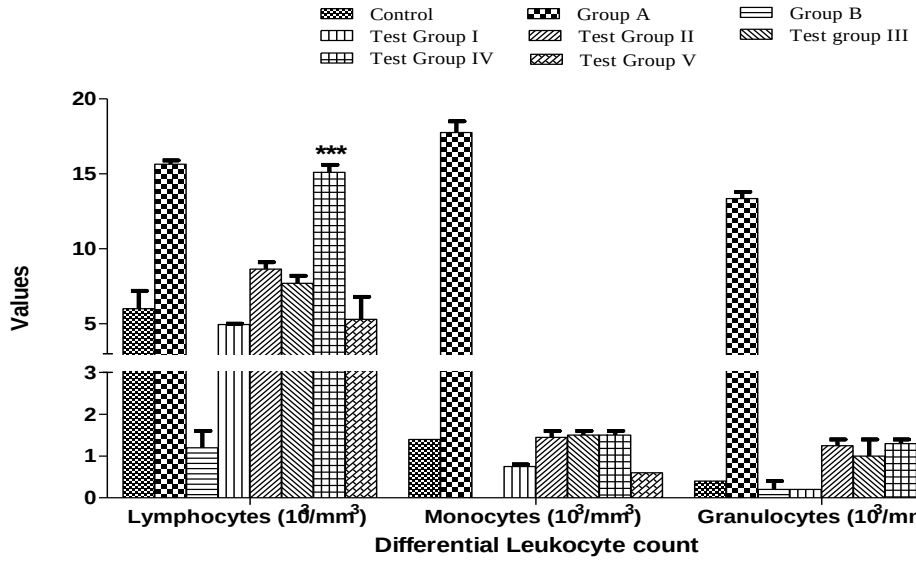

Figure 2: Effect of kakoli extracts on differential leukocyte count. Control (no treatment); Group A: levamisol 50 mg per kg; Group B: cyclophosphamide 30 mg per kg; Test group I: EAEK 200 mg per kg; Test group II: CHEK 200 mg per kg; Test group III: AEK 200 mg per kg; Test group IV: EEK 200 mg per kg; Test group V: HAEK 200 mg per kg. All values are expressed in Mean ± SD with six mice in a group. ***p<0.001, experimental group compared with control.

found to be very high in ethyl acetate extract. Kakoli extracts were also found to contain steroids but the yield recorded were minimal (0.16±0.001 mg/g - 0.221±0.002 mg/g).

Acute toxicity study for Kakoli and Kshirkakoli extracts.

Administration of the stepwise doses of extracts of Kakoli and Kshirkakoli from 5 – 2000 mg/kg of body weight showed no considerable sign of toxicity in the tested animals.The experimental mice treated with acute oral limits dose of 5000 mg/kg body weight of different extracts of Kakoli and Kshirkakoli displayed appreciable changes in physical activity and signs of apparent toxicity symptoms.

Effects of kakoli and kshirkakoli extracts on delayed type hypersensitive reaction (DTH)

The cell mediated immune response was assessed by DTH reaction, i. e. foot pad reaction. EEK showed most effective and produced a significant increase in DTH

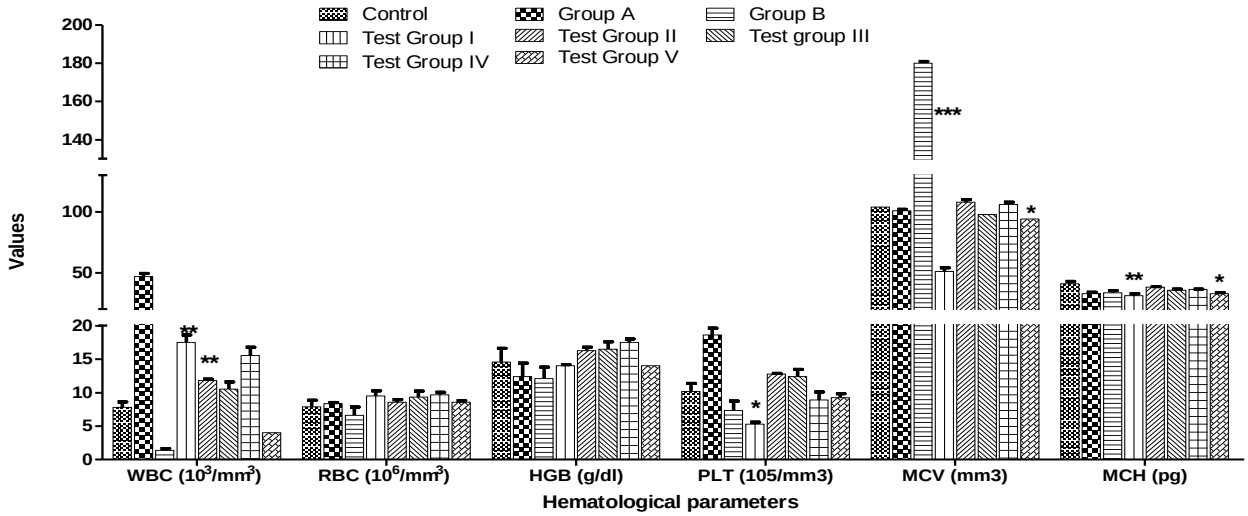

Figure 3: Effect of kshirkakoli extracts on haematological parameters. Control (no treatment); Group A: levamisol 50 mg per kg; Group B: cyclophosphamide 30 mg per kg; Test group I: EAEKS 200 mg per kg; Test group II: CHEKS 200 mg per kg; Test group III: AEKS 200 mg per kg; Test group IV: EEKS 200 mg per kg; Test group V: HAEKS 200 mg per kg. All values are expressed in Mean ± SD with six mice in a group. *p<0.05, **p<0.01, ***p<0.001, experimental group compared with control

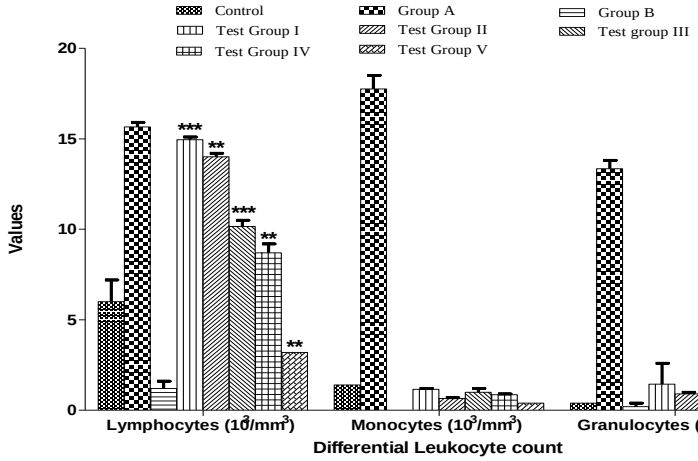

Figure 4: Effect of kshirkakoli extracts on differential leukocyte count. Control (no treatment); Group A: levamisol 50 mg per kg; Group B: cyclophosphamide 30 mg per kg; Test group I: EAEKS 200 mg per kg; Test group II: CHEKS 200 mg per kg; Test group III: AEKS 200 mg per kg; Test group IV: EEKS 200 mg per kg; Test group V: HAEKS 200 mg per kg. All values are expressed in Mean ± SD with six mice in a group. *p<0.05, ***p<0.001, experimental group compared with control.

reactivity (1.16±0.03 mm**) in mice when compared with control (0.53± 0.15 mm). Increase in DTH reaction in mice in response to T cell dependent antigen revealed the stimulatory effect of EEK. (**P<0.01) (Table 2).

Effect of kakoli and kshirkakoli extracts on haematological parameter

Effect on haematological parameters is given in Table 3 and Table 4. EEK showed significant increase in WBC, platelet count and lymphocyte count while there is a decrease in MCH in mice compared to control (***P<0.001, *P<0.05, ***P<0.001, *P<0.05 respectively). (Figure 1 and Figure 2) Treatment with EAEKS increase the WBC count and lymphocyte count (**P<0.01 and **P<0.001 respectively) while it causes the decrease in platelet count, MCV and MCH significantly (*P<0.05, ***P<0.001, **P<0.01 respectively). The CHEKS also showed significant increase in WBC count (P<0.05) in experimental animals (Figure 3 and Figure 4).

Effect of kakoli and kshirkakoli extracts on lymphoid organ weights.

None of the kakoli and kshirkakoli extracts showed toxicity or mortality in extracts treated animals. No significant body weight gain differences were recorded in various groups of animals (Table 5). There was an increase in weight of thymus in AEK, EEK and EAEKS treated animals (0.3±0.0** g/100 g of body weight, 0.25±0.02* g/100 g of body weight and 1.05±0.16 g/100 g of body weight respectively) when compared to control (0.13±0.01 g/100 g of body weight). The size and weight of spleen was also enhanced significantly by the administration of CHEK, AEK, EEK, HAEK and EAEKS (0.55±0.02** g/100 g of body weight, 0.63±0.03** g/100 g of body

Table 4: Effects of kakoli and kshirkakoli extracts on differential leukocyte count

Groups		Lymphocytes ($10^3/mm^3$)	Monocytes ($10^3/mm^3$)	Granulocyte ($10^3/mm^3$)
Control		6±1.69	1.4±0.0	0.4±0
Plant A	Test Group I	4.95±0.07	0.75±0.07	0.2±0
	Test Group II	8.65±2.1	1.45±0.21	1.25±0.21
	Test group III	7.7±0.70	1.5±0.14	1±0.56
	Test group IV	15.1±0.63***	1.5±0.14	1.3±0.14
	Test group V	5.3±2.12	0.6±0	0.5±0.14
	Test Group I	14.95±0.21***	1.15±0.07	1.45±1.62
Plant B	Test Group II	8.7±0.70**	0.85±0.07	1±0.0
	Test group III	14±0.28***	0.65±0.07	0.9±0.14
	Test group IV	10.15±0.49**	1±0.28	0.7±0.42
	Test group V	3.2±0**	0.4±0	0.4±0

Control (no treatment); Plant A: Kakoli; Plant B: Kshirkakoli; Test group I: ethyl acetate extract 200 mg per kg of b.w.; Test group II: chloroform extract 200 mg per kg of b.w.; Test group III: acetone extract 200 mg per kg of b.w.; Test group IV: ethanol extract 200 mg per kg of b.w.; Test group V: hydroalcohol extract 200 mg per kg of b.w. Values are expressed in mean ± SD of six mice in a group.**p<0.01;***p<0.001, experimental group compared with control.

Table 5: Effect of kakoli and kshirkakoli extracts on organ weighing in mice

Groups		Liver (g/100g of bw)	Thymus (g/100g of bw)	Spleen (g/100g of bw)
Control		5.56±0.43	0.13±0.01	0.275±0.02
Plant A	Test Group I	5.42±0.127	0.2±0.04	0.32±0.09
	Test Group II	5.68±1.66	0.21±0.01	0.55±0.02**
	Test Group III	5.83±0.28	0.3±0.00**	0.63±0.03**
	Test Group IV	5.02±0.21	0.25±0.02*	0.69±0.02***
	Test Group V	5.53±1.01	0.19±0.02	0.55±0.01**
Plant B	Test Group I	5.26±0.15	1.05±0.16***	0.73±0.15**
	Test Group II	5.63±0.02	0.125±0.007	0.41±0.07
	Test Group III	5.52±0.07	0.225±0.03	0.41±0.03
	Test Group IV	5.72±0.48	0.225±0.04	0.385±0.06
	Test Group V	5.7±0.56	0.08±0.0	0.225±0.02

Control (no treatment); Plant A: Kakoli; Plant B: Kshirkakoli; Test group I: ethyl acetate extract 200 mg per kg; Test group II: chloroform extract 200 mg per kg; Test group III: acetone extract 200 mg per kg; Test group IV: ethanol extract 200 mg per kg; Test group V: hydroalcohol extract 200 mg per kg. Values are expressed in mean ± SD of two mice in a group. * p<0.05, ** p<0.01, *** p<0.001;experimental group compared with control.

weight, 0.69±0.02*** g/100 g of body weight, 0.55±0.01** g/100 g of body weight and 0.73±0.15** g/100 g of body weight) when compared to control (0.275±0.02 g/100 g of body weight). There was no significant change in weight of liver in any treated group. (*P<0.05, **P<0.01, ***P<0.001).

DISCUSSION

Immunoregulation is a complex balance between regulatory and effector cells any imbalance in the immunological mechanism can lead to pathogenesis. In present study, the immunomodulatory activity of kakoli and kshirkakoli an important plant of asthvarga group was explored. Administration of EEK and EAEKS individually found to increase total WBC's significantly, indicating that extract could stimulate haematopoetic system. Both innate and adaptive immunity depends on the activity of WBC's. The present study includes the use of Levamisole and cyclophosphamide as a reference standard[23]. Levomisole resulted in significant increase in white blood cells and platelet count, while use of cyclophospamide lowered the white blood cell count and haemoglobin. The extract not only potentiate non-specific immune response, but is also effective in improving humoral as well as cell-mediated immunity. Administration of EEK was found to enhance the delayed type hypersensitivity in mice as compared to untreated control, indicating the stimulatory effect on lymphocytes and necessary cell types required for expression of the reaction. Moreover, EEK and EAEKS was found to increase the weight of spleen and thymus indicating that their extract stimulated the production of immune cells.

CONCLUSION

In conclusion, the increased immumodulatory potential of EEK and EAEKS over the other extracts after successive extraction procedure could be due to partial purification achieved by it, which might be resulted in increase in degree of polymerization and segregation of secondary metabolites suggesting that metabolites such as alkaloids, flavanoids, steroids etc present in EEK and EAEKS may play important role in its immunomodulatory potential.

The conclusions dealing with our experimental data also revealed increase in activity which can be extrapolated to a human situation or backchecked with Ayurvedic formulations and treatment schedules, reconfirming the immunomodulatory activity which is not directly mentioned in Ayurveda.

ACKNOWLEDGEMENTS

The authors acknowledge and thankful to Dr. P. R. Itankar for their valuable suggestions and University Grant Commission, New Delhi, India for providing financial support to carry out this work, through Junior Research Fellowship.

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