In *vitro* & *in vivo* immunomodulatory effect of decoction of some commonly used herbs.

SHORT TITLE: Immunomodulatory effect of decoction of some herbs.

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Abstract:

OBJECTIVES:

1. To assess the effect of decoction of herbs on immunity by using *invivo* sepsis model in mouse.
2. To explore mechanism of action of decoction of herbs as well as individual herbs present in it.

METHODS: Immunostimulatory action of decoction of herbs was explored by using *invivo* sepsis model in mouse. The mechanism of action was investigated by using spleen, thymus & bone marrow of mice who were survived from sepsis. This study also evaluated immunostimulant activity of individual herbs present in the test drug by using *invitro* model in rats.

RESULTS: The test drug has inhibited the destruction of T & B cell areas of spleen & markedly enhanced myeloid cells in bone marrow. Some of the individual herbs present in the test drug have shown immunostimulant activity.

CONCLUSION: This study has shown statistically significant immunostimulant activity of the test drug.

Key words: bone marrow, decoction of herbs, immunomodulation, phagocytic activity, spleen, thymus

Introduction:

Alteration of the immunity is appearing as a major area in therapeutics, particularly in cases where uninvited suppression of immunity occurs due to some drug therapies. The immune response can be altered by using medicinal plant products which has become a subject of active therapeutics especially in recent swine flu & covid-19 pandemics[1]. Indian traditional medicines also have emphasized on enhancing body's innate immunity to prevent occurrence of infections. There are reports about many herbs having immunomodulatory activity e.g. *Tinospora cordifolia, Piper longum, Emblica officinalis, Ocimum sanctum, Azadirachta indica, Terminalis arjuna, Aloe vera, Allium sativum (garlic), Zingiber officinale, Withania somnifera and Panax ginseng*. Antioxidant, antineoplastic, antiulcer, anti-inflammatory and immuno-stimulating potential of plants has been discovered in many compounds (e.g. alkaloids, flavonoids, quinones, terpenoids) after screening[2-4].

Taking this into consideration, this study was planned to evaluate immunomodulatory activity of some herbal preparations, commonly used or recommended to augment immunity. Decoction of herbs used in this study, is a preparation recommended by Dr. Shree Balaji Tambe, during the swine flu epidemic in 2010 to prevent an attack of swine flu.

Marked inflammation is the characteristic response in sepsis & require immediate medical aid. It can worsen eventually & result into multiple organ failure if left untreated. The death rates in patients with sepsis is up to 50%[5]. *Escherichia coli* is the common causative agents involved in intraperitoneal infections[6]. These patients were presented with depletion of T and B lymphocytes due to apoptosis of lymphocytes in various lymphoid organs[7].

Ayurvedic or herbal products contain many ingredients & often a query is raised as to which ingredient is responsible for this particular action. Therefore individual herbs present in the test drug were studied for its immunomodulatory action using *in vitro* phagocytosis model[8]. Dunn D. *et. al.*[9] have reported that phagocytosis of bacteria is an important mechanism of host defense.

Materials and Methods:

Approval of Institutional Animal Ethics Committee (IAEC) was taken before starting the experiment.

A. *Invivo* method: Swiss Albino mice of weight 20-25 g of either sex were used

1. **Septilin syrup** was of The Himalaya Drug Company.
2. **Decoction of commonly used herbs:**

   All herbs were procured from local market. 100 ml of purified water was boiled. All herbs were added from **Table I** in prescribed quantity. The heating was continued till the

decoction reduced to half. Then it was cooled & filtered. **The decoction was prepared fresh daily.**

Table I: components of decoction

Embelia ribes seeds	10 nos
Cymbopogon citratus(lemon grass)	2 leaves
Zingiber officinale (ginger)	1/2″
Ocimum Sanctum leaves	10 leaves

Three groups were made, containing thirty mice per group.

Group A: Control (Negative control)

Group B: Septilin Syrup (Dose 2 ml/ kg) (Positive control).

Group C: Decoction of herbs (Dose 10 ml/ kg) (Test drug).

Drug/vehicle administration was done orally daily for 28 days. Sepsis was induced in all mice on 29th day by intraperitoneal injection with 3 x 10^{10} *E. coli* (hospital strain) suspension. All were observed for 7 days for survival. On the 8th day, survived mice were sacrificed. Spleen & thymus were isolated for histopathology. To score immunostimulant response in spleen, marginal zone (MZ), follicles and periarterial lymphoidal sheath (PALS) was screened [11]. To score immunostimulant response in thymus, cellularity in medulla and cortex was screened [11]. To calculate the myeloid (granulocytic) cells, femur bone marrow slides were prepared, stained with May Gruenwald's Giemsa stain[12].

B. *Invitro method:* Immunostimulant activity of individual herbs using polymorphonuclear (PMN) cells: of Sprague Dawley rats weighing 200-250 g of either sex.

Preparation of decoction of individual herb:

The ingredients 1- 4 were procured from local market. 50 ml of water was boiled. To this was added one of the herbs from **Table II** in prescribed quantity. The heating was continued to reduce decoction to half. The decoction was cooled and then filtered. The same procedure was followed for preparing individual decoctions of other herbs of **Table II.**

Table II:

Name of the herb	**Quantity**
Embelia ribes seeds	10 no
Cymbopogon citratus(lemon grass)	2 leaves
Zingiber officinale (ginger)	1/2″
Ocimum sanctum leaves	10 leaves

Animals were anaesthetised. Two drops of fresh whole blood was collected by retro –orbital puncture on a glass slide which was kept in a moist chamber. After 7-8 minutes the clot was gently removed. The slide was covered with test solution and again incubated for 60 minutes. Then it was drained, covered with Candida albicans (10^6CFU/ml) suspension and again

incubated for 60 minutes. Then fixed with methanol and stained with Giemsa stain. Phagocytic activity and phagocytic index were calculated[9].

STATISTICAL ANALYSIS:

GraphPad was used to analyse the results. The *invivo* test results were analysed using the Chi-square test. The histopathology results of spleen & thymus were analysed using kruskal wallis test followed by Dunns test for multiple comparison. The bone marrow & *invitro* test results were analysed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. For all tests $p<0.05$ was considered as statistically significant.

RESULTS:

Table III: % survival of *invivo* sepsis model

Treatment	Dead	Alive	% survival
Control (Negative control)	25	05	16.60
Septilin syrup (standard drug) (Positive control)	20	10	33.00***
Decoction of herbs (Test drug)	17	13	43.00***

*** compared with negative control group ($p<0.0003$)

Decoction of herbs treated group has shown remarkable protection ($P< 0.0003$) from sepsis as compared to negative control. The survival of test drug was comparable with standard drug therapy ($P>0.0003$) **Table III, Fig.1**

Fig. 1 Percent survival from sepsis

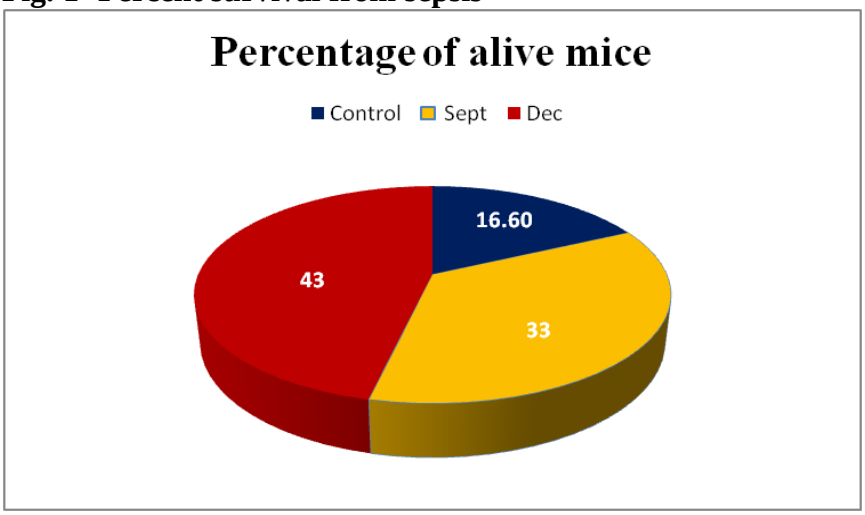


Table IV: Effect on Periarterial lymphoidal sheath (PALS) of spleen in protected mice.Results expressed in median.

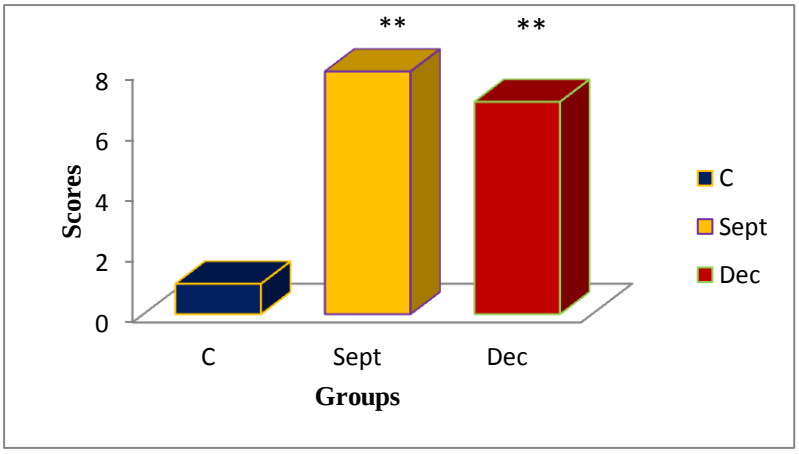
	Control (Negative control)	Septilin syrup (Positive control)	Decoction of herbs (Test drug)

Number, size & lymphocytes	1.00	8.00 **	7.00**
N	5	10	13

**P< 0.01- compared with negative control

Decoction of herbs treated group has shown remarkable enhacement (P<0.01) in number, size as well as in lymphocytes of periarterial lymphoidal sheath (PALS) of spleen when compared to negative control. The results were comparable with standard drug therapy (P>0.01) **Table IV, Fig. 2**.

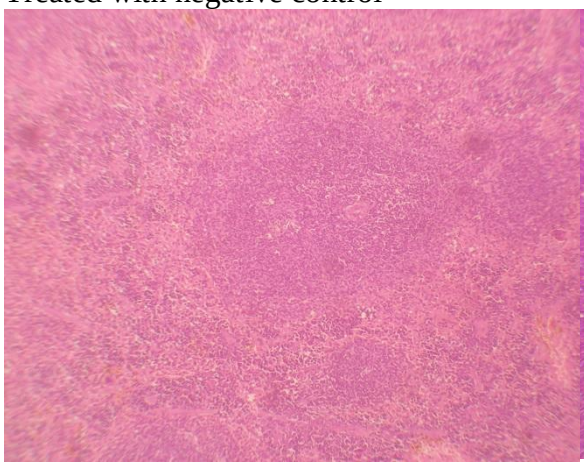
Fig. 2: Effect on periarterial lymphoidal sheath (PALS) of spleen



**P< 0.01 - compared with negative control

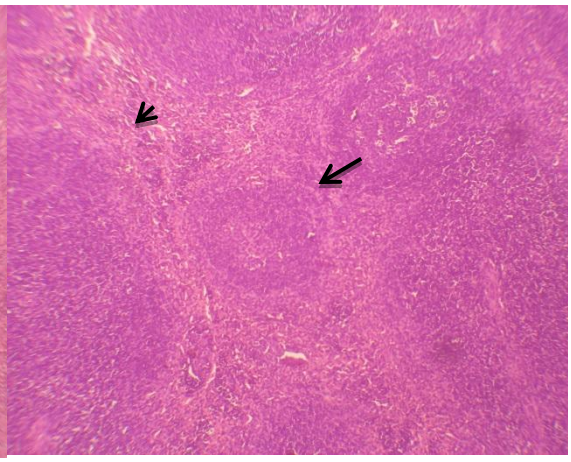
Photomicrographs showing effect of treatment on PALS of spleen:

Treated with negative control



Photomicrograph of spleen showing normal histology of spleen. Few PALS are seen with quiescent marginal zone & germinal centres. (H & E, x 100)

Treated with decoction of herbs



Photomicrograph of spleen showing enlarged PALS (arrow head) with stimulated germinal centres (long arrow) due to activation of lymphocytes. (H & E, x 100)

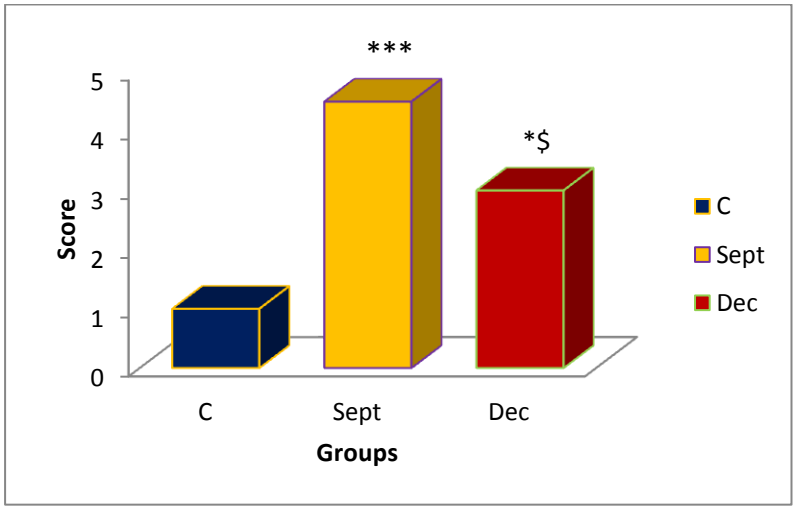
Table V: Effect on marginal zone (MZ) of spleen in protected mice
Results expressed in median .

	Control (Negative control)	**Septilin syrup (Positive control)**	**Decoction of herbs (Test drug)**
Size & lymphocytes	1.00	4.50 ***	3.0*$
N	5	10	13

*** P< 0.001 compared with negative control, * P< 0.05 compared with negative control, $ P< 0.05 compared to positive control

The test drug has shown significant increase in size & lymphocytes of marginal zone of spleen as compared to negative control (p<0.05). Score of decoction of herbs treated group was significantly less as compared to standard drug ie positive control group - septilin syrup (p<0.05) **Table V, Fig.3.**

Fig 3 : Effect on (MZ) of spleen



*** P< 0.001- compared with negative control,* P< 0.05 - compared with negative control, $ P< 0.05- Compared to positive control

Table VI: Effect on follicles of spleen in protected mice
Results expressed in median.

	Control (Negative control)	**Septilin syrup (Positive control)**	**Decoction of herbs (Test drug)**
Number , lymphocytes & germinal centre	2.00	9.00 ***	6.00$
N	5	10	13

***P< 0.001 compared with negative control, $ P< 0.05 compared to positive control

The test drug decoction of herbs treated group has not shown stimulation of lymphocytes & germinal centre of follicles of spleen as compared to control group & was less as compared to standard drug (P<0.05) **Table VI.**

Table VII: Effect on cortex and medulla of thymus in protected mice. Results expressed in median.

	Control (Negative control)	**Septilin syrup (Positive control)**	**Decoction of herbs (Test drug)**
Size & Number	1.00	4.0***	3.0$
N	5	10	13

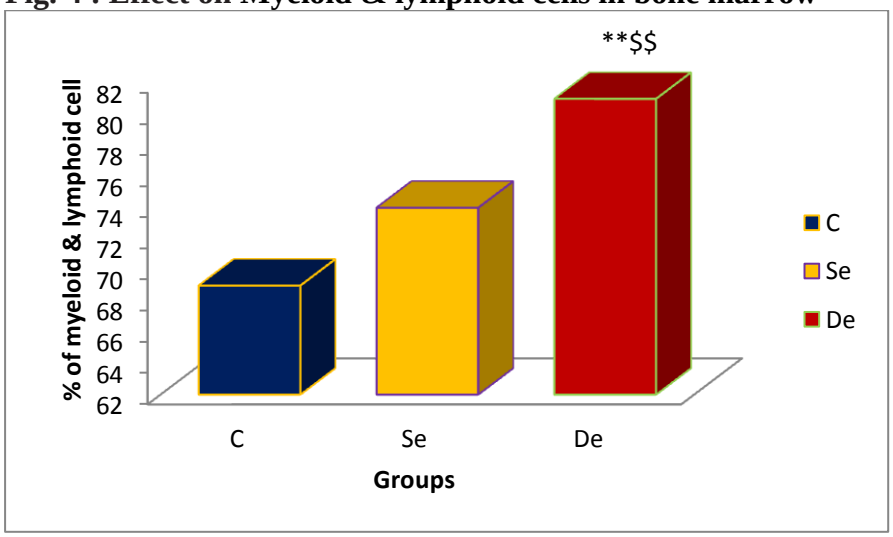
***P< 0.001 compared with negative control group, $ P< 0.05 compared to positive control
The test drug decoction of herbs treated group has shown increase in the size & number of lymphocytes in cortex as well as medulla of thymus as compared with control group but it was less as compared to standard therapy. **Table VII**.

Table VIII: Effect on myeloid cells of bone marrow in protected mice
Results expressed in mean ±SD.

	Control (Negative control)	**Septilin syrup (Positive control)**	**Decoction of herbs (Test drug)**
Size & Number	69±4.4	74±6.1	81±7.1 **$$
N	5	10	13

** P< 0.001 compared with negative control, $$ P< 0.001 compared with positive control
Decoction of herbs treated group has shown remarkable enhancement in myeloid cells as compared to negative control (P<0.001) & positive control (P>0.001) **Table VIII Fig.4** .

Fig. 4 : Effect on Myeloid & lymphoid cells in bone marrow

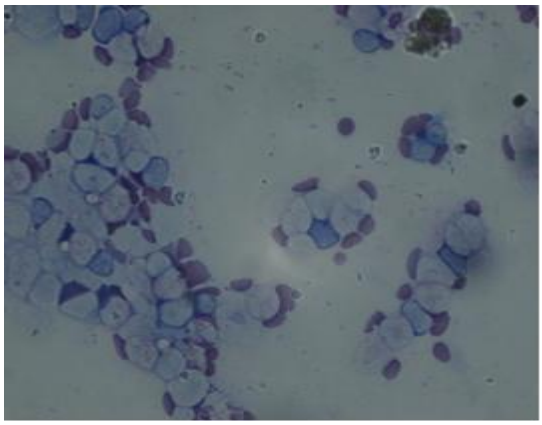


** P< 0.001 compared with negative control, $$ P< 0.001 when compared with septilin

Effect of treatment on lymphoid & myeloid cells of bone marrow:

[photograph showing lymphoid + myeloid cells stained as light blue to dark blue (arrow) & erythroids cells stained in pinkish colour (arrow head)]

Treated with negative control Treated with decoction of herbs



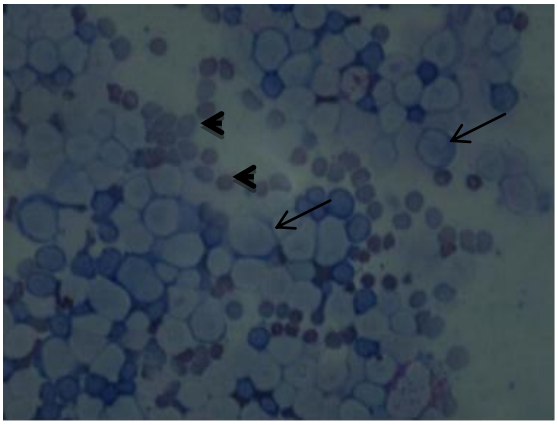


Table IX: Effect of individual herbs present in test drugs on Phagocytic activity (n= 6 per group) mean ±SD

	C	Sept	Decoction of herbs			
			Em ribes.	Cym cit	Ginger	Basil
Phag	40±11	53± 5.3	68±12**	44 ± 19	62 ±11*	65± 8.6**
PI	1.4±0.11	1.5±0.33	1.9±0.30	1.6 ± 0.78	1.7 ± 0.31	2.4±0.82*

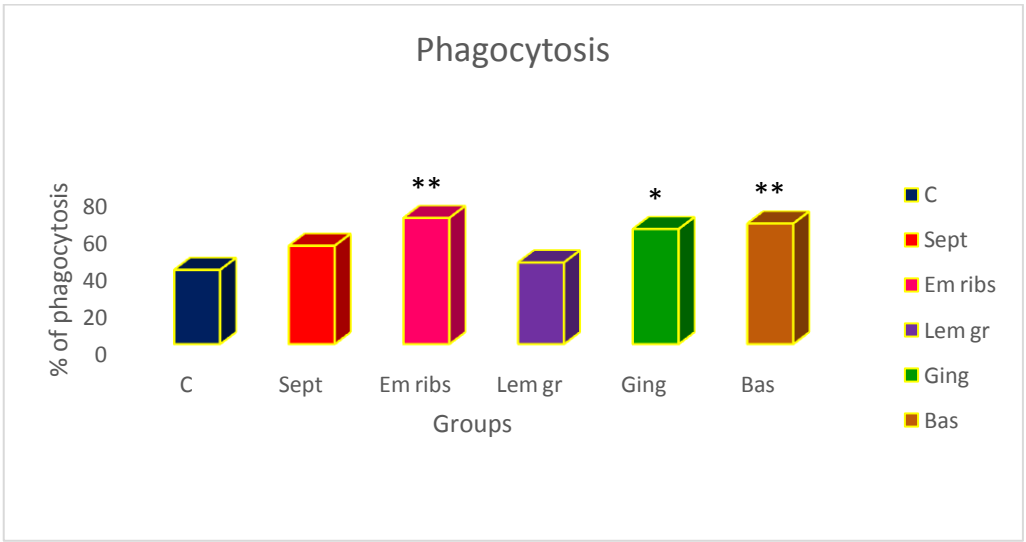
PI: Phagocytic index , Phag: PMN cells showing phagocytosis

*P<0.05 **P< 0.01 compared with negative control group

Em ribs: Embelia ribes seeds, Cym cit: Cymbopogon citratus (lemon grass)

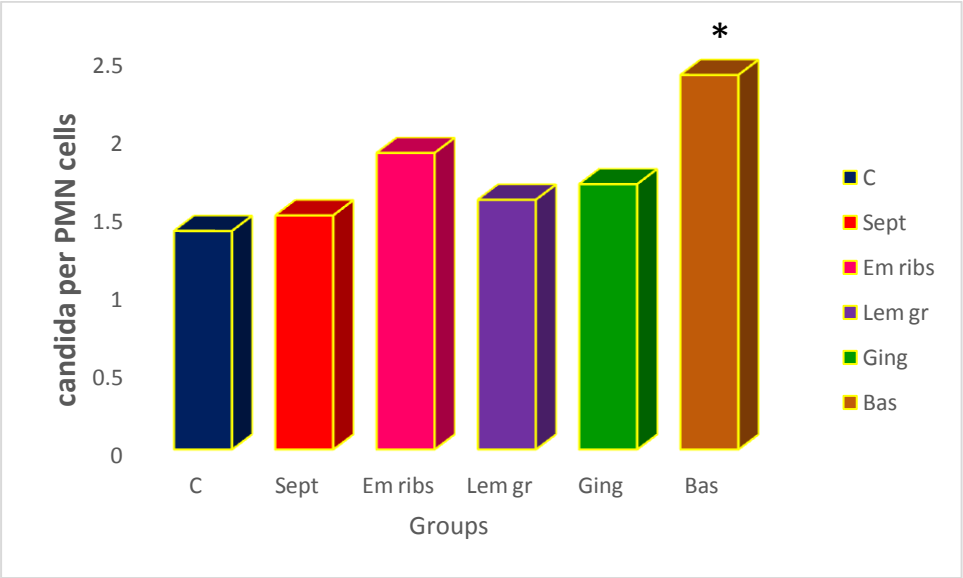
There was statistically significant increase in phagocytosis seen with embelia ribes (P<0.01), ginger (P<0.05) & basil (P<0.01) of decoction of herbs as compared to negative control & was comparable with standard therapy (P>0.05). However the statistically significant increase in phagocytic index was seen only with basil of decoction of herbs when compared with negative control(P<0.05). **Table IX, Fig. 5 & 6**.

Fig. 5 Effect of individual herbs present in test drugs on Phagocytosis



*P<0.05, **P< 0.01 compared with negative control

Fig. 6 Effect of individual herbs present in test drugs on Phagocytic index



* P<0.05 compared with negative control

DISCUSSION:

Expansive loss of T & B cells results in tremendous suppression of immunity in patients with sepsis[7]. Even though it is consuming vast healthcare resources, mortality in sepsis patients is very high. This indicates the need of effective adjuvant therapies with imunno-stimulant properties[5].

The present study shows that the pre-treatment with decoction of herbs has shown protection against sepsis & increased the survival in mice. This confirms its immunostimulant activity.

The spleen is the substantial second lymphoid tissue and contains one-fourth of the body's lymphocytes and responsible for initiating immune responses to blood-borne antigens[13]. In rodents, it is a major tissue of hematopoiesis. Present study shows stimulation of periarterial lymphoidal sheath (PALS) areas & increased synthesis of lymphocytes in marginal zone of spleen with decoction of herbs treated group as compared with negative control group. The results were comparable with the standard therapy. T lymphocytes are mainly found in PALS & B lymphocytes in MZ of spleen[14]. This indicates that decoction of herbs stimulates both cellular as well as humoral immunity.

Another important organ of immunity is thymus whose main role is to mature lymphocytes[15]. T cell are synthesized in the bone marrow but its differentiation, selection and maturation takes place in thymus[16]. They are responsible for providing cellular immunity against intracellular microorganisms like bacteria, viruses. Present study shows increased number of lymphocytes in medulla & cortex of thymus as compared to negative control group but the difference was not statistically significant.

Chief lymphoid organ is bone marrow & leading site for granulopoiesis & lymphopoiesis[17]. Our study shows remarkable enhancement of myeloid & lymphoid cells of bone marrow in decoction of herbs treated group when compared with negative control as well as standard drug. This indicates that decoction of herbs stimulates production of leucocytes in bone marrow.

Many scientists have reported that strengthening of the phagocytic activity indicates the improved phagocytic function of macrophages & neutrophils. It indicates nonspecific immune response. This type of immune response of macrophages has utmost importance as it plays key role in eradicating foreign bodies from the blood[18-19].

The *in-vitro* study that we have carried out has confirmed that some of the individual herbs present in the decoction of herbs have immunostimulant activity. The individual herb basil present in the decoction of herbs shows increase in phagocytic activity when compared with negative control.

Therefore we suggest that there is enhancement of specific as well as nonspecific immunity with the pretreatment of the test drug.

CONCLUSION:

The decoction of herbs has shown immunomodulatory activity which may be due to the presence of the herb ie basil (tulsi) in it. Pretreatment with decoction of herbs may have impact on the survival of the patients whose immunity is compromised due to sepsis, AIDS, cancer chemotherapy. It may also help to prevent the occurrence of infection.

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