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Research Article

Preparation and Immunocharacterization of Probiotic DNA Loaded Chitosan Nanoparticles: An *In Vitro* and *In Vivo* Study

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ABSTRACT
The rise in infectious diseases as well as non infectious immune related disorders demand the need for the development of efficient immunomodulators. The chemical based drugs employed to cure these diseases may have the side effects and are costly. In the present era, consumer awareness about the harmful effects of chemical drugs raised a need to search for natural/alternative therapy for the disease treatment. Immunotherapy is one of the alternative ways of management/modification of diseases. Probiotics have been proved to be beneficial for human beings. Even its components such as cell wall, genomic DNA, etc act as foreign antigen to eukaryotic organisms and are immune enhancers. The immune enhancing efficacy of probiotic DNA might be increased by nanoparticle assisted delivery. Chitosan nanoparticles (chitosan NP) have been greatly explored and developed for pharmaceutical applications. Hence, the present study was conducted to prepare and characterize the probiotic genomic DNA loaded chitosan nanoparticles (DLCNP) and to compare their immunomodulatory potential with *Lactobacillus acidophilus* NCDC343 (LA 343) whole cell and their isolated genomic DNA (LA DNA) *in vitro* as well as in experimental animals. The characterization studies revealed that nanoparticles size ranging from 350 to 515nm were prepared with a positive zeta potential in between +8.71 to +17.7mV. *In vitro* experiments proved that LA 343, LA DNA, DLCNP showed immune enhancing activity; maximum being shown by DLCNP. Further, *in vivo* experiments demonstrated that DLCNP show significantly higher activity than LA DNA. Moreover, study on routes of administration indicated that i.m route is best for giving LA DNA whereas, i.p route is better for nanoparticles.

Keywords: Probiotics, Probiotic DNA, Chitosan Nanoparticles, Chitosan DNA Nanoparticles, Immunotherapy.

INTRODUCTION

The ever increasing knowledge about the mechanisms of immune response in modulating many diseases has raised interest in application of probiotics as immunomodulators. The health effects of probiotic micro-organisms are well known and many probiotic products are commercially available now as nutraceuticals. Though, probiotics have GRAS (Generally Regarded as Safe) status, yet their use might be limited and concentration dependent in aged and immunocompromised persons. Recent advances in immunology and molecular biology lead towards finding that the DNA of the bacteria is capable of immune activation. The vertebrate immune system recognizes 'CpG motifs' (Oligodeoxynucleotides containing CpG motifs) of probiotic DNA as foreign and trigger protective immune responses which are strongly Th1-based[1]. The structural differences between bacterial and eukaryotic DNA basically makes the bacterial DNA capable of serving as an immune activating agent. Moreover, the preliminary experiments revealed that probiotic genomic DNA is a better immune enhancing agent than its whole cell[2,3]. Although, nucleic acids (such as DNA, siRNA, CpG-ODNs) are being used as drugs, either as one of the components of adjuvants of vaccine or in gene therapy procedures, yet their efficacy is often limited by their lower potential in reaching the site of therapeutic action. Many of the issues faced by the oral gene delivery are that the naked nucleic acids are unstable, prone to degradation by serum nucleases and thus have reduced bioavailability in intestine. This problem of instability and DNA degradation can be overcome by the use of nanoparticles (NPs). Various nano sized materials such as cationic liposomes, polyethylenimine, poly(L-lysine), carbon nanotubes, etc and their various derivatives are frequently used for delivering nucleotides[4]. Out of these nano materials, chitosan has gained more attention as drug delivery carrier. Chitosan being positively charged binds to negatively charged nucleic acids and protects the nucleic acids from *in vivo* harsh environment containing various kinds of nucleic acid degrading enzymes[5]. In the present study, experiments were designed to find out whether the immuno modulatory efficacy of genomic DNA can be improved by chitosan nanoparticle delivery.

MATERIAL AND METHODS

Probiotic strain

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Lactobacillus acidophilus NCDC343 (LA 343) was procured from NDRI, Karnal. The culture so obtained were given two revival cycles in de Man–Rogosa–Sharpe broth (MRS broth) at 37°C and maintained for further use.

Isolation of Genomic DNA from LA 343

For genomic DNA preparation, cells were grown in the corresponding medium containing 1.5 % glycine to facilitate cell lysis. Genomic DNA was isolated and its quality was checked by agarose gel electrophoresis and quantity was determined spectrophotometerically[6].

Preparation of Chitosan Nanoparticles (CNP) and DNA loaded Chitosan Nanoparticles (DLCNP)

Chitosan (deacetylation degree ≥75%) was purchased from HiMedia Laboratories Pvt Ltd. It was purified by reprecipitation from the filtered 1% acetic acid solution with 1N Sodium hydroxide till pH of 8.5 reached. The precipitate was washed with deionized water and vacuum-dried[7]. The purified chitosan (0.25%) was dissolved in 1% acetic acid (pH varying from 3.5 to 5.5) with gentle heating on magnetic stirrer at 1300rpm till a homogeneous solution was obtained[8].

DNA sample was dissolved in 20% sodium sulphate solution. For the formation of DLCNP, 1 part of sodium sulphate solution containing DNA was added dropwise to 3 parts of chitosan solution and mixed on magnetic stirrer at 500 rpm till a hazy solution was formed. DLCNP were recovered by centrifugation and after washing with deionized water, stored in deionized water at 4°C for further study[9]. The solution was sonicated for 8 min at 50W amplitude with 10sec ON and 5sec OFF cycle before conducting characterization studies, *in vitro* and *in vivo* experiments.

Characterization of Nanoparticles

Characterization of CNP

Morphological examination: Nanoparticles were observed under the Scanning electron microscope (SEM) and transmission electron microscope (TEM).

Dynamic Light Scattering (DLS) study: Sizing of the nanoparticles was performed by photon correlation spectroscopy on the Zetasizer 3000 (Melvern Instruments Ltd., UK) to obtain average nanoparticle size measurements. Zeta potential of the nanoparticles was measured in demineralised water at neutral pH by laser Doppler anemometry using Zetasizer 3000 (Malvern Instruments Ltd., UK).

Characterization of DLCNP

Alongwith morphological examination and nanosoize as well as Zeta potential studies, following parameters were studied for characterization of DLCNP.

Determination of DNA complexed: The amount of complexed DNA (in DLCNP) was determined by agarose gel electrophoresis of nanoparticles versus naked DNA and supernatant after nanoparticles formation[7]

Loading capacity and Loading efficiency: The loading degree was determined by quantifying the non bound DNA in supernatant with a spectrophotometer. Both loading capacity (LC) and loading efficiency (LE) were calculated by using following equations[10]

$$\text{LC (\%)} = \frac{[(\text{total amount of DNA}) - (\text{non-bound DNA})] \times 100}{\text{Weight of Chitosan DNA nanoparticles}}$$

$$\text{LE (\%)} = \frac{[(\text{total amount of DNA}) - (\text{non-bound DNA})] \times 100}{\text{Total amount of DNA}}$$

In vitro DNA release study at physiological pH (7.4) and at stomach acidic pH (2.1): DLCNP were suspended in PBS (pH – 7.4 and pH – 2.1) separately in two flasks. The suspension was shaken in a water bath at 37°C. At defined time intervals, the supernatant was collected by centrifugation and nanoparticles were re-suspended in fresh buffer. Genomic DNA released into the supernatant was quantified by measuring with a spectrophotometer at OD_{260}[11].

Characterization of immunomodulatory potential of whole cell probiotic LA 343, LA DNA and DLCNP in vitro and in vivo

In vitro characterization

Spelnocytes isolation

Splenocytes were isolated from spleen by teasing the tissue. Cells were centrifuged (400 × g for 10 min at 4°C) and lysed by ACK lysis buffer (0.5M NH_4Cl, 10mM $KHCO_3$ and 0.1 mM disodium EDTA, pH 7.2). Splenocytes obtained were washed thrice in PBS, counted and adjusted ($2x10^6$ cells ml^{-1}) in Minimal Essential Medium (MEM) and was incubated for 2 hours at room temperature with the respective test samples i.e. LA 343; LA DNA; DLCNP and CNP. The splenocyte suspension was further subjected to NBT (Nitroblue Tetrazolium dye) reduction, iNOS (inducible Nitric oxide synthase enzyme) activity and Phagocytic activity tests.

NBT test[12]

NBT is based upon principle that whenever any foriegn particle is ingested by a phagocyte, a respiratory burst is induced; which could be measured by change in yellow colored NBT dye into blue colored formazon complex.

iNOS activty[13]

iNOS is based upon the principle that when macrophages are activated they together with T-cell derived IFN-gamma begin to express iNOS which oxidizes L-Arginine to citrulline and nitric oxide, The dark purple color so formed is detected by spectrophotometer.

Phagocytic activity[14]

Phagocytic activity is one of the important parameter to measure the phagocytic function of splenocytes. Phagocytic activity is measured by incybating splenocytes, test samples and *E.coli* together. Decrease in the formation of *E.coli* colonies on Nutrient agar, indicates the activation of splenocytes.

In vivo characterization

Experimental animals

Balb/c female mice (18-22gm) maintained on standard laboratory diet (Kisan Feeds Ltd., Mumbai, India) and water ad libitum were employed in the present study. The animals were divided into respective groups each of minimum six animals, housed individually in the departmental animal house and were exposed to 12 hr cycle of light and dark. The experimental protocol was approved by Institutional Animal Ethical Committee (Registration No: 107/99/CPCSEA/2013-24) were carried out as per the guidelines of committee for

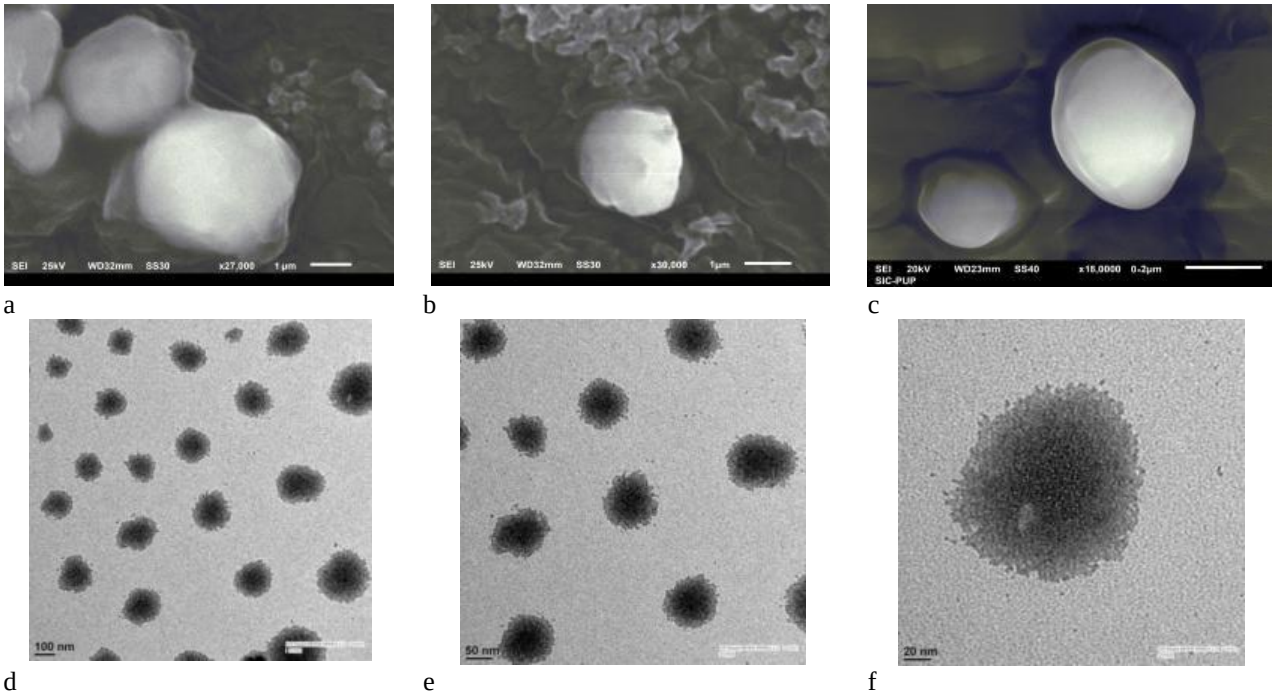

Figure 1: (a), (b) and (c) - Scanning Electron Microscopy of DLCNP. (d), (e) and (f) - Transmission Electron Microscopy of nanoparticles.

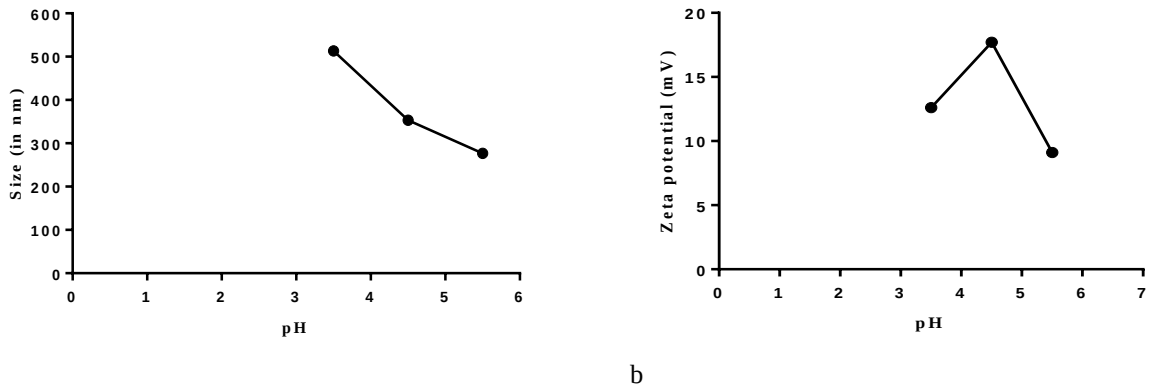

Figure 2: Change in DNA loaded Chitosan Nanoparticle's (a) Size (nm) and (b) Zeta Potential (mV) with increase in pH. The above data is represented as Mean ± S.E.M (n=3).

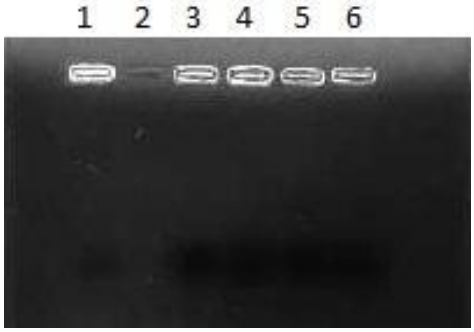

Figure 3: Agarose Gel Electrophoresis 0.8% agarose gel analysis of DLCNP (Well 1, 3, 4, 5 and 6 prepared at 0, 6,12, 18, 24th day respectively) and Supernatant (after the formation and before the washings of DLCNP) (Well 2).

Purpose of Control and Supervision of Experimental on Animals (CPCSEA) Ministry of Environment and Forest, Government of India.

Experimental groups of animals

Animals were divided into seven major groups; including Positive and Untreated control. The test animals were given the treatment with LA DNA, DLCNP and CNP. Two different routes of injection i.e. intraperitoneal (i.p) and intramuscular (i.m) route

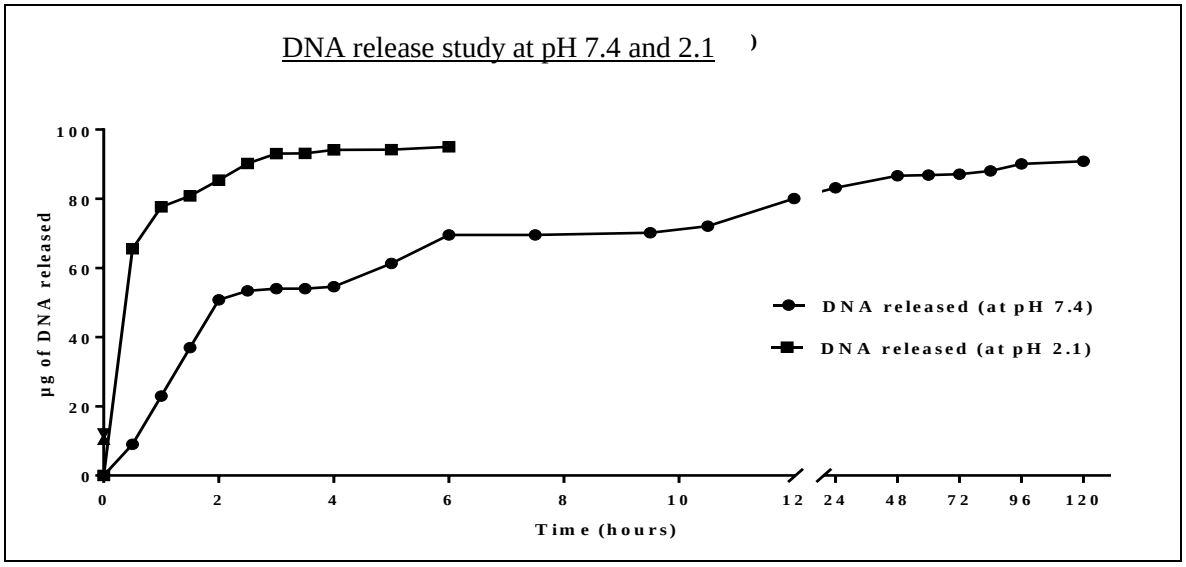

Figure 4: *In vitro* DNA release study at physiological pH – 7.4 and at stomach acidic pH – 2.1. The above data is represented as Mean ± S.E. M (n=3).

Table 1: *In vitro* immunomodulatory potential of probiotic cells, isolated genomic DNA and DNA loaded Chitosan nanoparticles.

Groups	NBT reduction (Mean%)	iNOS activity (Mean%)	Phagocytic activity (Mean%)
Untreated Control	12.51 ± 0.49	10.70 ± 0.38	11.60 ± 0.97
LA 343	44.57 ± 0.70	26.13 ± 0.27	35.35 ± 0.61
LA DNA	56.51 ± 0.49	80.00 ± 0.44	68.26 ± 0.24
CNP	40.08 ± 0.28	13.61 ± 0.47	26.84 ± 0.56
DLCNP	71.19 ± 0.89	95.97 ± 0.63	83.58 ± 0.35

Data are Mean value ± S.D (n=3)

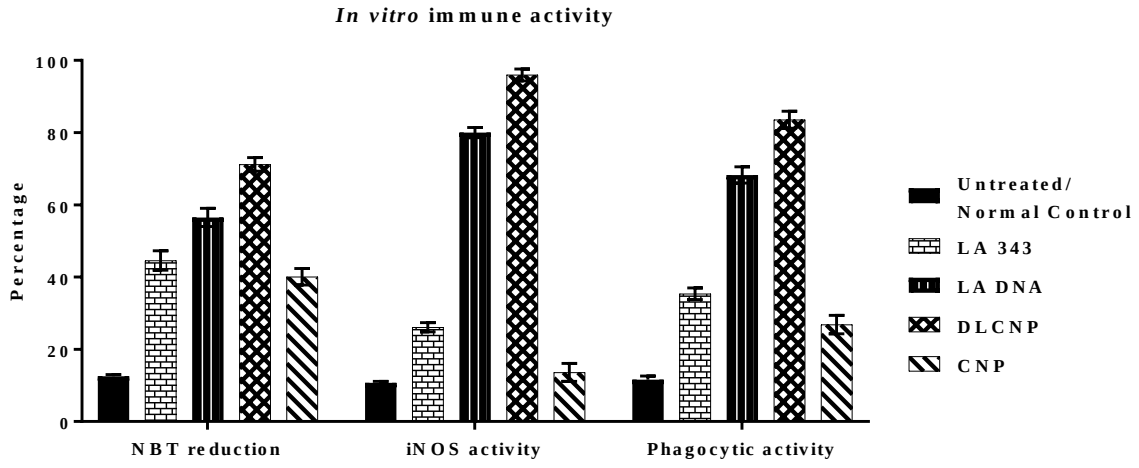

Figure 5: *In vitro* immunomodulatory evaluation of LA 343, LA DNA, CNP and DLCNP. The above data is represented as Mean ± S.E.M (n=3). p< 0.05 as compared to Normal Control

were examined for LA DNA delivery. The details of groups and their treatment is as given below.

Group I: Untreated Control (not subjected to any treatment i.e. kept only on diet)

Group II: Positive control (25mg kg^{-1} Levamisole, i.p for 17 consecutive days)

Group III: Immunized control (mice sensitized with SRBC, i.p and kept on normal diet)

Group IV: Mice treated with LA DNA*, i.m at the rate of 75µg DNA per mouse.

Group V: Mice treated with LA DNA**, i.p at the rate of 75µg DNA per mouse.

Group VI: Mice treated with DLCNP**, i.p at the rate of 80µg DNA $mouse^{-1}$.

Group VII: Mice treated with CNP**, i.p. *4 injections, intramuscularly in left tibialis anterior muscle[15] at the interval of 6 days between two doses.

Table 2: Effect of probiotic cells, isolated genomic DNA and DNA loaded Chitosan nanoparticles on NBT reduction, iNOS activity and phagocytic potential in Balb/c mice

Groups	NBT reduction (Mean%)	iNOS activity (Mean%)	Phagocytic activity (Mean%)
Normal Control	71.70 ± 1.63	40.74 ± 1.43	33.83 ± 1.66
Positive Control	89.82 ± 3.85 [a,b]	90.98 ± 1.67 [a,b]	95.92 ± 2.26 [a,b]
Immunized Control	65.83 ± 2.46 [a]	52.01 ± 1.76 [a]	63.41 ± 1.60 [a]
LA DNA (i.m)	87.99 ± 2.99 [a,b]	89.24 ± 1.32 [a,b]	93.39 ± 1.06 [a,b]
LA DNA (i.p)	75.04 ± 2.26 [a,b]	82.58 ± 1.60 [a,b]	88.59 ± 1.11 [a,b]
DLCNP	92.40 ± 1.97 [a,b]	93.56 ± 1.00 [a,b]	95.05 ± 0.66 [a,b]
CNP	71.11 ± 2.02 [a,b]	52.55 ± 1.92 [a,b]	62.19 ± 1.75 [a,b]

Data is represented as Mean ± S.D (n=6).
[a]p<0.001 in comparison to normal control and [b]p<0.05 in comparison to immunized control

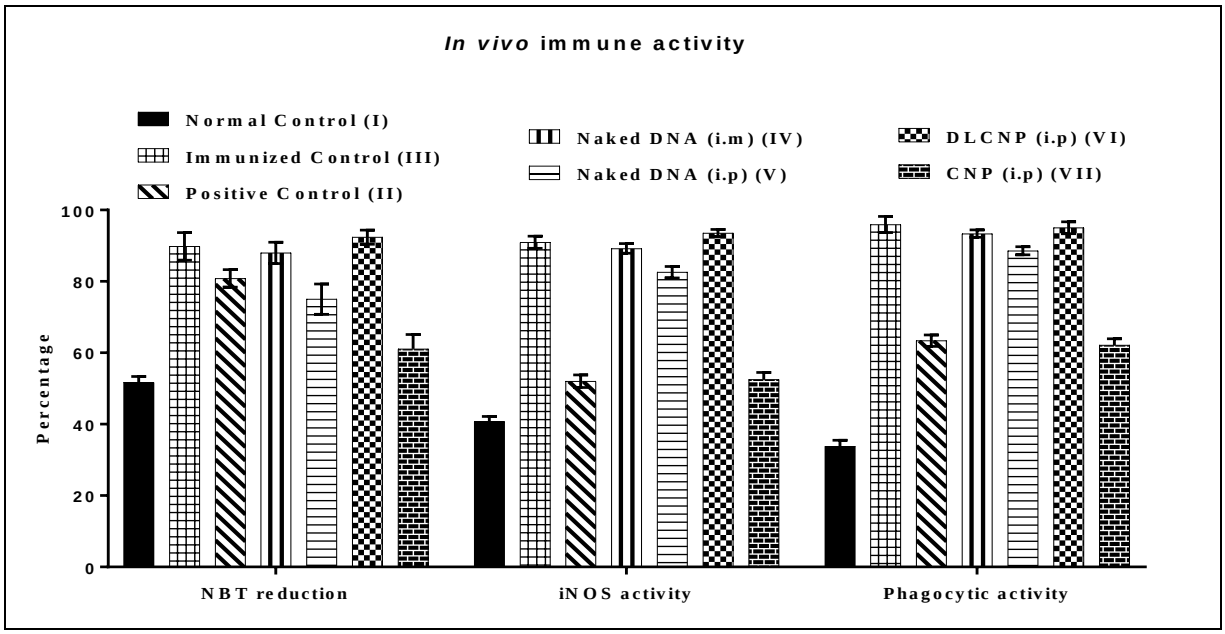

Figure 6: Comparative *in vivo* NBT reduction, iNOS activity and Phagocytic activity of LA 343, LA DNA, CNP and DLCNP. The above data is represented as Mean ± S.E.M (n=6).

** 4 injections, intraperitoneally[16] at the interval of 6 days between two doses.

All the animals were immunized with SRBC on 15th day and sacrificed on 21st day of experiment. Spleen was exiced from all the animals and processed individually to check cell mediated immune response. Blood was withdrawn for checking humoral immune response.

Immunization

Sheep blood was collected in Alsever's solution in the ratio 1:2 and was centrifuged at 400 × g for 10 min at 4°C. The erythrocyte pellet obtained was washed and suspended in PBS (0.1 M, pH 7.2) for further use[17]. All mice were antigenically challenged intraperitoneally with a single dose (100µl ml^{-1} of 1 × 10^7 cells/ml) of sheep red blood cells (SRBC).

Cell mediated immune response

Splenocytes were isolated from spleen by teasing the tissue and processed for NBT test, iNOS and Phagocytic

Delayed Type Hypersensitivity assay

Delayed Type Hypersensitivity response (DTH) was checked by foot pad swelling method[18]. All SRBC primed groups were challenged intradermally on day 15th with SRBC suspension (1 × 10^7 100µl $saline^{-1}$) in the hind footpad. The control lateral paw was given equal volume of saline. Paw thickness was measured with micro-calliper at 24h interval up to 72h. The difference in paw thickness compared to control was taken as a measure of DTH and expressed in millimeter. Results are expressed as mean ± S.E.M. of footpad thickness up to 72h.

Humoral Immune Response

To assess the humoral immune response, blood was withdrawn from retro-orbital plexus of all SRBC antigenically challenged animals on day 0 (pre-immunized), 8th, 13th and 20th (post immunization). The serum was separated and assayed by direct haemagglutination[12]. Titer was described as the highest dilution capable of visible agglutination. The results were expressed as mean ± S.E.M. log titre of individual animals.

*Statistical Analysis*All the results were expressed as mean ± S.E.M. Data of tests were statistically analyzed using one-way ANOVA. The data were considered to be statistically significant if the probability had a value of 0.05 or less.

RESULTS

Characterization of CNP and DLCNP

Table 3: DTH response of LA 343, LA DNA, CNP and DLCNP.

Groups	0 hour	24 hour	48 hour	72 hour
Normal Control	1.63 ± 0.02	1.63 ± 0.02	1.63 ± 0.02	1.63 ± 0.02
Positive Control	1.66 ± 0.04	1.86 ± 0.04 [a,b]	1.99 ± 0.05 [a,b]	1.75 ±0.02
Immunized Control	1.65 ± 0.03	1.68 ± 0.03 [a]	1.75 ± 0.06 [a]	1.66 ± 0.02
Naked DNA (i.m)	1.62 ± 0.03	1.8 ± 0.04 [a,b]	2.05 ± 0.03 [a,b]	1.64 ± 0.03
Naked DNA (i.p)	1.61 ± 0.03	1.86 ± 0.04 [a,b]	2.14 ± 0.04 [a,b]	1.57 ± 0.02
DLCNP (i.p)	1.63 ± 0.02	1.91 ± 0.04 [a,b]	2.19 ± 0.02 [a,b]	1.7 ± 0.02
CNP (i.p)	1.6 ± 0.02	1.79 ± 0.03 [a,b]	1.92 ± 0.04 [a,b]	1.65 ± 0.02

The above data is represented as Mean ± S.E.M (n=6).
[a]p<0.05 in comparison to normal control, [b]p<0.05 in comparison to sensitized control

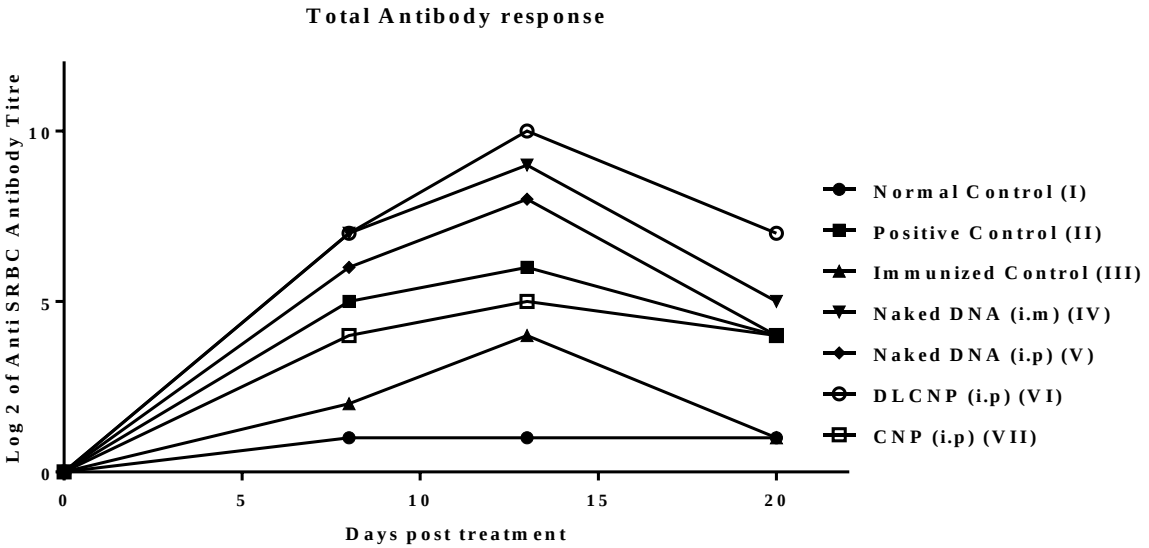

Figure 7: Effect of different groups on production of anti- SRBC antibody titre on pre-immunization(0^{th} day) and post-immunization (8^{th}, 13^{th} and 20^{th} day).

The conditions for the nanoparticle preparations were optimised by observing Nano-size and zeta potential, by light microscopic examination (i.e. SEM and TEM), as well as by agarose gel electrophoresis, Laoding effciency & Loading capacity and DNA release study under *in vitro* conditions.

Morphological examination

The morphological examination of the microspheres was performed by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). Figure: 1 (a to f) shows the observed results. Morphological examination showed that DNA loaded chitosan nanoparticles formed are spherical/oval shaped. SEM study indicate that naoparticles formed have a size ranging in between 350nm to 515nm, whereas TEM data revealed that size ranged in between 100-350nm.

Dynamic Light Scattering (DLS) study

Nano-size and Zeta potential of CNP at pH 4.5 was 342.7 nm and +17.2 mV respectively; whereas the size and zeta potential of DLCNP (prepared in acetic acid) was measured at three different pH- 3.5, 4.5, 5.5.

The presence as well as amount of DNA did not affect the zeta potential value. Nanoparticles had a positive surface charge at all pH values. Zeta potential first increased from +12.6 mV to +17.7 mV as the pH changed from 3.5 to 4.5 and further decreased to +9.11mV at pH 5.5, as depicted in figure 5.11. Size of nanoparticles decreased gradually with increase in pH, being 513.2nm, 353.4nm, 276.5nm at pH value of 3.5, 4.5, and 5.5 respectively of the preparing solution as shown in Figure 2(a) and (b).

Analysis of DNA-Chitosan complex

The optimised chitosan–DNA nanoparticles were analysed by agarose gel electrophoresis along with non-complexed DNA and supernatant (left after DLCNP formation) samples as controls.

Figure 3 clearly indicate that DNA was completely retained in the loading well after sonication of DLCNPs. Moreover, sonication of DLCNPs did not cause any shearing of DNA, as no trailing of DNA was observed in wells loaded with DLCNP in gel electrophoresis. This confirmed that the DNA was fully complexed into chitosan–DNA nanoparticles and supernatant loaded into the well, did not show any fluorescence, suggesting 100% complex forming efficiency, which could be further confirmed by loading capacity and efficiency.

Loading capacity and Loading efficiency

Loading efficiency and loading capacity are very significant parameters in preparation of chitosan/DNA nanoparticles. Loading capacity illustrates how strong is the capacity of chitosan for encapsulating DNA.

Loading capacity determined was 0.18% and loading efficiency was 97%.

In vitro DNA release study at physiological pH – 7.4 and at stomach acidic pH – 2.1

It was observed during *in vitro* DNA release experiment that DNA release occurs gradually for 3-4 days at pH 7.4 (physiological pH) as compared to the fast release of upto 90% in first 3 hours of incubation in acidic (stomach pH 2.1) conditions, as shown in Figure 4.

In vitro immunomodulatory potential of whole cell probiotic LA 343, LA DNA and DLCNP

In vitro testing of immunomodulatory activity of whole cell probiotic (LA 343), its isolated genomic DNA, DNA loaded onto Chitosan nanoparticles and vehicle (Chitosan Nanoparticles) was determined. NBT, iNOS and phagocytic activity is depicted in Table 1. Results indicated that all the test samples showed immune having immune enhancing potential. Isolated probiotic DNA was observed to be more immune active as compared to whole cell probiotic cells, whereas DLCNP had maximum immune enhancing potential. The results of NBT reduction observed for DLCNP treated cells was 71% as compared to 56% in LA DNA treated cells. DLCNP was 27% more efficient than in reducing NBT as comapred to LA DNA.

Similar pattern of results was observed for iNOS as well as for phagocytic activity, maximum activity being shown by DLCNP, followed by naked DNA treated cells and whole cell probiotic. DLCNP showed 20% and 22% more iNOS and phagocytic activity than LA DNA respectively. Figure 5 shows the graphical representation of NBT reduction, iNOS activity and phagocytic activity of all the test sample treated groups.

In vivo immunomodulatory potential of whole cell probiotic LA DNA and DLCNP

Cell mediated immune response

NBT reduction, iNOS and Bactericidal activity

Probiotic genomic DNA and DLCNP significantly ($p< 0.05$) increased NBT reduction as compared to immunized control group. Naked probiotic DNA administerd through i.m route showed NBT reduction of 88% which was higher than shown by DNA administered through i.p route (i.e. 75%). NBT reduction by increased by 4%, when probiotic genomic DNA was administered after loading onto chitosan nanoparticles as depicted in Table 2. However, among all the treated groups, DLCNP was showing maximum NBT reduction efficacy of 92.4%. Pattern of iNOS activity in various groups was similar to that observed in NBT reduction. DLCNP treated group showed maximum iNOS activity which was significantly higher than that observed in untreated normal control ($p< 0.001$). DLCNP was showing 3% higher activity than positive control and 4% higher iNOS activity than Naked DNA administered through i.m route.

Like other two parameters i.e. NBT and iNOS, the bactericidal activity against *E.coli* also followed the same trend as shown in Table 2 and Figure 6. The effect of probiotic DNA on bactericidal activity was studied in terms of decrease in number of colony forming units (CFU) of *E.coli* (1×10^6). The treatment of animals both with DLCNP and DNA treated group reduced the number of colonies and thus enhanced the bactericidal activity as compared to normal control animals. Naked DNA administered through i.m route showed 93.3% phagocytic activity, which was higher than the group in which DNA was administered through i.p route (88.5%). Maximum phagocytic potential was shown by DLCNP treated group (i.e. 95%).

Delayed Type Hypersensitivity assay

The effect of the isolated DNA and DNA loaded nanoparticles was assessed by the footpad swelling, a measure of Delayed type hypersensitivity as mentioned in Table 3.

The effect was compared with immunized control and with untreated normal control group. In normal control group, no rise in footpad thickness was observed. In DLCNP and LA DNA treated group significant elicitation of the T-cells response was evident by an increase in footpad thickness as compared to CNP and immunized control groups after 24 hours and 48 hours. It was found that DLCNP and LA DNA treated groups showed more rise in footpad thickness as compared to positive control group i.e. Levamisole treated after 48 hours. The maximum thickness of footpad was observed at 48 hours in the animals treated with DLCNP and LA DNA treated group respectively.

Humoral Immune Response

Anti SRBC- antibody titre by direct haemagglutination assay for each group was done on 0^{th}, 8^{th}, 13^{th} and 20^{th} day post immunization. No anti SRBC antibody-titre was observed on day 0. Higher titre was observed in the DLCNP treated group as compared to LA DNA and CNP treated group on all days of treatment (as seen in Figure 7). On 13^{th} day, the highest antibody-titre in various groups was 1:1024, 1:256, 1:32, 1:64, 1:16 in DLCNP, LA DNA, CNP treated, positive and immunized control groups respectively.

In positive control group i.e. Levamisole treated group, 1:64 titre was observed whereas in immunized control group titre was found to be 1:16 on 13^{th} day. On 20^{th} day DLCNP treated group showed an anti-SRBC antibody titre of 1:128 followed by 1:32 in LA DNA (i.m) and 1:16 in LA DNA (i.p), Positive control and CNP treated groups.

DISCUSSION

The side-effects of chemical based drugs such as dizziness, impotence, weakness and nausea[19] for many lifestyle diseases prevailing mnowadays like like metabolic diseases, asthma, heart diseases, inflammatory bowel disease, neurological diseases, rheumatoid arthritis, diabetes etc., as well as consumer awareness is leading the people towards alternative natural and safe therapies for treatment of diseases. Immunotherapy through probiotics[20] or medicinal plants[21,22] is one of the best natural ways of treating

diseases. The present work was conducted to find out the immunomodulatory efficacy of probiotic DNA loaded onto chitosan nanoparticles both *in vitro* and *in vivo*. Previous studies report probiotics as efficient immunomodulators. Moreover, probiotics have different components which increase the immune response of the host. The immune effects are exerted by live live bacteria[23] and inactivated or heat killed bacteria[24] or cell components such as peptidoglycan, lipoteichoic acid and DNA, Unmethylated CpG DNA motifs and short sequences of DNA also act as immunomodulatory[25,26]. Because of the GRAS status and beneficial effects of probiotics and their components, probiotic genomic DNA was selected in the present study. Further probiotic genomic DNA was loaded onto the chitosan nanoparticles and was subjected to various *in intro* and *in vivo* immunological tests to evaluate its immunomodulatory potential. The loading of DNA onto nanoparticles was carried to protect it from the nucleases degradation[5]. Different materials are available these days to make nanoparticles such as alginate, dextran, pullulan, etc[4]. The most compatible material to form DNA complex is chitosan because of its properties such as biocompatibility, non-toxicity, high mechanical strength and susceptibility to chemical modifications. Chitosan is a naturally existing polymer and is cost-effective[27].

SEM studies show that DNA loaded chitosan nanoparticles formed are spherical/oval shaped with a size ranging between 350nm to 515nm. The TEM images revealed that the complexes had well-formed spherical shape and compact structure. Moreover, the size of nanoparticles as depicted by TEM data was ranging in between 100 – 350nm. Study on nanoparticle shape's internalization efficiency showed that spherical particles are being internalized 500% more efficiently than the particles having rod-shaped structure of similar size[28]. The nanoparticles upto the size of 400nm are displayed to B-cells or T-cells (approx. 8µm in size), leading to release of cytokines and chemokines[29].

DLS study revealed that the DNA loaded Chitosan Nanoparticles had a Zeta potential of +17.7mV ± 5.07 and a size of 313nm ± 7.54. The net positive charge of the particles is needed to prevent the aggregation of particles and to promote electrostatic interaction with the cell membranes (being negatively charged). Particle size and charge play important roles in transferring the DNA into cells and they also have a greater impact on particle distribution in the body. The smaller sized particles can enter the cells through endocytosis and/or pinocytosis, which increases the transfection rate[30].

Sun et al.[11] suggested that at lower pH, most of the amino groups of chitosan are protonated, enabling the chitosan molecule with an extension confirmation due to strong charge repulsion. With increasing pH, the size gets reduced due to relieve in extended confirmation. The reason for initial increase in zeta potential at pH 4.5 and then decrease at pH 5.5 respectively, is not very clear so far. This process implies that the positively charged surface of the chitosan particles is shielded at pH 4.5, which can be a result of reorganization of the molecule structure[11]. Loading capacity and efficiency results are supported by the earlier studies done on loading of DNA onto nanoparticles showing 100% loading efficiency and 0.1- 0.8% loading capacity. More than 90% loading efficiency indicates best DNA-vehicle interaction[11]. Moreover, a higher concentration (approximately 0.2%) of chitosan, confer stability of nanoparticles inside body systems[7].

Before conducting the experiments for immune enhancing activity, the DNA release experiments were carried out *in vitro* to find out the most effcient route for DLCNP delivery in body. As DNA release is affected by pH, experiments were carried out at two differnet pH; i.e. physiological pH 7.4 (same as of peritoneum) and stomach pH 2.1. Results showed that DNA release occurs gradually for 3-4 days at physiological pH as compared to the fast release of upto 90% in first 3 hours of incubation in acidic conditions. Hence, i.p route was selected and followed for administration of DNA loaded chitosan nanoparticles in experimental animals. *In vitro* immunocharacterization results indicated that the DNA loaded chitosan nanoparticles showed maximum immune enhancing activity. Based on the results of *in vitro* experiments, the *in vivo* experiments were designed using Balb/c mice as experimental model to find out the immune efficacy of DLCNP. In *in vivo* experiments, various test groups alongwith suitable controls were planned and divided into seven groups. The results of nitroblue tetrazolium reduction (NBT) test, iNOS , phagocytic activity, DTH response and humoral immune response showed that the DLCNP was better immune stimulator than all the other groups. NBT is an indirect marker of the oxygen dependent bactericidal activity of phagocytes[31]. The NBT dye after phagocytosis is reduced to formazon, which is measured spectrophotometrically[32]. The functional ability of phagocytes was evident from increased expression of iNOS that oxidizes L-arginine to citrulline and nitric oxide. This activity is correlated to the phagocytic activity of macrophages and has been employed to measure the immunomodulatory potential[33,34].

The footpad thickness was observed maximum in the DLCNP treated group after 48 hours of treatment. DTH is a good measure of cell mediated immune response and is assessed by foot pad swelling assay which is an expression of T- cell response[35,36]. In humoral immune response, the SRBC mediated immune response is a highly sensitive indicator of integrity of immune system cells, which also requires the coordinated interaction of various cells and their products[37]. Hence, all the cell mediated and humoral immunological tests showed that DLCNP is better immunopotentiator than LA DNA.

The results indicate positive interaction between the probiotic DNA and the chitosan nanoparticles. The underlying mechanism behind formation of DNA-Chitosan complex is the DNA interaction with the

cationic polysaccharide, Chitosan. After complete binding of the DNA with the polycation ligand, the DNA-Chitosan complex crosses through the cell membrane of the host cell and enters endosome[38]. Chitosan nanoparticles exhibit proton sponge effect and swelling occurs due to the acidic pH inside the endosome[39], which are further released into cytoplasm. Through the cytoplasm the DNA-Chitosan complex enters into the nucleus through trafficking, leading to the production of the cytokines. The balance of Th1 & Th2 cytokines play a role in the activation of the various immune cells in enhancing the immunomodulatory potential for treating various diseases. The present study makes us to conclude that loading of DNA onto nanoparticles enhances its immune efficacy and can be applied as immunotherapeutic agent to manage/modify the immune related disorders.

REFERENCES

1. Krieg AM. Cp G motifs in Bacterial DNA and their immune effects. Annu Rev Immunol. 2002; 20: 709-760.
2. Randhawa MK, Bhatia A, Ali SA, Balgir PP. *In vivo* comparison of probiotic bacteria's with their DNA: As augmenter of immune efficacy. Adv Appl Sci Res. 2012; 3: 826-832.
3. Bhatia A, Kaur M, Kaur H. Comparison of Immunomodulatory Activity of Probiotic Bacteria and their DNA: A Study Conducted with *L. acidophilus* NCDC343 & *L. casei* Isolated from Yakult. Int J Pharm Res Scholars. 2015; 4:120-126.
4. Tiyaboonchai W. Chitosan Nanoparticles : A Promising System for Drug Delivery. Naresuan Univ J. 2003; 11: 51-66.
5. Rajalakshmi R, Indira MY, Aruna U, Vinesha V, Rupangada V, Krishna moorthy SB. Chitosan Nanoparticles - An Emerging Trend In Nanotechnology. Int J Drug Deliv. 2014; 6: 204-229.
6. Atashpaz S, Khani S, Barzegari A, Barar J, Vahed SZ, Azarbaijani R, Omidi YA. Robust Universal Method for Extraction of Genomic DNA from Bacterial Species. Microbiol. 2010; 79: 538–542.
7. Chew JL, Wolfowicz CB, Mao HQ, Leong KW, Chua KY. Chitosan nanoparticles containing plasmid DNA encoding house dust mite allergen, Der p 1 for oral vaccination in mice. Vaccine. 2003; 21: 2720–2729.
8. Silva VJDD. Preparation and characterization of chitosan nanoparticles for gene delivery, Technico Lisboa. 2013; 1-10.
9. Akbuga J, Turan SO, Erdogan N. Plasmid-DNA loaded chitosan microspheres for in vitro IL-2 expression. Eur J Pharm Biopharm. 2004; 58: 501–507.
10. Dastan T and Turan K. *In Vitro* characterization and delivery of Chitosan-DNA microparticles into mammalian cells. J Pharm Pharm Sci. 2004; 7: 205-214.
11. Sun Y, Zhang S, Peng X, Gong Z, Li X, Yuan Z, Li Y, Zhang D, Peng Z, Preparation, characterization and transfection efficacy of chitosan nanoparticles containing the intestinal trefoil factor gene. Mol Biol Rep. 2012; 39: 945–952.
12. Hudson L and Hay FC. In: Handbook of Practical Immunology, 3rd ed. Oxford: Blackwell Scientific Publications, London; 1989.
13. Stuehr DJ and Marletta MA. Synthesis of nitrite and nitrate in murine macrophages cell lines. Cancer Res. 1987; 47: 5590-5594.
14. Raghuramula N, Nair KM, Kalyansundharam S. In: A manual of laboratory techniques, NIN, ICMR, Silver prints, Hyderabad; 1983.
15. Millan CLB, Weerantna R, Krieg AM, Siegrist CA, Davis HL. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. Proc Natl Acad Sci. 1998; 95: 15553–15558.
16. Gao X and Huang L. Potentiation of cationic liposome-mediated gene delivery by polycations. Biochem. 1996; 35: 1027–1036.
17. Alsever JB and Ainsile RB. A new method for the preparation of dilute blood plasma and the operationof a complete transfusion service. NY State J Med. 1941; 41: 126-131.
18. Titus RG and Chiller JM. A simple and effective method to assess murine delayed type hypersensitivity to proteins. J Immunol methods. 1981; 45: 65-78.
19. Ryan G, Briscoe TA, Jobe L. Review of pramlintide as adjunctive therapy in treatment of type 1 and type 2 diabetes. Drug Des Devel Ther. 2009; 2: 203–214.
20. Nandakumar NS, Srinivasan P, Mohan KM, Jayakanthan K, Ramakrishna BS. Effect of Vibrio cholerae on chemokine gene expression in HT29 cells and its modulation by *Lactobacillus* GG. Scand J Immunol. 2009; 69: 181-7.
21. Bhatia A and Mishra T. Hypoglycemic activity of *Ziziphus mauritiana* aqueous ethanol seed extract in alloxan-induced diabetic mice. Pharma Biol. 2010; 48: 604-610.
22. Bhatia A, Anand M, Singla R, Sharma A. Antioxidant activity of native and micropropagated *Tylophora indica* leaves extract: A comparative study. J Nat Prod Plant Resour. 2013; 3: 1-7.
23. Ménard S, Laharie D, Asensio C, Vidal-Martinez T, Candalh C, Rullier A, Zerbib F, Mégraud F, Matysiak-Budnik T, Heyman M. *Bifidobacterium breve* and *Streptococcus thermophilus* secretion products enhance T helper 1 immune response and intestinal barrier in mice. Exp Biol Med. 2005; 230: 749-56.
24. Ou CC, Lin SL, Pasi JJ, Lin MY. Heat killed lactic acid bacteria enhance immunomodulatory potential by skewing the immune response towards Th1 polarization. J Food Sci. 2011; 76: 260-267.
25. Krieg AM., Motifs: the active ingredient in bacterial extracts. Nat. Med. 2003; 9: 831-835.
26. Zhong Y, Huang J, Tang W, Chen B, Cai W. Effects of probiotics, probiotic DNA and the CpG oligodeoxynucleotides on ovalbumin-sensitized Brown-Norway rats via TLR9/NF-κB pathway. FEMS Immunol Med Microbiol. 2012; 66: 71-82.

27. Kaur M, Sethi D, Bhatia A. Chitosan Nanoparticles: A Therapeutic Carrier for Delivery of DNA, siRNA and CpG-ODNs. Nanosci Nanotechnol Asia. 2016; 6: 92-104.
28. Verma A and Stellacci F. Effect of surface properties on nanoparticle-cell interactions. Small. 2010; 6: 12–21.
29. Smith DM, Simon JK, Baker JR. Applications of nanotechnology for Immunology. Nature Rev. Immunol. 2013; 13: 592–605 .
30. Oh N and Park JH. Endocytosis and exocytosis of nanoparticles in mammalian cells. Int J Nanomed. 2014; 9: 51–63.
31. Hellum KB. Nitroblue Tetrazolium test in bacterial and viral infections. Scand J Infect Dis. 1977; 9: 269-276.
32. Szczylik C, Gornas P, Carewicz R. NBT reduction test methods and practical use. Diagn Lab. 1979; 1: 35-40.
33. Park KM, Kim YS, Jeong TC, Joe CD, Shin HJ, Lee YH, Nam KY, Park JD. Nitric oxide is involved in the immunomodulating activities of acidic polysaccharide from *Panax ginseng*. Planta Med. 2001; 67: 122-126.
34. Frankova D and Zidek Z. IFN-gamma induced TNF-alpha is a prerequisite for in vitro production of nitric oxide generated in murine peritoneal macrophages by IFN-gamma. Eur J Immunol. 1998; 28: 838-843.
35. Mu HH and Sewell WA. Regulation of DTH and IgE responses by IL4 and IFN- gamma in immunized mice given pertussis toxin, Immunol. 1994; 83: 639-645.
36. Suvas S, Singh V, Sahdev S, Voha H, Agrewala JN. Distinct role of CD80 and CD86 in the regulation of the activation of B cell and B cell lymphoma. J Biol Chem. 2002; 8: 7766-7775.
37. Koganei A, Tsuchiya T, Samura K, Nishikib M. Use of whole sheep red blood cells in ELISA to assess immunosuppression *in vivo*. J Immunotoxicol. 2007; 4: 77-82.
38. Behrens I, Pena AIV, Alonso MJ, Kissel T. Comparative uptake studies of bioadhesive and non-bioadhesive nanoparticles in human intestinal cell lines and rats: the effect of mucus on particle adsorption and transport. Pharm Res. 2002; 19: 1185-1193.
39. Ishii T, Okahata Y, Sato T. Mechanism of cell transfection with plasmid/chitosan complexes. Biochem Biophys Acta. 2001; 15: 51-64.