Evaluation of In-Vitro Immunomodulatory Activity of Hydroalcoholic Extract of *Ceropegia Bulbosa Roxb.*

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Abstract: **The *Ceropegia bulbosa Roxb* belongs to the Asclepiadoideae family and it is found in western ghats of Kolhapur and Rajasthan in India. The tubers and leaves of Ceropegia bulbosa R are eaten and considered to be tonic and digestive. Preliminary Phytochemical analysis of hydro alcoholic extract of Ceropegia bulbosa tubers was done and showed the presence of carbohydrates, saponins, tannis, glycosides, alkaloids, phytosterols, flavonoids. The present study was intended to evaluate the *in-vitro* immunomodulatory activity of hydro alcoholic extract of dried bulbs of *Ceropegia bulbosa Roxb* . Effect of the extract was evaluated at various concentrations (832 µg/ml to 6.5 µg/ml) for secretion of mediators like nitric oxide, superoxide, lysosomal enzyme and myeloperoxidase activity of isolated murine peritoneal macrophages. The extract showed in-vitro phagocytic stimulation of nitric oxide, lysosomal enzyme and myeloperoxidase activity in peritoneal mouse macrophages. In-vitro phagocytic index showed significant results and thus proving the need for confirmation through in-vivo studies.**

Keywords: ***Ceropegia bulbosa Roxb*, peritoneal murine macrophages, nitric oxide, superoxides, phagocytic index.**

I. INTRODUCTION

The immune system is known to be involved in the etiology as well as pathophysiological mechanisms of many diseases[1]. Ayurveda gives emphasis on promotion of health – a concept of strengthening host defenses against different diseases[2]. Rasayana plants are particularly recommended for the treatment of immune disorder [3], Ayurveda (with particular reference to plants) may play an important role in modern health care, particularly where satisfactory treatment is not available. Development of agents, capable of moving patient's immune system from a state of immune deficiency to normal function, would likely to have a significant impact on patient's disease condition. . Such agent would not be a cure, but would control the manifestation and course of disease[4]

Ceropegia bulbosa R belongs to the Asclepiadoideae family. Cerpegin [5] is a rare naturally occurring pyridine alkaloid and has been reported to be isolated from Ceropegia bulbosa. The aqueous extract of edible Ceropegia bulbosa contains polyphenols, potassium steroids and sugar [6].It contains starch, gum, albuminoids, fats, crude fibre. The n-hexane, ethanolic and water extract were screened for various constituents (alkaloids, saponins, tannins, anthraquinones, sterol, flavonoids, terpenoids, glycosides ,simple sugars) using standard protocol.[7]

Ceropegia bulbosa is a useful medicinal plant. Traditionally, it is used for diarrhoea, dysentery kidney stone, to increase fertility and vitality.[8]The tubers and leaves of Ceropegia bulbosa are eaten and considered to be tonic and digestive. In Rajasthan this species is known as Khadula (Hindi). It is used here for many purposes, for example the seeds, brayed to a paste, are used to cure deafness, while this paste is dropped into the ears. An decoction made from the tubers is used, orally to ease dropping out urinary bladder stones[9].Raw tubers are cooked and eaten by ladies to enhance fertility and vitality.[10].Study on animal model of urolithiasis.[7]and study of anti-ulcer activity -Pylorus ligation method [11]were done for Ceropegia bulbosa R but so far no reports were found on immunomodulatory activity of Ceropegia bulbosa R so present study was done to explore the immunomodulatory activity as it was used as a tonic by the people of Rajasthan.

II. MATERIALS AND METHODS

Plant Material and Preparation of Extract

Tubers of C*eropegia bulbosa R*, were obtained from western ghats of Kolhapur, identified by botanist Dr. Rao in August 2012 and authenticated by Blatter Herbarium, Department of Botany ,St. Xavier's college, Mumbai. The voucher specimen (No.NI-2140 of N.A.Irani) was deposited in the Institute for future reference. The fresh tubers were cut into small pieces and dried at controlled temperature 45^0C and powdered. The powder was then extracted with ethanol and water (in equal volume) under soxhlation to give hydro alcoholic extract of

tubers of C*eropegia bulbosa R* (CBR) .The extract was filtered and evaporated to dryness with a dryer.

Preliminary Phytochemical Screening

The hydro alcoholic extract of tubers of C*eropegia bulbosa R* was subjected to Preliminary phytochemical screening [12] for the detection of various plant constituents.

Chemicals

Nitroblue Tetrazolium (NBT) and Tetramethyl Benzedrine Hydrogen Peroxide (TMB/H_2O_2) were procured from Bangalore Genei, India. Streptomycin, penicillin, Roswell Park Memorial Institute (RPMI) 1640 medium and HEPES buffer were procured from Himedia Pvt. Ltd. India. Fetal bovine serum (FBS) and Phytohemagglutinin-M (PHA) were procured from Sigma Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Isolation of peritoneal macrophage and culture conditions

Peritoneal macrophages were isolated from mice which were injected intraperitoneally (i.p.) with 2 ml of 4% (w/v) fluid thioglycollate medium 3 days prior to peritoneal lavage with 10 ml of RPMI 1640 medium. The collected cells were washed with RPMI 1640 and cultured in RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, 100 U/ml penicillin, and 100µg/ml streptomycin (complete RPMI). The macrophage count was determined by using haemocytometer and cell viability was tested by trypan-blue dye exclusion technique. Then the cells were adjusted to required cell count per ml and plated on a 96-well flat-bottom culture plate (Tarsons Products Pvt. Ltd., India) and then incubated for 2 hr at 37^0C in a 5% CO_2 humidified incubator. After removing the nonadherent cells, the mono-layered macrophages were treated with both the extracts separately (832-6.5µg/ml) dissolved in complete RPMI medium containing 20% DMSO and maintained for 24 h at 37^0C in a 5% CO_2 humidified incubator [13]

Following ***in vitro*** assays were performed on these incubated cells. PHA (10µg/ml) was used as a positive control. All the experiments were performed in triplicate.

Nitrite Assay

Nitrite accumulation was used as an indicator of nitric oxide (NO) production in the medium as per the procedure described earlier[14]. Nitric oxide production was determined by assaying culture supernatants for nitrite using griess reagent. isolated murine peritoneal macrophages (5×10^5 cells/ well) were cultured in complete rpmi 1640, incubated for 2 hr at 37ºc in a 5% co_2 atmosphere.The test extracts dissolved in complete rpmi-1640 containing 10% DMSO were added to cultured wells at various concentrations (832-6.5 µg/ml) in triplicate. the plates were incubated for 24 h at 37ºc in a 5% co_2 atmosphere in co_2 incubator. Cell-free supernatant (50µl) from 24 hr incubated macrophages ($5x10^5$ cells/ml) was mixed with 50µl of Griess reagent (1% sulfanilamide, 0.1% napthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. The optical density (OD) was measured at 540 nm with a microplate reader (ELX800MS, BioTek Instruments Inc., USA). Nitrite concentrations were determined from standard curve of sodium nitrite in culture conditions. Stimulation index (SI) for nitrite release was calculated as the nitrite concentrations ratio of the treated and control macrophages.

NBT Dye Reduction Assay

The NBT dye reduction assay was carried out as described previously [15].Briefly, 50µl of 0.3% NBT solution in PBS (phosphate buffered saline, pH 7.4) was added to the 24 hr incubated cells (1×10^6 cells/ml) with CBR and the mixture was further incubated in CO_2 incubator. After incubation for 1hr, the adherent macrophages were rinsed vigorously with complete RPMI medium, and washed four times with 200µl Methanol. After air-drying, formazan-deposits were solubilized in 120µl of 2M KOH and 140µl of DMSO. After homogenization of the contents of the wells, the OD was read at 630 nm by using a microplate reader. Stimulation index (SI) was calculated as the OD ratio of the treated and control macrophages

Cellular Lysosomal Enzyme Activity

The cellular lysosomal enzyme activity of macrophages was evaluated by measuring acid phosphatase activity as described earlier [15]. Briefly, 24 hr after incubation of macrophages with CBR extract at 37°C in 5% CO_2, the supernatant was removed by aspiration and 20µl of 0.1% Triton X-100 (Himedia, India) were added to each well. After 15 minutes incubation, 100 µl of 10 mM *p*-nitrophenyl phosphate (*p*NPP) and 50 µl of 0.1 M citrate buffer (pH 5.0) were added. Further the plates were incubated for 1 hr and 0.2 M borate buffer (150 µl, pH 9.8) was added. The OD was measured at 405 nm by using a microplate reader. The Phagocytic stimulation index (SI) was calculated as the OD ratio of the treated and control macrophages .

Myeloperoxidase Activity Assay

Myeloperoxidase activity was evaluated on isolated macrophages as per the earlier procedure [16]. Briefly, 24 hr incubated macrophages ($5x10^5$cells/ml) with test extract(CBR) was washed three times with fresh complete RPMI medium. Then the mixture (100 µl) of *o*-phenylenediamine (0.4g/ml) and 0.002% H_2O_2 in phosphate-citrate buffer (pH 5.0) was added to each well. The reaction was stopped after 10 min using 0.1 N H_2SO_4 and OD were measured at 490 nm. The myeloperoxidase stimulation index (SI) was calculated as the OD ratio of the treated and control cells.

Statistical Analysis

Results are expressed as Mean ± SEM. Data was analyzed by one way ANOVA followed by Dunnets Multiple Comparisons Test. **P** value less than 0.05 was taken as the criteria for significance.

III. RESULTS

Extraction

The yield of CBR extract obtained was 8.25 w/w . The extract showed presence of carbohydrate, phytosterols, saponins, proteins, alkaloids, tannins, glycosides and flavonoids.

Nitrite Assay on Isolated Peritoneal Macrophages

The nitrite level (nitric oxide) produced in cell culture supernatants was measured at 24 hr of treatment, showing that CBR extract induced nitrite production in statistically significant **P** value. (*P<0.05*) at 832µg/ml (SI 1.701), 416µg/ml (SI 1.501), 208µg/ml (SI 1.360). PHA (positive control) also showed significant increase (*P<0.05*) in nitrite release (SI 2.232).

Phagocytic Assay on NBT Dye Reduction

The *in vitro* phagocytic effects of different concentrations of CBR extract on the reduction of NBT dye on macrophages are presented in Fig. 1. The effect of CBR extract showed significant stimulation (*P<0.05*) on NBT reduction at 832µg/ml (SI 1.489) , 416µg/ml (SI 1.393), 208µg/ml (SI 1.315), 104µg/ml (SI 1.276), 52µg/ml (SI 1.245) & 26 µg/ml (SI 1.174)PHA showed significant stimulation (*P*<0.05) of NBT reduction (S.I. 1.910).

Cellular Lysosomal Enzyme Activity

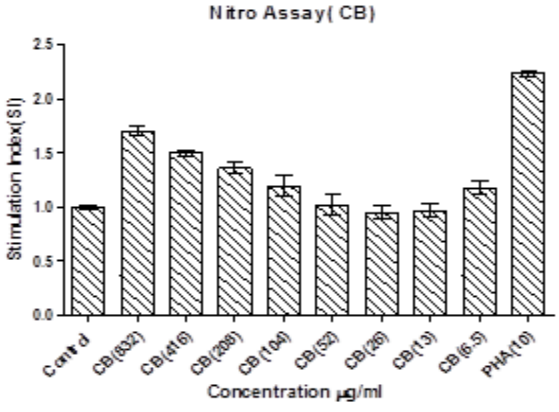
In case of lysosomal enzyme activity CBR extract (fig 1) showed at 832µg/ml (SI 1.558), 416µg/ml (SI 1.345), 208µg/ml (SI 1.259).PHA showed significant stimulation (*P*<0.05) of lysosomal enzyme release (SI 1.937) for both extract.

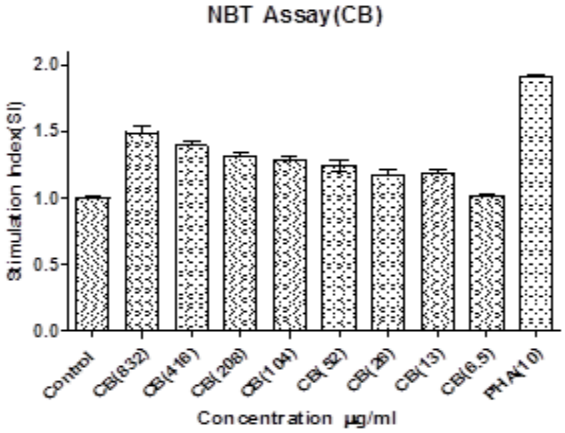
Myeloperoxidase Activity Assay

The effect of CBR extract on myeloperoxidase activity of macrophages is presented in Fig. 1 & Fig 2 respectively. The CBR extract showed significant (*P*<0.05) stimulation of myeloperoxidase activity of macrophages at 832µg/ml (SI 1.640), 416µg/ml (SI 1.483), 208µg/ml (SI 1.326), 52µg/ml (SI 1.229), .Positive control, PHA showed significant stimulation with SI value (1.862).

TABLE 1.In-vitro effect of CBR on release of nitric oxide, NBT reduction, Lysosomal and Myeloperoxidase activity of isolated macrophages

Sr No.	Concentration (µg/ml)	Stimulation Index(SI)			
		NO	NBT	Lysosomal	MPO
1	**Control**	1.000±0.01	1.000±0.01	1.000±0.03	1.000±0.01
2	**CBR(832)**	1.701±0.04	1.489±0.05	1.558±0.08	1.640±0.01
3	**CBR (416)**	1.501±0.03	1.393±0.03	1.345±0.03	1.483±0.03
4	**CBR (208)**	1.360±0.05	1.315±0.03	1.259±0.06	1.326±0.05
5	**CBR (104)**	1.194±0.10	1.276±0.03	1.162±0.06	1.229±0.04
6	**CBR (52)**	1.023±0.10	1.245±0.04	1.053±0.02	1.079±0.05
7	**CBR (26)**	0.952±0.06	1.174±0.04	1.078±0.02	1.182±0.03
8	**CBR (13)**	0.968±0.06	1.184±0.02	1.126±0.04	1.078±0.02
9	**CBR (6.5)**	1.178±0.07	1.013±0.02	1.116±0.01	1.097±0.03
10	**PHA(10)**	2.232±0.03	1.910±0.02	1.937±0.06	1.862±0.02





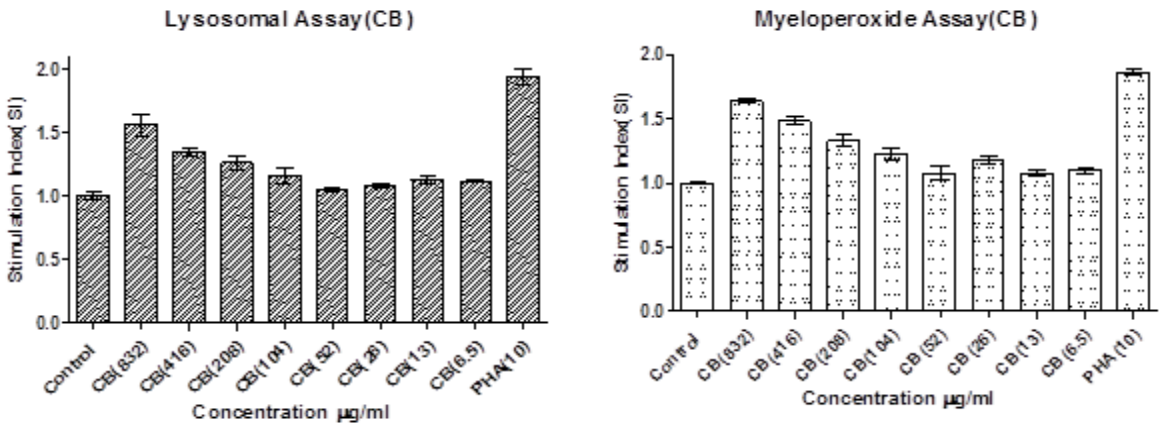


Figure 1

IV. DISCUSSION

Immunomodulation through stimulation or suppression may help in maintaining a disease-free state. Agents that activate host defense mechanisms in the presence of an impaired immune responsiveness can provide supportive therapy to conventional chemotherapy [17]. There is a growing interest in identifying herbal immunomodulators ever since their possible use in modern medicine has been suggested [18]. The main objective of the study was to investigate the immunomodulatory effects of hydro alcoholic extract of Ceropegia bulbosa R..

Macrophages have been known to play an important role in the host protection against a wide range of tumors and microorganisms. Macrophages also presents antigen to lymphocytes during the development of specific immunity and serve as supportive accessory cells to lymphocytes. When activated, macrophages increase the phagocytic activity and release various materials such as cytokines and reactive intermediates and then carry out non-specific immune responses. There has been great interest in reactive nitrogen intermediates, nitric oxide (NO), because of its antibacterial and antitumor effect[19]. NO mediates diverse functions, including vasodilatation, neurotransmission and inflammation[20]. A very high NO production indicates increased phagocytosis and bactericidal activity, which is supported by the data, presented in Fig. 1. Macrophages play an important role in defense mechanism against host infection and in killing tumour cells. Higher reduction of NBT dye by CBR extract represents a higher activity of oxidase enzyme, reflecting stimulation of phagocytes in proportion to intracellular killing. For lysosomal enzyme activity, the transformation of *p*-NPP to coloured compound by the acid phosphatase of the stimulated macrophages correlates to the extent of degranulation in phagocytosis[21]. Phagocytosis of particles by macrophages is usually accompanied by a burst of oxidative metabolism allowing the generation of reactive oxygen species which can be detected through an assay based on the reduction of NBT [22].The effect of various concentrations of CBR extract on the reduction of NBT dye and lysosomal enzyme activity response of macrophages were studied for phagocytic assay. CBR extract appeared to produce phagocytic stimulation with dose response relationship in lysosomal enzyme activity evaluation.

Myeloperoxidase, a heme protein secreted by neutrophils and macrophages, which uses the oxidizing potential of H_2O_2 to convert chloride ion into hypochlorous acid (HOCl). A potent bactericidal agent, HOCl is a critical component of host defenses against invading bacteria, fungi, and viruses [23].The increase in the stimulation index of myeloperoxidase by the exposure of CBR extract indicates enhanced defense capability of these cells to pathogenic organisms. The release of immune mediators from murine peritoneal macrophages was significantly stimulated by the exposure of the extract. Murine isolated peritoneal macrophages incubated with the CBR extract at different concentrations ranging between 832 – 6.5 µg/ml for 24h, showed a significant activation of macrophages by modulating the secretion of various mediators including nitric oxide (NO), lysosomal enzyme and myeloperoxidase activity. This suggests that CBR extract can effectively strengthen innate immunity against foreign particles [24]

The present investigation suggests that *Ceropegia bulbosa R* hydro alcoholic extract had stimulated the phagocytic index in murine peritoneal macrophages.

V. CONCLUSION

The studies have demonstrated immunostimulating properties of hydro alcoholic extract of Ceropegia bulbosa R fruits in various *in vitro* experimental methods. Further studies to elucidate the exact immunostimulatory mechanism of *Ceropegia bulbosa R* need to be explored. In-vivo studies also need to be done to correlate with the in-vitro results.

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REFERENCES

[1]. Sharma P. 1983 Charak Samhita. Chikitasasthana. Chaukhamba Orientalia, Varanasi , India . P. 54
[2]. Thatte U. M., Dahanukar S.A.,1986, Ayurveda and contemporary scientific thought , trends in pharmacological sciences 7, 247.
[3]. Wagner H. 1999 In: Dhanukar S. A., Thatte U. M., Rege N. M., Birkhauser (Eds.), Immunomodulatory agents from plants, Verlag, Basel. Switzerland, pp. 289-323.
[4]. Gottlieb A.A., Gottlieb M.S. Scholes V.E. 1987, Reconstitution of immune functions in AIDS /ARC. Concepts in Immunopathology 4, 261-274.
[5]. Sivakumar, K.; Eswaramurthy, S.;Sabramanian, K.;Natarajan,S. Acta Crystallogr.1990, C46, 839-841
[6]. Prachi khare, Vinod kumar Mishra, Kakkar arun, Neetu bais, Rajendra singh , study on in vitro anti-lithiatic activity of phyllanthus niruri linn. leaves by homogenous precipitation and turbiditory method, Vol 6, Issue 4, 2014.
[7]. Jain Monika, Bhandari Anil, Bhandari Aakanksha, Patel Priyanka "Isolation,Characterization and Invitro Antiurolithiatic activityof Cerpegin Alkaloid from Ceropegia bulbosa var.Lushii root" Int. J. Drug Dev. & Res., October-December 2012, 4(4): 154-160.
[8]. Upadhyay B, Dhaker PA, Kumar A. Ethnomedicinal and ethnopharmaco-statistical studies of Eastern, Rajasthan, India. J. Ethnopharmaco 2010; 129: 64-86. Swarnkar S, Katewa SS. Ethnobotanical observation on tuberousplants from tribal area of Rajasthan (India). Ethnobotanical Leaflets 2008; 12: 647-66.Choudhary K, Singh M, Pillai U. Ethnobotanical Survey of Rajasthan - An Update. Am-Euras J Bot 2008; 1 (2): 38-45.
[9]. Bioorganic and medicinal chemistry vol-18, Issue-11,1/6/2010 page no.3940-3945
[10]. Pakistan Journal of biological science 9(14) 2546-2555,2006
[11]. Pelagiaresearchlibrary.com/der-pharmacia.../DPS-2012-3-1-148-152.pdf
[12]. Trease, G.E., Evans , M. C. , 1983 , Text book of Pharmacognosy, 12th edition , Balliere, Tindall, London pp 343 -383.
[13]. Choi C. Y, Kim J. Y., Kim Y. S. , Chung Y. C. Hahm K. S., Jeong H. G. Augmentation of macrophage functions by an aqueous abstract isolated from Platycodon grandiflorum, Cancer letter 166, 2001, 17-25
[14]. Lee S., Suk K. Heme oxygenase-1 mediates cytoprtective effects of immunostimulation in microglia; Biochemical Pharmacology 74; 723-729).
[15]. Manosroi A, Saraphanchotiwitthaya A, Manosroi J., Effects of Pouteria Cambodiana extracts on invitro immunomodulatory activity of mouse immune system ; Fitoterapia 77, 2006, 189-193)
[16]. Choi E . M ., Hwang J.K. Effects of methanolic extract and fractions from Litsea cubeba bark on the production of inflammatory mediators in RAW264.7 cells, fitoterapia, 75, 2004, 141-148)
[17]. Wagner H. Immunostimulatory drugs of fungi and higher plants. In: ss
[18]. Tiwari U, Rastogi B, Singh P, Saraf DK, Vyas SP., Immunomodulatory effects of aqueous extract of Tridax procumbens in experimental animals; Journal of Ethnopharmacology, 92, 2004, 113–119.
[19]. Lowenstein C.J, Snyder .H., Nitric oxide, a novel biologic messenger. Cell 70, 1992, 705.
[20]. Aurasorn S, Kornkanok I, Pattana S., Effect of Bacopa monniera Linn. Extract on Murine Immune Response In Vitro; Phytotherapy Research; 22, 2008, 1330 –1335.
[21]. Benencia F, Courreges MC, Coulombie FC, Effect on the phagocytic activity and respiratory burst response of peritoneal macrophages; Immunopharmacology, 41, 1999, 45–53.
[22]. Brown KE, Brunt EM, Heinecke JW, Immunohistochemical detection of myeloperoxidase and its oxidation products in kupffer cells of human liver, Am. J. Pathol., 159, 2001, 2081-2088.
[23]. Xie O.W, Cho H.J., Calaycay J, Mumford RA, Swiderek KM, Lee TD, Ding A, Troso T, Nathan C., Cloning and characterization of inducible nitric oxide
[24]. Miller L. E. (1991) In: Ludke, H.R., Peacock, J.E., Tomar, R.H. (Eds.), Manual of Laboratory Immunology, Lea and Febiger, London; 1–18.