

# Assessment of Immunopotential Action of Standardized Indian Herbal Formulation (Body Revival)

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**Abstract**—Immunity is a prime indicator of the balance between health and disease. Malnutrition, aging, chronic stress, chronic infections, antibiotics and chemotherapeutic agents can suppress the immune function. Body Revival (BR) an herbal preparation was standardized by physicochemical parameters and HPLC. Body Revival pretreatment significantly and dose dependently (200-400 mg/kg) improved the leukocytes, granulocytes and lymphocytes counts in peripheral blood and cellularity in bone marrow in Cyclophosphamide induced immune suppressive mice. Therefore, Body Revival has immunopotentiating action and can be therapeutically useful for acute and chronic infections or other immune suppressive diseases.

**Index Terms**—Chemotherapeutics; Cyclophosphamide; HPLC; Immunopotential.

## I. INTRODUCTION

Immune systems protect our body against any kind of infections from deadly virus, bacteria and other harmful microorganisms. The immune system develops appropriate tolerance to avoid unwanted response to healthy tissues. This system is more and more found to be involved in the development of several chronic illness including Alzheimer's disease, type 1 diabetes and hepatocellular carcinoma [1]. Innate and adaptive immunity mainly depends on the activity of white blood cells. Innate immunity largely depends upon granulocytes, macrophages and dendritic cells; while adaptive immune response depends upon lymphocytes for providing long term immunity [2]. The innate immune system also consists of interferons (IFNs) and pro-inflammatory cytokines or interleukins. During the viral threat IFNs acts to prevent the spreads of virus in the host [3]. It is general believes that older and younger people are susceptible to infectious diseases, particularly viral and bacterial infections due to their poor/weak immune system in their body.

Immunomodulators could act (stimulate or suppress) on both the innate- and adaptive immune systems to exert positive effects on the host defence mechanisms or enhance

the host's ability to tolerate damages caused by toxic compounds (such as chemotherapeutics) [4]. Consequently, research efforts largely focus on identifying and investigating specific groups of herb-related compounds (such as flavonoids, polysaccharides, lactones, alkaloids, diterpenoids, and glycosides) and their potential implication in immunomodulation [5],[6]. Drugs of natural origin have also demonstrated their usefulness against a wide variety of viral infections and diseases [7]. These groups of herbal medicines are gaining popularity as a means to control viral infections due to their safety and low incidence of side effects [8],[9]. Some of the important herbs having immunomodulation, antibacterial, antiviral, antioxidant and anti-inflammatory properties are *Panax ginseng*, *Withania somnifera*, *Ocimum sanctum*, *Rubia cordifolia*, *Phyllanthus emblica*, *Aegle marmelos*, *Glycyrrhiza glabra*, *Blumea lacera*, *Symplocos racemosa* *Acorus calamus*, *Cucumis melo*. Mehrotra et al., (2003) have been reported the anticellular and immunosuppressive properties of *A. calamus* [10]. Badam (1995) reported *A. calamus* has antiviral activity against *Herpes simplex* virus HSV-1 and HSV-2 [11]. Marmelide, a coumarin derivative isolated from *Aegle marmelos* proved to be highly potent compound against human coxsackieviruses B1-B6 compared to ribavirin [12]. *Blumea lacera* reported for its antimicrobial and anti cancer activities [13]. Antimicrobial and antiviral actions of *Cucumis melo* and *Rubia cordifolia* have been established [14],[15]. *Symplocos racemosa* has also antiviral and anticancer activity [16]. *Withania somnifera* is known as Indian adaptogen and its role on immunity and cancer research is well documented [17],[18]. Perhaps, it is assumed that these herbal and natural medicines drugs are devoid from drug resistance and drug dependence.

It is assumed that mixed herbal formulations are more therapeutically potent than any single herb, because of their synergistic actions. In this context Body Revival (BR), a new formulation, prepared with the active parts of herbs reported for antimicrobial, antiviral, anticancer and detoxification activities was screened for its immune potentiating action, although it has already been reported for effective in cardio protective function [19]. Therefore, the present study was aimed to validate the herbal formulation in the view point of pharmaceutical approaches and also establish its immunotherapeutic role against chemotherapeutic immunosuppressive experimental model.

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## II. MATERIALS AND METHODS

### A. Animals

Swiss mice, body weight between 20-30g of either sex were housed 4-6 in groups on polypropylene cages with steel nozzle water bottle. The sterile cutting straw was used as matting substances and was changed every day. The room temperature was maintained at  $25^{\circ}\pm 2^{\circ}\text{C}$ , humidity between 40 and 60% and 12 h light cycle (7 a.m. to 7 p.m. illumination) [20]. Mice were fed balanced diet for animal and water *ad libitum*. The permission from Institutional Animal Ethic Committee was also obtained prior to experiments.

### B. Chemicals

Fine chemicals like cyclophosphamide (CPx), quercetin, gallic acid, catechin, coumarin, rutin, p-coumaric acid, myrecetin, chlorogenic acid, caffeic acid, apigenin, naringenin, kaempferol etc. were purchased from Sigma Aldrich (USA). All other common chemicals and reagents were AR/GR grade.

### C. Test Drug

BR was prepared and supplied by M/s Health Reactive, Kota, India. Each 5 ml of BR contained active dry extracts of *Aegle marmelos* (150 mg), *Acorus calamus* (175 mg), *Withania somnifera* (325 mg), *Blumea lacera* (115 mg), *Rumex vesicarius* (240 mg), *Rubia cardifolia* (200 mg), *Cucumis melo* (200 mg), *Symplocos racemosa* (95 mg) and honey. The test drug was store in a brown bottle and kept in refrigerator at  $4^{\circ}\text{C}$  before use.

### D. Physicochemical Analysis

#### Specific gravity.

5 ml of BR was poured carefully into pre-weighed pycnometer. The weight of the samples was taken and specific gravity was calculated by comparing the weight of equal volume of water [21].

#### Ash Content.

5 g of BR was placed in silica crucible and heated in muffle furnace for 5h at  $500^{\circ}\text{C}$ . It was cooled in desiccators and weighed [21].

#### Crude Lipid.

10 g of BR was extracted with petroleum ether ( $60-80^{\circ}\text{C}$ ) in a soxhlet apparatus for about 6h. The residual petroleum ether extract was filtered and the filtrate was evaporated in a pre-weighed beaker. Increase in weight of beaker gave crude lipid [22].

#### Crude Fibre.

10 g of BR was treated with 200 ml of 1.25%  $\text{H}_2\text{SO}_4$  and the residual part was neutralized with 1.25% NaOH. Then it was the filtered, washed with hot water and mixed with 1%  $\text{HNO}_3$ . The washed residue was dried in oven at  $130^{\circ}\text{C}$  to constant weight. The residue was scraped into a pre-weighed porcelain crucible, weighed, ashed at  $550^{\circ}\text{C}$  for two hours, cooled in a dessicator and reweighed. Crude fibre

content was expressed as percentage loss in weight on ignition [22].

#### Crude Protein.

The crude protein was determined using micro Kjeldahl method [22]. 10 g of BR was decomposed by digestion with concentrated sulphuric acid in the presence ammonium sulphate. An excess of sodium hydroxide solution was added to dilute the reaction mixture. The liberated ammonia was distilled in steam and absorbed in standard sulphuric acid. Titration of the residual mineral acid with standard sodium hydroxide gives the equivalent of ammonia obtained from the weight of the sample taken. From this the percentage of nitrogen in the compound was calculated. The average nitrogen (N) content of proteins was found to be about 16%, which led to use of the calculation [ $\text{N}\times 6.25(1/0.16=6.25)$ ] to convert nitrogen content into protein content.

#### Carbohydrate.

The presence of carbohydrate was determined by the following formula [22]:

$$100 - (\text{ash}\% + \text{fat}\% + \text{protein}\% + \text{crude fibre}\%).$$

#### Nutritive Value (Energy Content).

1 g carbohydrate and protein yield 4 kcal energy; whereas 1 g lipid yields 9 kcal energy. The energy content of BR was determined by multiplying the values obtained for protein, fat and available carbohydrate by 4, 9 and 4 respectively and adding up the values [22].

#### Mineral Contents.

5 g BR was taken in a silica crucible and heated in a muffle furnace at  $400^{\circ}\text{C}$  till there was no evolution of smoke. The carbon-free ash was moistened with concentrated sulphuric acid and heated on a heating mantle till fumes of sulphuric acid ceased to evolve. The crucible with sulphated ash was then heated in a muffle furnace at  $600^{\circ}\text{C}$ . 1 g of sulphated ash obtained above was dissolved in 100 ml of 5% HCl to obtain the solution ready for determination of mineral elements (sodium, potassium and calcium) through atomic absorption spectrophotometer (Agilant, USA) [23].

I: PHYSICO-CHEMICAL ANALYSIS OF BODY REVIVAL

Parameters	Mean $\pm$ SD
Specific gravity (%)	1.31 $\pm$ 0.002
Ash content (%)	8.35 $\pm$ 0.02
Crude fibre (%)	9.16 $\pm$ 0.01
Crude lipid (%)	0.0004 $\pm$ 0.00006
Crude protein (%)	0.18 $\pm$ 0.01
Carbohydrate (%)	82.26 $\pm$ 0.08
Nutritive value (Kcal/100g)	330.79 $\pm$ 0.65
Sodium (mg/100 g)	4.18 $\pm$ 0.005
Potassium (mg/100 g)	1.15 $\pm$ 0.01
Calcium (mg/100 g)	3.76 $\pm$ 0.03

N=6 in each experiment

#### HPLC Analysis.

HPLC fingerprint of BR was performed with Dionex Ultimate 3000 liquid chromatograph (Germany) with solvent delivery system (LPG 3400 SD) including a diode array detector (DAD 3000) and Chromeleon 6.8 system manager as data processor. The separation was achieved by

a reversed-phase AcclaimTM120 C18 column (5 µm particle size, i.d. 4.6 x 250 mm). The test sample and standards were prepared and filtered through micro filtration unit. The mobile phase contains 1% aqueous acetic acid solution (Solvent A) and acetonitrile (Solvent B), the flow rate was adjusted to 0.7 ml/min, the column was thermostatically controlled at 28°C and the injection volume was kept at 20 µl. Total analysis time per sample was 115 min. HPLC Chromatograms were detected using a photo diode array UV detector at 280 nm according to absorption maxima of analysed compounds. Each compound was identified by its retention time and by spiking with standards under the same conditions. The quantification of the sample was done by the measurement of the integrated peak area and the content was calculated using the calibration curve by plotting peak area against concentration of the respective standard sample [24].

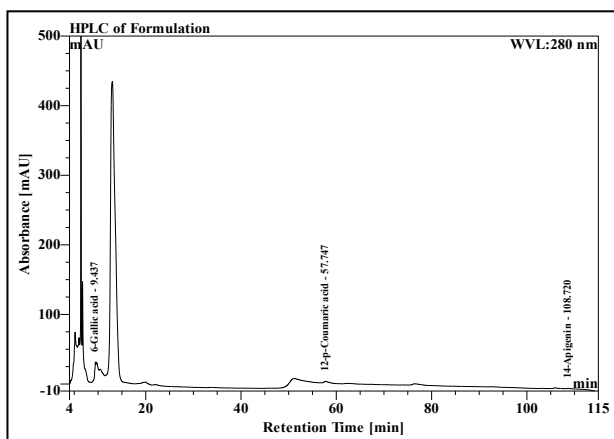


Fig. I: HPLC chromatogram of Body Revival.

### E. Pharmacological Studies

#### Acute Toxicity Studies.

BR was examined for its safety measure following the OECD guidelines No. 423 [25]. BR was given to 18 h fasted female Swiss mice (20-30g body weight) as arithmetically progressive manner by oral route at 0.5 ml/100 g, 1 ml/100 g, 1.5 ml/100 g and 2.0 ml/100 g, in a single dose and observed for 3 days. The rate of mortality up to 3 day was recorded for the selection of 50% lethal dose of BR.

#### Immunopotential Studies

Swiss male mice (20-30 g) were divided into four groups of six animals each: Group I and Group II animals were pretreated orally with normal saline (5 ml/kg), while, Group III with BR (200 g/kg) and Group IV with BR (400 g/kg) orally for 7 consecutive days. On day 7, CPx (Sigma, St Louis, MO) at the dose of 300 mg/kg was given subcutaneously to all mice, except Group I. The treatments were continuing in all groups for another 10 days [26]. Total count of leukocytes was determined on the day of CPx injection (day 0), day 5 and day 10 using hemocytometer after the red blood cell lysis. Differential counts were determined on Leishman's stained blood smears made from whole blood. Absolute granulocyte and lymphocyte counts were calculated from the total leukocytes and the differential

count. Bone marrow cellularity was determined by the method of Mehra and Vaidya (1993) [27]. Briefly, on day 10, all mice were sacrificed under deep anaesthesia (sodium hexobarbitone 40 mg/kg, i.p) and the bone marrow cells were collected carefully from both femurs and suspended in RPMI media in cold condition. The suspended cells were washed with the medium and centrifuged. The suspended cells were reconstituted with the medium and their numbers was counted microscopically using hemocytometer and expressed as total number of cells/femur.

### F. Statistical Analysis

The data were expressed as mean ± standard deviation (SD). The differences between the groups were analyzed statistically (t-test) using software (spss v20, IBM, USA). The level of significance was considered at less than 0.05.

## III. RESULTS

### Physicochemical Analysis.

Body Revival was a thick dark brown suspension of eight different medicinal plants and honey. The basic physicochemical characteristics are given in Table I. It contained nearly 9.16% fibre and practically lipid free. It is also a moderate calorie product.

### HPLC Chromatographic Analysis.

HPLC analysis revealed out of twenty four known standard polyphenolics, only three important biomarkers gallic acid, p-coumaric acid and apigenin were matched and quantified in Body Revival [Fig. I, Table II].

II: POLYPHENOLIC COMPOUNDS BY HPLC

Parameters	Mean ± SD
Gallic acid (µg/g)	18.40±0.015
p-Coumaric acid (µg/g)	2.41±0.02
Apigenin (µg/g)	0.29±0.0004

N=6 in each experiment

### Pharmacological Studies.

No mortality was observed in BR treated mice up to the maximum dose limit *i.e.*, 2ml/100g body weight for 3 days and considered safe for oral use.

CPx treatment gradually decline the number of peripheral leukocytes compared to non-immune suppressive mice (Table III). Pretreatment with test drug, BR at the dose of 400 mg/kg body weight restore the number up to 65% within 10 days.

III: LEUKOCYTES IN PERIPHERAL BLOOD ON CPx INDUCED IMMUNOSUPPRESSIVE MICE

	Leukocytes (per mm <sup>3</sup> ) in blood		
	Day 0	Day 5	Day 10
Normal	6540±30.2	6480±36.8	6530±32.5
CPx	6450±32.7(a)	2860±28.3(a)* [-59%]	3190±31.4(a)* [-51%]
CPx+BR 200 mg/kg	6560±35.6(b)	4090±36.7(b)* [43%]	4930±38.1(b)* [54%]
CPx+BR 400 mg/kg	6570±35.6(b)	4350±33.2(b)* [52%]	5280±43.6(b)* [65%]

N=6; Mean±SEM; student t-test; (a) Normal vs. CPx; (b) CPx vs. BR; \* indicate <0.05; % change in parentheses

Furthermore, CPx injection gradually diminished the granulocytes and lymphocytes numbers 54% and 44% respectively within 10 days [Table IV-V]. On the other hand, pretreatment with BR at the dose of 400 mg/kg

significantly restored the numbers of granulocytes and lymphocytes up to 77% and 49% than immune suppressive mice.

Moreover, 10 days after injection of CPx, bone marrow cells were declined to 61% in femur. But, pretreatment with BR dose dependently (45% and 72%) and significantly improved the cellularity of bone marrow in femur than CPx immune suppressive mice [Fig. II].

IV: GRANULOCYTES IN PERIPHERAL BLOOD ON CPx INDUCED IMMUNOSUPPRESSIVE MICE

	Granulocytes (per mm <sup>3</sup> ) in blood		
	Day 0	Day 5	Day 10
Normal	830±10.8	836±9.9	842±11.6
CPx	840±11.3(a)	310±14.9(a)* [-63%]	390±17.2(a)* [-54%]
CPx+BR 200 mg/kg	836±10.8(b)	420±15.1(b)* [35%]	570±13.8(b)* [46%]
CPx+BR 400 mg/kg	852±10.6(b)	490±12.7(b)* [58%]	690±14.9(b)* [77%]

N=6; Mean±SEM; student t-test; (a) Normal vs. CPx; (b) CPx vs. BR; \* indicate <0.05; % change in parentheses

V: LYMPHOCYTES IN PERIPHERAL BLOOD ON CPx INDUCED ON IMMUNOSUPPRESSIVE MICE

	Lymphocytes (per mm <sup>3</sup> ) in blood		
	Day 0	Day 5	Day 10
Normal	5250±30.5	5130±29.8	5280±27.4
CPx	5130±31.9(a)	2440±28.3(a)* [-52%]	2930±33.7(a)* [-44%]
CPx+BR 200 mg/kg	5060±28.6(b)	2740±30.5(b)* [12%]	3510±34.2(b)* [19%]
CPx+BR 400 mg/kg	5080±29.8(b)	3020±26.9(b)* [23%]	4360±32.7(b)* [49%]

N=6; Mean±SEM; student t-test; (a) Normal vs. CPx; (b) CPx vs. BR; \* indicate <0.05; % change in parentheses

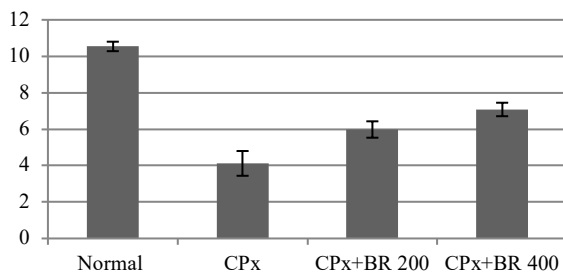


Fig. II: Bone marrow cells (10<sup>5</sup>/femur) on CPx induced immunosuppressive mice

IV. DISCUSSION

The body's immunity has been shown to be suppressed in conditions such as malnutrition, under nutrition, aging, chronic stress, chronic infection, side effect of antibiotics and chemotherapeutic agents etc. Any imbalance occurring between regulatory and effectors cells of immune system can also lead to immunological breakdown and pathogenesis. Cyclophosphamide (CPx) is well-documented antineoplastic agent and enlisted on World Health Organization's (WHO) list of essential medicines [28]. It is a potent immunosuppressive agent and in high-dose CPx is increasingly used to treat both autoimmune and alloimmune conditions. It is also the most commonly used drug in blood and marrow transplantation [29]. It is basically nitrogen mustard and can transfer alkyl radicals, that react with the

nucleic acid bases and inhibit DNA synthesis and also bring about cross-linkage of DNA strands in resulting as well as in dividing cells and thus interfere with cell replication [30]. At over dosage, CPx produces acute myelosuppression and thereby suppressing both cellular and humoral immunity [31]. For this, CPx is used as a pharmacological tool for searching new Immunomodulators.

The concept of immunomodulation relates to nonspecific activation of the function and efficiency of macrophages, granulocytes, complement, natural killer cells and lymphocytes and also to the production of various effectors molecules generated by activated cells. The test drug, Body Revival (BR) is consisted with the active parts of eight medicinal plants and honey. These individual component like, *Aegle marmelos*, *Acorus calamus*, *Withania somnifera*, *Blumea lacera*, *Rumex vesicarius*, *Rubia cardifolia*, *Cucumis melo*, *Symplocos racemosa* and honey have been used in traditional and complementary medicine for their anti-inflammatory, antimutagenic, immunostimulant, antimicrobial, antiviral, adaptogenic and rejuvenation properties, particularly detoxifying actions during pathophysiological situations or disease conditions. But their role in a combination could not be work out. Thus, we investigated whether the herbal formulation Body Revival could improve down-size leukocyte populations in CPx treated hosts.

In the present study, initial dose of CPx injection quickly reduced the number of leukocytes within five to ten days. More specifically, it significantly diminished the numbers of granulocytes and lymphocytes in the peripheral blood and suppressed the macrophage proliferative action significantly in bone marrow. Earlier it was reported by Jang and his colleagues (2013) that CPx treated mice exhibited significant reduction in natural killer cell (NK cell) in splenocytes [32]. It can influence the immune function that include Th2/Th1 shifts or repressed in cytokine production, like TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\alpha$ , IL-2, IL-6, IL-12 in serum suggesting that CPx is a potent immunosuppressive agent [33]. It was noted that, CPx infected animals exhibited signs of sickness and lethargies and that was reversed in BR pretreated animals.

Physicochemical analysis revealed that BR is rich in calcium, fibre and polyphenolics, mainly gallic acid, p-coumaric acid and apigenin. It is well established facts that polyphenols have anti-inflammatory and immunomodulatory effects and their antioxidant properties are mainly mediated through down-regulate the nuclear factor NF-kB, modulating important cell signalling pathways involved in inflammation even in cancer [34],[35]. The antioxidants properties of gallic acid are p-coumaric acid facilitated in the modulation of immune function either prevent the expression of inflammatory mediators including cytokines and histamines [36],[37]. Immunostimulatory potential of gallic acid against CPx immunosuppressant mice has also been reported [38]. In the present study, BR at the dose of 400 mg/kg drastically recovered the peripheral

leukocytes to 52% within five days and to 65% within ten days after CPx injection. Moreover, BR has also capability to restore the granulocytes and lymphocytes after CPx, indicating its role on both inner and adaptive immune function. Bone marrow cells, however, are also an integral part of innate immunity, and lack of these cells, regardless of a normal level predisposes hosts to infections. In the present study, pretreatment with BR exhibited enhancement of cell population in bone marrow compared to CPx mice. Safety studies in animals also confirmed its non hazardous action. Considering these observations it may be inferred that pretreatment with BR has effectively improved the immune suppression induced by CPx. The major ingredients of BR especially *W. somnifera*, *A. marmelos*, *A. calamus*, *R. cardifolia* and honey have already been reported for their protections in deadly infectious diseases. The test product, BR has the ability to overcome the untoward situation due to viral/microbial infections or chemotherapeutic regimens in tumour/cancer treatment.

## V. CONCLUSION

Body Revival pretreatment undoubtedly improves the health condition during weak/suppressive immune state, either by regulating the signalling pathways of inflammations or modifying the cellular mechanisms to regenerate/restore the cell functions. Eventually, it may be effective to combat acute and chronic infections from environmental pathogens or drug/chemically induced immune suppressive conditions.

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