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Chitosan nanoparticles as Carrier of Immunorestoratory plant extract: synthesis, characterization and Immunorestoratory efficacy

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Abstract

Various medicinal plants are rich in certain bioactive compounds that can help in restoration of immune system but these compounds may be unstable in gastro intestinal tract due to elevated pH and harsh conditions that render the biotherapeutic compound ineffective in GI tract. Various particulate systems such as nanoparticles had been used to improve the pharmacokinetic and pharmacodynamic properties of various drugs. In our present research we have used chitosan, a biodegradable polymer as carrier of plant extract (i.e Ethanolic extract of leaves of *Ziziphus mauritiana*) having immunomodulatory property and checked its immunorestorative efficacy in immunosuppressed host

It was found that the oral administration of leaf extract of plant loaded in chitosan nanoparticles is an efficient immunorestorer in Swiss albino mice whose immune system had been suppressed by giving intraperitoneal injection of hydrocortisone(10mg/Kg body weight).

The nanoparticles system enhances stability in harsh conditions in GI tract and may be a better vehicle in future for efficient drug delivery

Keywords: Chitosan, nanoparticles, immunorestoration, Ziziphus mauritiana, Hydrocortisone, Immunosuppression

Introduction

For the past few decades there is a considerable research interest in the area of drug delivery systems as carriers for small and large molecules. Nanotechnology has been increasingly employed in drug delivery as it increases the drug dissolution rate, leading to enhanced drug absorption and bioavailability. Polymeric nanoparticles (NPs), prepared via coprecipitation of drugs and polymers are promising drug delivery vehicles for treating diseases with improved efficacy and reduced toxicity [1]. Particulate systems are found to be very efficient in drug delivery. DNAzyme and si RNA is used in gene therapy by encapsulating it in chitosan nanoparticles [2]. Water soluble chitosan nanoparticles are also used as carrier of proteins [3]. Particulate system like nanoparticles [4,5] has been used as a physical approach to alter and improve the pharmacokinetic and pharmacodynamic properties of various drug molecules. There are many materials known to be used for preparation of nanoparticles like cyclodextrins, chitosan, ceramics etc. but all the materials cannot be employed in *in vivo* systems because of their toxicity. Formulation of doxorubicin-polylactide nanoconjugates is employed for cancer drug delivery [6]. Bioadhesive nanoparticles of fungal chitosan are used for oral DNA delivery [7].On the other hand plants have been reported to have medicinal values, but still a number of plants remain unexplored for their immunomodulatory

property. Moreover, there is hardly any report where a bioactive plant extract has been delivered in nanoparticles. In our lab previous studies revealed that leaf extract of *Ziziphus mauritiana* was immunomodulatory. The present study was conducted with the aim to find out whether the extract efficacy can be enhanced by its delivery through non toxic biodegradable chitosan nanoparticles.

Methods

Preparation of Extract

Aqueous: Ethanolic (1:1) extract of shade dried crushed leaves of *Ziziphus mauritiana* percolated in solvent and extract was filtered (whatmann paper), centrifuged (4000 rpm, 10 min) and concentrated under reduced pressure in a thin film evaporator at 50 ± 5 °C.

Preparation of chitosan nanoparticles [8]

Chitosan (0.17-0.18 % w/v) added to distilled water containing 2% acetic acid and 1.2 % Tween 80 . The solution was placed on a magnetic stirrer for 68- 72 h with an intermittent sonication every 24 h in a bath sonicator. Sodium Sulphate 17% (w/v) was added and stirred for 1 h on magnetic stirrer and sonicated again for 15 min . The nano sized particles were precipitated out. The size was found to be 395 nm.

Loading of nanoparticles with leaf extract

100 ml of nanoparticles solution was centrifuged at 5000 rpm for 15 min. The wet pellet (6mg/ml) was taken and suspended in normal saline containing 0.5 mg/ml of (protein) powdered extract. The suspension was stirred on a magnetic stirrer at 300 rpm - 400 rpm for 4 h and later centrifuged to get loaded nanoparticles and protein content of supernatant was determined before and after loading of nanoparticles with plant extract by Lowry method. The protein content of supernatant was found to be 0.1 mg/ml which was initially 0.5 mg/ml. Loading efficiency (LE) was determined using the formula

$$LE = \frac{\text{Initial protein content in suspension} - \text{Final protein content in suspension}}{\text{Initial protein content in solution}} \times 100$$

The loading efficiency was found to be 80%.

Animals

Swiss albino mice (18-22 gm) of either sex were maintained on standard laboratory diet (Kisan Feeds Ltd., Mumbai, India) and having free access to tap water were employed in the present study. They were housed in the departmental animal house and were exposed to 12 hr cycle of light and dark.

Induction of immunosuppression.

Immunosuppressed state was produced by giving 1 ml Hydrocortisone intraperitonially

Experimental setup

The animals of either sex were divided into six groups (n = 6) viz.:

- Group I, untreated control;
- Group II, Hydrocortisone (HC) treated; (**HC only)**
- Group III, Plant extract (PE) treated (0.3 mg/ml); (**HC+P.E$_{300}$**)
- Group IV, Plant extract treated (0.2 mg/ml); (**HC+P.E$_{200}$**)
- Group V, Loaded nanoparticles (LNP's) treated (3mg/day); **(HC+LNP's)**
- Group VI, Unloaded nanoparticles (ULNP's) treated (3mg/day) ; **(HC+ULNP's)**

Follow up: All the groups were treated intraperitoneally with hydrocortisone **(**1ml /day) on day 0 and day 5th, and the respective drug orally. All the groups were immunized with 1% BSA (200 µl) intraperitoneally on day 7th, 12th and 17th of hydrocortisone treatment. All the animals were treated with 30 µl of carregeenan (20 mg/kg body weight/animal) dissolved in normal saline into the planter side of left foot and same volume of sterile normal saline was injected into the planter side of right foot Thickness of the footpad with vernier caliper was measured on day 19th, 20th and 21st . The animals were sacrificed

on day 21st of the Hydrocortisone treatment and their spleen was excised in MEM for immunological assays. The numbers of cells were adjusted to 2X 10^6 viable cells/ml. The cells were employed to assess the immune status of the animals employing the various techniques: Nitro blue Tetrazolium Chloride (NBT) reduction test [9], Inducible Nitric Oxide Synthase (iNOS) test [10], Bactericidal activity [11] and Delayed type of hypersensivity (DTH) response.

Results and Discussion

Characterization of nanoparticles

Stability of nanoparticles in suspension is directly proportional to zeta potential. The zeta potential of nanoparticles was found to be 30mV. The observed positive zeta potential might be due to residual amine group which act as carrier of plant extract.

Immunorestoration efficacy of plant extract delivered as such and through nano delivery

Delayed type hypersensivity

The delayed type hypersensivity response was more pronounced in all the groups after 48 hours (Table 1). The maximum response was observed in the animals treated with LNP's.

The unloaded particles showed the lower response as compared to only extract treated groups showing that the activity observed was due to plant extract as LNP's showed the maximum activity.

Nitro Blue Tetrazolium test

The maximum NBT index observed in LNP's treated group as compared to all other groups (Table 2). The plant extract treated groups also showed the rise in activity in a dose dependent manner. LNP's treatment increased the bioactivity by 2.3 folds as compared to PE 0.3 mg/ml group.

Inducible Nitric Oxide Synthase (INOs)

The results of INOS activity were parallel to those of NBT. The inducible nitric oxide synthase activity was highest in *Ziziphus mauritiana* leaf extract loaded chitosan nanoparticles group as compared to other groups (Table 2). This shows that leaf extract loaded on nanoparticles has better bioavailability as compared to when only plant extract is taken directly through oral route.

Bactericidal activity:

The bactericidal activity also followed the same trend. The LNP's showed the maximum activity as compared to all other groups (Table 2).

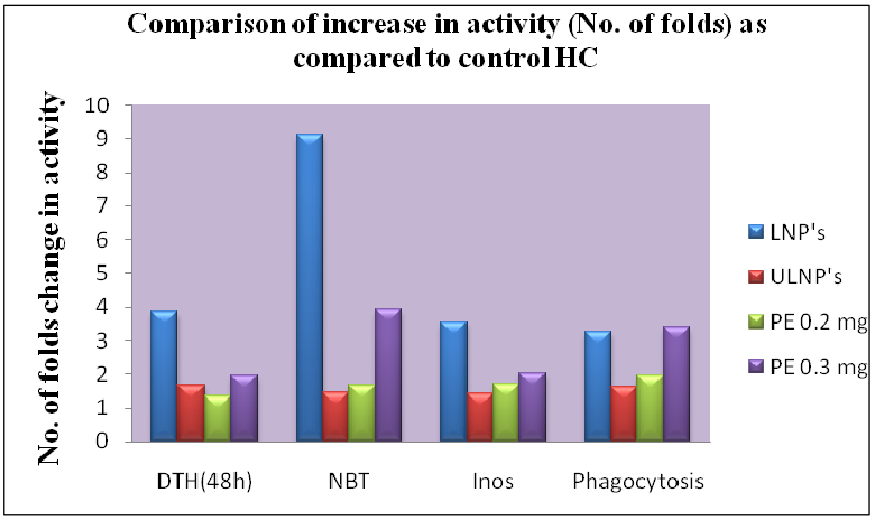

Figure 1: Comparison of increase in activity (number of folds) as compared to only HC treated control. DTH- Delayed type hypersensivity, NBT –Nitro Blue Tetrazolium,INOs- Inducible Nitric Oxide Synthase, PE – Plant Extract

Table 1: DTH response of different group of mice after 24h and 48h

Groups	Average footpad thickness after 24h (in mm)		Average footpad thickness after 48h (in mm)	
	Test	Control	Test	Control
(HC+P.E$_{200}$)	1.69↑	1.35	2.01↑	1.39
UNLP's +HC	2.04↑	1.68	2.36↑	1.61
(HC+P.E$_{300}$)	1.89↑	1.39	2.38↑	1.49
LNP's +HC	2.41↑↑↑	1.22	2.90↑↑↑	1.16
HC only	1.48↑	1.30	2.67↑	1.22

Table 2: Effect of test material on Immune response

Activity	HC	HC+ULNP's	HC+P.E$_1$	HC+P.E$_2$	HC+LNP's
NBT index	17.28	25.53 (↑1.47)	29.09 (↑1.67)	58.29 (↑3.95)	68.08 (↑9.14)
iNOS	-	30.76 (↑1.44)	41.93 (↑1.72)	50.86 (↑2.03)	71.87 (↑3.55)
Bactericidal	16.38	29.91 (↑1.62)	32.19 (↑1.96)	55.98 (↑3.41)	53.27 (↑3.25)

Legend: HC-only hydrocortisone treated**; HC+ULNP's-** hydrocortisone and unloaded nanoparticles treated**; HC+P.E$_1$-** hydrocortisone and 0.2 mg/ml plant extract treated**; HC+P.E$_2$-** hydrocortisone and 0.3 mg/ml plant extract treated**; HC+LNP's-** hydrocortisone and plant extract(300ppm) loaded nanoparticles treated**; (↑)** Number of times increase in activity as compared to only hydrocortisone treated group

The possible reason for this potentiation of bioactivity of extract by delivery through nanaoparticles might be due to the fact that when given as such (unloaded) the extract might get degraded or digested by the gastro intestinal juices or enzymes etc. Nanoparticles are stable under elevated pH range [12] of gut and might not get degraded by the digestive enzymes or it may be due to the reason that there residence time in the gut is very less that is why they did not give time to intestinal juices to act upon. The Immunorestoratory activity of loaded nanoparticles was found to be higher as compared to plant extract, showing that nanoparticles can be the better vehicles for localized drug delivery. The higher immunomodulatory efficacy may be due to control release of the plant extract locally. In short, the high diffusibility and better stability accounts for their overall immunomodulatory and Immunorestoratory efficacy.

Effective oral drug administration is desirable but challenging owing to the nature of gastro intestinal tract. The highly acidic pH in the stomach, the enzymes and mucus in the gastro intestinal tract are complicating factors for the effective particle delivery [11]. The recent development in the field of pharmacy and medicine reveals that drugs / bacteria delivered in nanoparticles show better activity in the body but none of the reports in literature shows the delivery of plant extract in nanoparticles system. It is for the first time that we have employed

plant extract loaded nanoparticles as therapeutic agents in animals.

In the present study, attempts were made to entrap leaf extract from a common medicinal plant *Ziziphus mauritiana* in nanoparticles made of chitosan, a biodegradable and biocompatible polymer and in vivo studies were conducted to see the Immunorestoratory efficacy of Ziziphus mauritiana leaf extract loaded nanoparticles in immunosuppressed mice**.**

Delayed type of hypersensivity, Nitro blue tetrazolium reduction and Inducible nitric acid synthase tests were used as parameters to study the immune status of host. Results revealed that immunological response was highly pronounced when plant extract was given loaded on nanoparticles as compared to plant extract alone.

Conclusion

Delivery of plant extract through nanoparticles as a vehicle is a better way to improve the efficacy of result in expression of bioactivity of plant extract as an immunomodulator as well as an immunorestorer.

Competing interests: No competing Interests as declared by authors

Authors Contribution: AB designed the experiment. Experimentation was done by PS. PS did manuscript drafting and carried out extraction of plant extract of Ziziphus mauritiana by the method given by TM and formulated nanoparticles by the method given by DC and characterization of nanoparticles such as particle Size ,zeta potential is also done by PS. AB directed the Study and drafting of manuscript by many revisions.

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