Int J Pharm Bio Sci 2013 Apr; 4(2): (P) 852 - 859

Research Article **Pharmacology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

IMMUNE RESPONSE MODULATION TO DPT (DIPHTHERIA, PERTUSSIS, TETANUS) VACCINE BY NDL FROM *CALOTROPIS PROCERA*

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ABSTRACT

Immunomodulation with adjuncts is novel pharmaceutical intervention which explores its new therapeutical dimensions in medical field. *Calotropis procera* (Family *Asclepiadaceae*) was evaluated in laboratory animals immunized with DPT (Diphtheria, Pertussis, Tetanus) vaccine for its immunopotentiation effect. NDL (non-dialyzable latex) latex of *Calotropis procera* (150 mg/kg/day, p.o.) was evaluated for its immunopotentiation effect. The immunostimulation was evaluated using serological and hematological parameters. Treatment of immunized animals with NDL (150 mg/kg/day, p.o.) for 15 days resulted in a significant increase of antibody titers to B. pertussis ($P < 0.05$). B. pertusis BLC-216 strain were used to immunized animals and after that animals were observed for 14 days (treated and untreated) were challenged ~~with~~ by B. pertusis BLC-216 and the animals were observed for two weeks. That indicate the NDL (150 mg/kg/day, p.o.) treated animals groups represent significant enhancement of antibody titers as compared to controlled / untreated animals after challenge ($P < 0.05$). Immune protection against intra-cerebral challenge of ~~live~~ B. pertussis live cells was evaluated on behailf of degree of sickness, paralysis and death. Reduced mortality accompanied with overall improved health status was observed in treated animals after intracerebral challenge of B. pertussis indicating the development of protective immune response.The NDL of *Calotropis procera* also offers direct therapeutic benefits resulting in reduced morbidity and mortality of experimental animals. Present study indicates application of NDL of *Calotropis procera* act as a mild immunopotentiating agent with possible applications in immunopharma industry.

KEYWORDS: vaccines, Immunoadjuvant, NDL of *Calotropis procera,* Immunomodulators.

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INTRODUCTION

Vaccines are now very well recognized are successful pharmaceutical interventions against the number of diseases which had a major contribution in the morbidity and mortality in the developing countries. Combined use of immunomodulators and vaccines is now considered as novel approaches in vaccine design and development.[1-2] Immunomodulators obtained from different sources like synthetic, bacterial, viral have been used for enhancement of immune response to vaccines. But there is a compromise on level of adjuvant and acceptable level of side effects for newer vaccines as they include synthetic, recombinant or highly purified subunit antigens which are weakly immunogenic, but as the vaccine formulations often require adjuvant as immunopotentiators for better immunological effect. [3-4] Hence, there is need of safe and effective adjutants to be used as immunopotentiators in vaccines.[5-7] Ayurveda is one of the ancient medicinal sciences practiced in all over the world.[15] Ayurveda also gives the principle of alleviation of disease by the modulation of immune response.[16] Herbals are the choice of immunomodulators for researchers from eons of time.[8-11] Medicinal herbs are also known for their immune responses in the various researches. (L-I) Moreover the co-administration of herbal extracts with vaccines has been shown to increase the antibody response and to enhance the proliferative response of T cells [L-2]. Various researches are focused in animal as well as in human to explore the usefulness of herbal extracts as adjuvant to vaccines. The plant *Calotropis procera* (Asclepiadaceae) is a non-cultivated shrub of geographic distribution covering Asia, Africa and Northeast of South America. Pharmacological properties of the latex include pro and anti-inflammatory, antinociceptive, antipyretic, antibacterial and antidiarrhoeal activities among others.[17-18] Plant have been used as purgative, anthelmintic and also in the treatment of diseases, such as leprosy, ulcers, tumors and piles (L-3). The Chemical constituents *Calotropis procera* are caoutchouc, calotropin, calotoxin 0.15% , calactin 0.15% , uscharin 0.45% , trypsin, voruscharin, uzarigenin, syriogenin and proceroside.[20-23] Protein analysis of NDL suggests that it contains three types of proteins PI, PII, and PIII which are biologically active.[24] The NDL has previously shown to display anti-inflammatory and analgesic activities by intraperitoneal route. Therefore the aim of the present study was to investigate the immunomodulating potential of NDL to DTP vaccine. This will further explore its adjuncts role with DTP vaccine and could be the novel therapeutically approach for mankind.

2. MATERIALS AND METHODS

Plant Materials

Fresh plants of *Calotropis procera* were collected in the month of Nov 2007 from Ujjain (M.P.), INDIA. The plant authenticated was carried by Horticulture & agriculture Department, Ujjain (M. P.)

Latex preparation

The crude latex of non-cultivated and healthy plants was collected in distilled water (ratio 1:1) in plastic tubes that were shaken gently, closed and maintained at environmental temperature (25–28 ◦C) until handled in the laboratory. The samples were initially submitted to centrifugation at 25 ◦C during 10 min in a bench centrifuge.The rubber precipitate was separated from soluble phase. In secondly soluble phase was submitted for dialysis with water and cut-off through 8000 mol. wt. membranes. The volume of water used for dialysis is equivalent to the volume of latex material filled in the dialysis tube. Dialyzed water was pooled and termed as dialyzable latex (DL). After completion of 60 hrs of continuous dialysis the retained material on membrane was freshly centrifuged as previous and water soluble very clean material was separated out of the new pallet and termed as non-dialyzable latex (NDL).Then this pallet was mixed to that of first centrifugation and termedrubber latex (RL). The three latex fractions were thus freeze-dried and

used in the further determinations.(Latex preparation)

Animals and immunization

Swiss albino mice of either sex weighing between 22–30gm were obtained from Govt. Veterinary college, Mhow –Indore (M.P.), India, and used. The animals were housed under standard laboratory conditions and maintained on natural light and dark cycle and had free access to food and water. Animals were acclimatized to laboratory conditions before the experiment. Each animal was used only once. All the animals were randomly distributed in total eight groups consisting four main groups and four replica groups (n = 8). Groups were maintained in replica in anticipation of the mortality associated with intracerebral challenge. For the practical purpose, data of all the surviving animals from each set of groups (main and replica) were used for analysis. Each group consists of minimum 8 animals. All the experiments were carried out between 0900 and 1500h. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) and conducted according to the animal regulatory body of the Indian Government, (CPCSEA).

Drugs and treatment

DTP vaccines obtained from Biological E. Limited, India. Each 0.5ml of vaccine contains Diphtheria toxoids: 02 lf to 20 LF, Tetanus toxoids: 05 lf to 25 LF, B. Pertus: 04 IU. Animals in all groups were vaccinated with DPT adsorbed vaccine. All animals received 0.5 ml of 1:26 diluted standard dose of vaccine, i.p. on day 0.

Challenge to vaccinated animals

The challenge dose was prepared as per standard W.H.O. protocol[26] The B. pertussis cells grown on Bordet-Gengou medium for less than 24 h were suspended in a diluent and cell density was adjusted to 100–1000 times median lethal dose (LD50) in 0.03 ml of diluents

Serology and hematology

The antibody response to D and T components of vaccine was evaluated by a single radial immunodiffusion (SRID) while the pertussis component of the vaccine was evaluated by micro-slide agglutination technique. The technique has advantages over tube agglutination technique, being faster and requiring smaller volumes of reactants.[27] This paper reports results for pertussis component of vaccine only. Level of antibody was expressed as titre, which is defined as the highest dilution of the test serum that gives a visible detectable reaction with MVP pool of B. pertussis vaccine containing 1, 2, 3 agglutinogens at 180 IOU/ml. This pool was obtained from BE Ltd. Hyderabad, (A.P.) that contains four different strains of B.pertussis. Four strains of B.pertussis are namely strain no 134 containing agglutinogens 1 and 3, strain no 509 containing 1 and 2, strain no 6229 containing 1,2 and 3 and strain no 25525 containing 1, 2 and 3.[28-30] Thus, total suspension contains agglutinogens 1, 2 and 3, which was used as an antigenic substance in slide agglutination for anti pertussis sera titre determination. The titers are expressed as Log2 values. Appropriate controls were kept during antibody assay to avoid misinterpretation by auto agglutinating cells if any. Blood was collected in anticoagulant for hematological parameter studies. The cytological examination was carried out using Leishman stained smears. The cell counts (red cells, total and differential white cells and platelets) and estimation of hemoglobin was carried out as per standard protocol.[27]

Mouse protection test

Test material was evaluated for its immunopotentiation potential in mouse protection test also known as intracerebral challenge test or Kendrick test, is one of the mandatory requirements for determination of potency of pertussis vaccines. This test is based on an immunization and challenge procedure. In this study, we have chosen a single optimal dose based on experience of multi dose potency tests routinely performed at the vaccine manufacturing industry. The study design has been planned to check the immunopotentiating activity of extract under study. Challenged animals were observed from day 14 to day 28 for sickness, paralysis and subsequent death, which is generally seen after

challenge.[31] Development of protective immunity against intracerebral challenge with live B. pertussis cells in treated animals, was evaluated using mortality rate and degree of sickness in animals.

Study design

Total duration of this study was of 28 days. On day zero, animals in all groups (I, IA, II, IIA III, IIIA, IV and IVA) received DPT vaccine (Table 1). From days 1 to 15, animals in treatment groups (II, IIA, IV and IVA) received test material (100 mg/kg/ day) while control groups (I, IA, III and IIIA) received vehicle 2% CMC per oral route. On day 14, blood was collected for serology from groups I and II while animals in groups III, IIIA, IV and IVA received an intracerebral challenge with B. pertusis BLC-216 . Morbidity (sickness and paralysis) and mortality were recorded among challenged animals from days 14 to 28 and animals dying within 3 days of challenge inoculation were not considered for the analysis.[32-33] An attempt was made to grade morbidity among the animals on the basis of severity of paralysis observed in animals after challenge. Animals were bled for hematology and serology on day 28. Weights of animals were recorded daily during the study period. Morbidity were analyzed statistically.

Scoring system

Scoring system was designed in order to record the morbidity observed after challenge. The animals were scored as Score 0: for normal animals, no paralysis; Score 1: for mild paralysis (swollen head, impaired movement, less food intake, mild weight loss (1–5%)); Score 2: for severe paralysis (swollen head, no movement, no food intake, severe weight loss (40–50%)) and Score 3: for death (Table2). Protective immunity developed to intra-cerebral challenge with live *B. pertussis* cells in treated animals was evaluated using above scores and mortality rate.

Statistical Analysis

Values are expressed as mean ± SEM. The behavioral assessment data were analyzed by a repeated measures two-way ANOVA with drug-treated groups as between and sessions as the within-subjects factors. The biochemical estimations were separately analyzed by one-way ANOVA. Post-hoc comparisons between groups were made using Tukey's test. The value $P < .05$ was considered significant.

3. RESULTS

Serology

Effect of NDL of Calotropis procera on unchallenged and unchallenged animals

In the present study the effect of with NDL of *Calotropis procera* (150mg/kg/day) in unchallenged animals after 14 days significantly increase the of antibody titers to B. pertussis as compared to control (Gr. 1 and Gr. II), moreover the result shows the same pattern of significantly increase the of antibody titers to B. pertussis after the 28 days in challenged also as compared to control (Gr. 1A and Gr. IIA). Further theirs was a significant increase in antibody titers to B. pertussis antibody titers in both untreated Gr. III, IIIA (Vaccine + Challenge) and treated Gr. IV, IVA (Vaccine + Challenge + Extract) animals as compaired to control and vaccine and challenge, which are because of boosting effect (Table 1).

Hematology

No significant effect on Hb, polymorphs, lymphocyte and W.B.C counts was observed at the end of study period (Table 1).

Morbidity and mortality

The mice were observed daily for morbidity (sickness and paralysis) and mortality. Deaths were recorded among challenged animals from day 14 to day 28. As per W.H.O protocol, mice dying in the first three days after challenge were not counted when computing the results, mild reduction in morbidity scores was observed in animals receiving treatment Gr. IV, IVA as compared to untreated Gr. III, IIIA animals (Table 2). Treatment with test material considerably lowered the incidence of mortality. The number of mice in control group surviving for 14 days was 07 out of 14 whereas in treatment group 09 could survive out of 15 animals (Table 1).

TABLE 1
Effect of test material NDL fraction from the latex of Calotropis procera on DPT immunized animals

Group*	Log2 average pertussis antibody titers	Hb Con.^{n} in g%	W.B.C (N_10^{3}/ Microlitre)	Polymorph Count (%)	Lymphocyte Count (%)	Total no. of Deaths (D)	Mortality %
Effect of test material on unchallenged animals ((Titre Values observed on day 14)							
Gr. I **Vaccine only**	6.26 ± 0.32	NA	NA	NA	NA	NA	NA
Gr. II **Vaccine & Extract**	5.41±0.46[a]	NA	NA	NA	NA	NA	NA
Effect of test material on challenged animals (Titre Values observed after challenge on day 28)							
Gr. I.A **Vaccine only**	6.31±0.60	10.9± 1.40	10000.67 ±798	7.80 ±1.82	94.48±2.18	NA	NA
Gr. II.A **Vaccine & Extract**	5.33±0.59[b]	11.01±2.20	9986±788	9.88±4.90	98.30±4.48	NA	NA
Gr.III &IIIA **Vaccine & Challenge**	6.80±0.44[c]	9.80±1.60	4100±216	8.26±5.22	87.50±4.75	6	42.8
Gr. IV &IVA **Vaccine, Challenge & Extract**	8.17±0.78[d]	10.30±2.43	4250±314	8.54±2.30	92.6 ±1.43	5	35.71

Tabular values represent mean ± standard deviation. N= no. of animals. NA= Not applicable
. [a] P<0.05 as compared to Gr. II, [b]P<0.05 as compared to Gr. 1A, [c]P<0.05 as compared to IA,
[d]P<0.05 as compared to Gr.III & IIIA,

TABLE 2
Morbidity observed with NDL of calotropis procers treated animals after challenge inoculation

Group III and IIIA		Group IV and IVA	
Animal no	**Morbidity Score**	**Animal no**	**Morbidity Score**
I	**3**	I	**3**
II	**3**	II	**3**
III	**3**	III	**3**
IV	**2**	IV	**0**
V	**D**	V	**2**
VI	**2**	VI	**3**
VII	**3**	VII	**1**
VIII	**D**	VIII	**2**
IX	**3**	IX	**0**
X	**3**	X	**3**
XI	**2**	XI	**3**
XII	**1**	XII	**0**
XIII	**1**	XIII	**2**
XIV	**0**	XIV	**1**
XV	**3**	XV	**0**
XVI	**0**	XVI	**D**
Mean ± SD	2.0714±1.1411	Mean ± SD	2.000±1.2799

D= Animals dying within 3 days of challenge inoculation hence not used for analysis.
**** Animals were graded on the basis of devised scoring system.***
Score 0: for normal animals, no paralysis;
Score 1: for mild paralysis [Swollen head, impaired movement, less food intake, mild weight loss (1– 5%)];
Score 2: for severe paralysis [Swollen head, no movement, no food intake, severe weight loss (40– 50%)] and
Score 3: for death.
Mean ± SD was estimated for Independent values. (Comparison between group III and IV).

4. DISCUSSION

Immune system affects in two ways in positive which we call immunostimulation this results in activation of specific and non-specific immunity and immunosuppressant which result in reduced resistance against infection and collectively this is known as Immunomodulation. The present study indicates that *Calotropis procera* is a potent immunostimulant, stimulating specific and non-specific immune mechanisms by demonstrating its mild stimulatory effect on serological parameters, however, unchanged effect on hematological parameters was observed which needs the further research. Mortality rate and degree of sickness in animals evaluation is also one of the known parameter for immunity evaluation. *Calotropis procera* treatment reduced the mortality rate and degree of sickness which could be concluded as the development of immune protection against intracerebral challenge with live B. pertussis cells. *Calotropis procera* slightly reduced the morbidity rate which justify its immunoprotective role *Calotropis procera.* Present study revel role of *Calotropis procera* as immune stimulator which possibly improves immunogenicity low and weak dose antigens. However the further studies are required to specify its immunocodynamics such as its antibody profiles, cytokine induction and regulation of immune response in terms of Th1 and Th2 needs to be undertaken to explore therapeutic and industrial applications.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. Kamalesh Dashora., HOD of Institute of Pharmacy, Vikram University, Ujjain (M.P.), INDIA; Thanks are also to Dr. Y. A. Jaaliwala, Professor, Indore Institute of Pharmacy, Indore (M.P.) INDIA, for his valuable support.

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