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# Immunostimulative effects of *Cyperus rotundus*, *Alpinia calcarata*, *Solanum surattense*, *Clerodendrum infortunatum* and *Croton laccifer* extracts combination on cyclophosphamide-induced immunosuppression in rats

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## ABSTRACT

**Objective:** The present work was undertaken to validate the combinatorial effect of the ethanol extractions of rhizomes of *Cyperus rotundus* Linn. ("Kalanduru") and *Alpinia calcarata* Rosc. ("Heen Araththa"), and root barks of *Solanum surattense* Burm.f. ("Elabatu"), *Clerodendrum infortunatum* Linn. ("Pinna"), and *Croton laccifer* Linn. ("Keppetiyā") as immunomodulators in rats. **Materials and Methods:** Wistar rats were orally fed with combined crude products of the five plant species (200 mg/ml) at each day for a period of 14 days while control group was orally fed with 1 ml of distilled water each day for the same period. Differential white blood cell (WBC) count, leukocyte adhesion, interleukin (IL-4), IL-10, and IL-12 levels were measured in all rats after the treatments. Subsequently, animals in each group were orally fed with 1 ml of cyclophosphamide solution at a concentration 20 mg/ml. **Results:** Hematological analyses revealed that total WBC and leukocyte adhesion were not significantly different in control and extract-treated groups. Expression of IL-4 and IL-10 was significantly different in treated and control groups while expression of IL-12 was not significantly different. Cyclophosphamide-induced immunosuppression of the control group caused moderate to severe skin lesions while the rats in the extract-treated group did not sustain any skin lesions. All the rats in the cyclophosphamide-treated control group died after three months while 83.33% of the cyclophosphamide + plant extract received group survived, indicating the ability of the plant combination to alleviate the immunosuppression induced by cyclophosphamide. **Conclusions:** Treating with ethanolic extract combination of above plant species might exert their immunomodulatory effect via cytokine expression and can attenuate the immunosuppression induced by cyclophosphamide.

**KEY WORDS:** Cyclophosphamide, cytokines, immunomodulation, immunosuppression, medicinal plants

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## INTRODUCTION

Development of plant-derived medicines targeted for immunotherapy is gaining attention [1]. Ayurvedic and traditional medical practitioners of Sri Lanka have been using *Cyperus rotundus* Linn ("Kalanduru," KD) since 1753, *Solanum surattense* Burm.f. ("Elabatu," EB) since 1768, *Alpinia calcarata* Rosc. ("Heen Araththa," HA) since 1807, *Clerodendrum infortunatum* Linn. ("Pinna," PN) since 1753, and *Croton laccifer* Linn. ("Keppetiyā," KP) since 1753 in their medicinal formulations to treat various ailments [2-9]. Extractions of these abovementioned plants have shown to induce a strong immunomodulatory effect in a rat model [10].

Based on these facts, it was hypothesized that the combinatorial effect of these plant extractions might alleviate the cyclophosphamide-induced immunosuppression by enhancing the immune function. Hence, the present study was undertaken to test the above hypothesis in a rat model.

## MATERIALS AND METHODS

### Plant Materials and Preparation of the Macerations (Ethanol Extractions)

Dried rhizomes of KD and HA, and dried bark of the roots of EB, KP, and PN were collected from the local areas of Galle

District, Sri Lanka. All the plant materials were authenticated according to the previous literature [2-10] and Professor Piyal Marasinghe, Chief Botanist of the Haldummulla Medicinal Plant Research Center, Sri Lanka. Authenticated specimens were deposited at Department of Zoology, Faculty of Science, University of Ruhuna, Matara Sri Lanka for any future reference (*A. calcarta*: AC/DZ/UoR1501; *C. rotundus*: CR/DZ/UoR/1502; *S. surattense*: SS/DZ/UoR/1503; *C. infortunatum*: CI/DZ/UoR/1504; and *C. laccifer* CI/DZ/UoR/1505).

The plant material was ground and extracted with 100% ethanol (800 ml). The mixture was filtered with Whatman No. 1 filter paper (Whatman International, Maidstone, UK). The filtrate was concentrated to dryness at 78°C under reduced pressure in a rotary vacuum evaporator [Table 1]. Dosage of each plant was determined as indicated by Ayurvedic practitioners and set as 200 mg/kg. Extractions were prepared separately. Test solution was prepared by mixing 800 mg of each extraction in 100 ml distilled water (40 mg/ml).

### Experimental Animals

6 months old, inbred healthy female Wistar rats weighing between 160 g and 200 g were obtained for the present study from Medical Research Institute, Colombo, Sri Lanka. The animals were housed in cages, and they were fed with standard laboratory diet and allowed free access to water and food. The animals were well-acclimatized to standard environmental conditions before starting the experiments. All the rats were pre-tested for their hematological parameters prior to the experiments.

### Preparation of Cyclophosphamide Solution

Four tablets of cyclophosphamide (400 mg) as an immunosuppressor were ground and mixed with 10 ml of distilled water in a sterilized beaker. Dosage was decided as indicated by the manufacturer.

### Administering Solutions

All solutions were administered orally via a gavaging needle (gastric feeding tube).

### Experimental Design

Rats were randomly divided into two groups ( $n = 8$  each), namely treatment and control groups. Test solution was administered for the treatment group for a period of 2 weeks (200 mg/kg) while the control group was given distilled

water (1 ml). On the 14<sup>th</sup> day at the end of the treatments, blood samples were collected from tail vein of all rats. After the collection of blood, all animals in the treatment and control groups were administered cyclophosphamide solution (100 mg/kg). Behavioral and morphological features of the experimental rats were observed during the study period.

### Measuring White Blood Cell (WBC), Leukocyte Adhesion, and Cytokines

Total leukocyte (WBC) count was analyzed using Automatic Veterinary Hematology Analyzer (GN-2200, Shanghai, PR China). After initial counts were obtained, leukocyte adhesion assay was carried out as described elsewhere [11]. Plasma interleukin (IL)-4, IL-10, and IL-12 were analyzed using Enzyme Linked Immunosorbent Assay kits as indicated by the manufacturer (Cusabio Biotech Co. Ltd., Wuhan, PR China).

Survival ratio of the rats was calculated after three months of administration of the cyclophosphamide solution.

Data were expressed as mean  $\pm$  standard deviation. The data were statistically analyzed using Minitab 14 (Coventry, UK) software. Total cell counts and the blood IL-4, IL-10, and IL12 of plant extract treated and control groups were statistically compared using Student's *t*-test.

All the experiments were conducted according to the guidelines approved by Faculty of Science, University of Ruhuna, Matara, Sri Lanka.

## RESULTS

### Lesions Appeared after Administration of Cyclophosphamide

Cyclophosphamide-treated control group rats sustained skin lesions at several locations of their bodies while the cyclophosphamide + plant extract-treated group did not show any signs of lesions [Figure 1].

### Blood Analysis

Total WBC count was not significantly different in the control and treated groups. Furthermore, adhesion percentages of leukocytes were not significantly different in control and treated groups. Expression of IL-4 and IL-10 was significantly different in treated and control groups while expression of IL-12 was not significantly different in control and treated groups [Table 2].

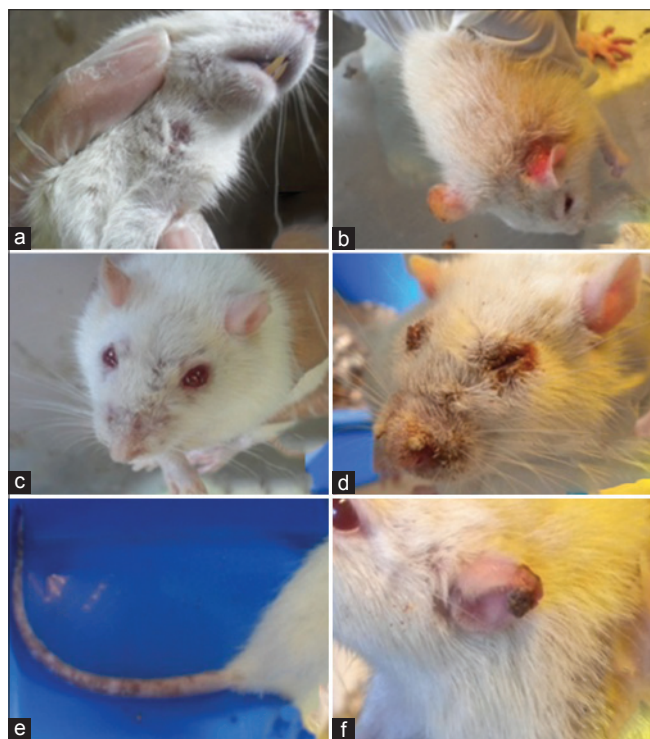
Table 1: Plant materials and nature of the extracts

Plant	Initial weight (g)	Product nature	Final weight (g)
<i>Cyperus rotundus</i> (Rhizome)	200	Dark brown semi-solid residue	12
<i>Solanum surattense</i> (Root bark)	200	Light brown semi-solid residue	10
<i>Alpinia calcarata</i> (Rhizome)	200	Brick-red powdered residue	12
<i>Croton laccifer</i> (Root bark)	150	Light brown powdered residue	08
<i>Clerodendrum infortunatum</i> (Root bark)	150	Light brown powdered residue	06

**Table 2: Blood analysis of control and treated groups after 14 days of the experiment (mean±SD)**

Cell type	Control group	Treated group	t-test (statistics)
WBC	15.86±3.98	12.33±4.14	1.44 ( $P=0.189$ )
Lymphocytes	13.76±2.89	10.83±3.57	1.5 ( $P=0.171$ )
Granulocytes	0.9±0.903	0.583±0.223	0.77 ( $P=0.487$ )
Leukocyte adhesion (%)	31.19±9.91	37.77±6.4	3.58 ( $P=1.3$ )
IL-4	4.14±0.472	9.817±0.605	17.47 ( $P<0.001$ )
IL-10	91.78±1.31	122.98±4.73	15.45 ( $P<0.001$ )
IL-12	127.48±4.1	129.86±7.1	0.7 ( $P=0.43$ )

WBC: White blood cells, IL: Interleukin

**Figure 1:** Lesions appeared in the cyclophosphamide-treated group: (a) ventral neck; (b) ventral ear; (c) face; (d) periphery of the eye and nose; (e) the tail; and (f) dorsal ear

### Survival Ratio

After three months of cyclophosphamide administration survival ratio of the plant extract treated group was 83.33% while none of the rats in the control group survived.

### DISCUSSION

Interestingly, total WBC count did not significantly differ in control and treated groups. Other hematological parameters such as platelet count, hemoglobin content, hematocrit, and the red blood cell count did also show no significant difference between control and treated groups (data not shown). In addition, the adhesion rate of leukocytes was also not significantly different in control and plant extract-treated groups. In an earlier study, it was shown that if rats were treated with these plant extracts separately, WBC count increased significantly, and rats were capable of exerting a strong inflammatory response in paw edema assay [10].

The effect of medicinal plant extracts as immunomodulators has been tested previously in the cyclophosphamide-induced immunosuppression animal model [12,13]. Following cyclophosphamide treatment control group rats those who were not treated with plant extracts sustained skin lesions, and all these rats died after three months of cyclophosphamide treatment. However, plant extract treated rats did not sustain skin lesions and survived even after three months of cyclophosphamide treatment. These data indicate a possible strong immune response exerted in the plant extract-treated rats.

The use of herbal medicines in modulating cytokine expression has been shown previously [1]. IL-4 is a major regulator in humoral and adaptive immunity [14]. Many medicinal plant extracts contain effects on at least one cytokine [1]. In the present study, expression of blood IL-4 and IL-10 levels increased significantly in the plant extract-treated group when compared to the control animals while IL-12 did not show any significant difference. Hence, IL-4 and IL-10 might play a role in immunomodulation. These observations may throw some light into the observation of the survival rate of immunosuppressed rats treated with plant extract.

Effectiveness of plant extract treated rats in overcoming the side effects of cyclophosphamide-induced immunosuppression provides evidence for balancing and adaptogenic effectiveness of the extracts used. The extract combination might have potentiated the non-specific immune response. This result may be attributed to different phytoconstituents [15]. However, further studies are needed in order to understand the exact mechanisms.

It has been shown that herbs play a major role in modification of bone marrow activity, and in most cases bone marrow is a sensitive target of herbal medicines [16]. Preliminary phytochemical analysis is needed to carry out in order to identify the active and toxic constituents of the ethanolic extract of the above plant species [17]. The results of the present study substantiate the belief that these plants are immune system boosters. The ethanolic extract of the above plants has protected the rats against cyclophosphamide-induced immunosuppression indicating its profound immunostimulatory activity possible via IL-4 and IL-10 modulation. Further experiments should be conducted to understand the dose-dependent activity, toxicological effects, and mechanism of action.

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