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SYNTHESIS AND CHARACTERIZATION OF MURVA ENCAPSULATED CHITOSAN NANOPARTICLES FOR THE TREATMENT OF DIABETES MELLITUS

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ABSTRACT

Design and development of herbal novel drug delivery system has become a frontier research in the novel drug delivery system arena. Murva is a potent phytomolecule obtained from *Maerua oblongifolia* of family capparaceae, has been traditionally used to cure various diseases. The limitations of herbal drugs are poor biodistribution and instability. In order to improve the therapeutic efficacy and stability, the herbal drug Murva was encapsulated in chitosan nanoparticles. Chitosan nanoparticles containing Murva (CNP1-CNP5) were synthesized by ionic gelation of chitosan with tripolyphosphate anions (TPP) resulting in particles size smaller than 650nm. The encapsulation efficiency of nanoformulations was over 41.5%. The nanoformulations exhibited slow and sustained *in vitro* release over 99% of drug from the Murva encapsulated chitosan nanoparticles after 24 hours. The synthesized nanoformulations were found to be stable, biocompatible and produced an enhancement of anti-diabetic activity.

KEYWORDS: Chitosan, Tripolyphosphate, Nanoparticles, Murva.

INTRODUCTION

Natural polymers (chitosan, dextran, gelatine, alginate, agar, etc) play an important role in biomedicine with applications in tissue engineering, regenerative medicine, drug-delivery systems and biosensors due to their unique properties such as non toxic, biocompatible and biodegradable properties.[1] Chitosan is a more versatile form of polysaccharide, derived from naturally occurring chitin. It is the second most abundant natural polymer on earth after cellulose. Its unique properties make it attractive for many industrial and biomedical applications (including controlled drug release, wound healing, nutrition supplements, water purification, removal of toxins, scaffolds for tissue engineering, and semipermeable membranes). An important application of chitosan is the development of drug delivery systems with a regulated drug release rate, enhancement of therapeutic effect, targeted drug delivery and reduced frequency of administration of the drug[2,3] due to its gel-forming ability in low pH range.[4] The hydrogen bonding and ionic interactions are responsible for the adhesive properties of chitosan and different substrates.[5]

The ionotropic gelation technique, is the most important technique for ionic cross-linking of chitosan with low molecular weight counterions, hydrophobic counterions and high molecular weight ions (e.g. sodium tripolyphosphate (TPP).[6,9] Liang *et al.*, (2011)[10] prepared chitosan nanoparticles loaded with tea polyphenol extract by this method. The particles have been proved to be good nanosystems for slow release, the polyphenolic material being actively maintained. A comparative analysis about the encapsulation of yerba mate extract was done by Harris *et al.*, (2011)[11] who analysed chitosan nanoparticles (ionic gelation) and microspheres prepared by spray-drying.

Murva (*Maerua oblongifolia*) is an important controversial drug used in various diseases like anaemia; fever; diabetes; stomach disorders; typhoid; urinary infection and cough.[12] In general, the herbal drugs lack in long term stability, this deficiency can be avoided by encapsulation methods described in the literature.[13] To increase the stability and to assess the enhancement of anti-diabetic activity of Murva in the form of nanoparticles (Murva encapsulated chitosan nanoparticles), this study was aimed to synthesize Chitosan nanoparticles loaded with Murva to improve the stability and anti-diabetic activity by evaluating their drug content, entrapment efficiency, particle size and *in-vitro* drug release.

MATERIALS AND METHODS

Materials

Chitosan, Glacial acetic acid, sodium tripolyphosphate were purchased from Otto Kemi, Mumbai. All the other reagents used in the experiments were of analytical grade.

Methods

Preparation of Murva encapsulated chitosan nanoparticles by ionic gelation method

Murva encapsulated nanoparticles were prepared by ionic gelation method as reported by Calvo. *et. al.*,(1997)[14] with slight modification. Briefly, chitosan solution was prepared by dissolving various concentrations of chitosan (0.1-0.5%) in glacial acetic acid. 10mg of drug was added to 0.1% TPP solution. This solution was stirred for 1500rpm for 30min on ultrasonicator (vibronics) and TPP solution was added drop wise and kept stirring for 3 hours on homogenizer. Nanoparticles were obtained upon the addition of a TPP aqueous solution to a chitosan solution. The NP suspension was then centrifuged at 15,000 rpm for 10 min using high- speed centrifuge (Sigma). The formation of nanoparticles results in interaction between the negative groups of TPP and the positively charged amino groups of chitosan.[15]

Table 1: Formula for preparation of Chitosan nanoparticles.

S.No	Formulation Code	Drug (mg)	Chitosan (%)	Tripolyphosphate solution (ml)	0.1% Acetic acid solution (ml)
1	CNP1	10	0.1	100	100
2	CNP2	10	0.2	100	100
3	CNP3	10	0.3	100	100
4	CNP4	10	0.4	100	100
5	CNP5	10	0.5	100	100

Drug Content

The drug content in each formulation was determined by weighing nanoparticles equivalent to 10mg of drug and dissolving in 100 ml of 7.4 pH phosphate buffer, followed by stirring. The solution was filtered through a 0.45µ membrane filter, diluted suitably and the absorbance of resultant solution was measured spectrophotometrically at 366 nm using 7.4 pH phosphate buffer as blank. The drug content of the prepared nanoparticles was determined by the formula:

$$\text{Drug content} = \frac{\text{Weight of the drug in Nanoparticles (mg)}}{\text{Weight of Nanoparticles (mg)}} \times 100$$

The results were given in results and discussion section.

Drug Entrapment Efficiency

The entrapment efficiency is also known as Association Efficiency. The drug loaded nanoparticles are centrifuged at a high speed of 3500-4000 rpm for 30 min and the supernatant is assayed for non-bound drug concentration by UV spectrophotometer.[16] The percentage Drug Entrapment Efficiency was calculated as follows:

$$\text{Encapsulation efficiency} = \frac{\text{Entrapped drug (mg)}}{\text{Total amount of drug added (mg)}} \times 100$$

Particle Size analysis and zeta-potential

The particle size analysis and zeta-potential measurement were analyzed by Zeta sizer Nano ZS (Malvern Instruments, UK). For the analysis, the nanoparticles sample of the desired concentration was flushed through a folded capillary cell (DTS1060) and the measurement was carried out on the second filling; a sufficient sample volume was used to completely cover the electrodes of the cell. The sample was injected slowly and analysis was carried out if there were no visible air bubble inclusions present. After inspection, the cell was placed into the Zetasizer and equilibrated at for 2 min prior to the particle size measurements.

***In-vitro* Release Studies**

Dissolution studies were carried out by using USP dissolution test apparatus. Capsule filled with nanoparticles equivalent to 10 mg of drug was placed in dissolution media in dissolution apparatus. In order to simulate the pH changes along the GI tract, three dissolution media with pH 1.2, 7.4 and 6.8 were sequentially used referred to as sequential pH change method. When performing experiments, the pH 1.2 medium was first used for 2 hours (since the average gastric emptying time is 2 hrs.), then removed and the fresh pH 7.4 phosphate buffer saline was added. After 3 hours (average small intestinal transit time is 3 hrs.), then the medium was removed and colonic fluid pH 6.8 buffer was added for subsequent hours. 900ml of the dissolution medium was used at each time.

Rotation speed was 100 rpm and temperature was maintained at 37±0.5oC. 5 ml of dissolution media was withdrawn at predetermined time intervals and fresh dissolution media was replaced. The samples were withdrawn at specified intervals and analyzed at 366 nm by UV absorption spectroscopy and the cumulative percentage release was calculated over the sampling times.

Accelerated stability study

The nanoparticles from the selected and optimized batch was studied for stability and kept under the accelerated conditions of temperature and moisture (humidity) for the period of six months. This nanoparticles stability was

studied at three different temperature conditions, i.e 2-8, 25 and 40°C for 6 months. Every sample separately weighed and enclosed by aluminium foils and sealed. The samples were kept in specified conditions at humidity chamber for six months. The samples were collected at specific time intervals and measured for the amount of drug release using UV-Spectrophotometer at 365nm.

Anti-diabetic activity studies

Male wistar strain rats with normal blood sugar level weighing between 120-180g were used for investigation. The animals were injected with alloxan monohydrate dissolved in distilled water at a dose of 150mg/kg body weight intraperitoneally to induce diabetes. After three days of injecting alloxan monohydrate, diabetes was confirmed by testing blood glucose sugar level using Abbott glucometer. The animals with blood glucose level more than 200mg/dl were selected for evaluation of anti-diabetic activity.

RESULT AND DISCUSSION

Chitosan Nanoparticles – Formulation and Evaluation

Chitosan Nanoparticles were successfully prepared and evaluated. All the formulations were white in colour. The particles obtained were of smooth and free flowing. Chitosan nanoparticles were prepared by ionic gelation technique. The chitosan nanoparticles were prepared based on the ionic interaction of a positively charged chitosan solution and negatively charged TPP solution. The charge density of both chitosan and TPP solution has a great effect on the ionic interaction. No visible impurity was seen the prepared chitosan nanoparticles.

Drug Content and Entrapment Efficiency of Chitosan Nanoparticles

The drug content of the chitosan nanoparticles varied from 86.3±0.6% to 88.1±1.2%. The drug content was decreased with increase in chitosan concentration. This may be due to loss of drug during manufacturing stage or increase in entrapment efficiency, so that drug is not available for estimation.

The entrapment efficiency of chitosan nanoparticles increased with increase in polymer concentration. The entrapment efficiencies were found to be minimum and maximum of 41.5±2.6% and 87.3±0.8% respectively. From the drug content and entrapment efficiency results, the formulation CNP5 was considered as an optimum trial. The results were given in the table 2.

Table 2: Drug content and Entrapment efficiency of chitosan nanoparticles.

S.No	Formulation Code	Drug content (%)	Entrapment efficiency (%)
1	CNP1	88.1±1.2	41.5±2.6
2	CNP2	87.9±2.4	57.5±2.4
3	CNP3	87.5±2.6	64.7±3.7
4	CNP4	86.9±3.2	75.6±0.8
5	CNP5	86.3±0.6	87.3±0.8

n=3 mean±SD

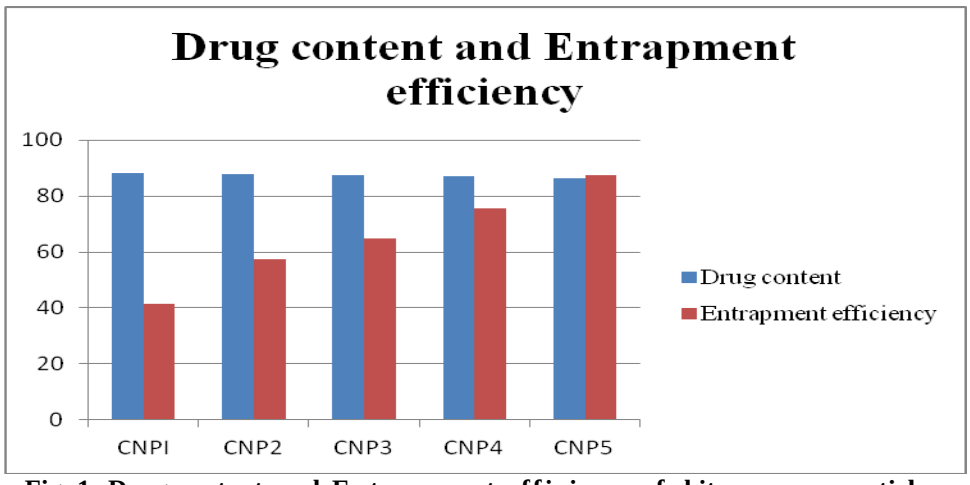

Fig. 1: Drug content and Entrapment efficiencyof chitosan nanoparticles.

Particle Size of Chitosan Nanoparticles

The particle size of chitosan nanoparticles varied from 360±12nm to 622±42nm. The mean particle size of chitosan nanoparticles was reduced from CNP1 (622±42nm) to CNP5 (360±12nm) with increase in polymer concentration. This may be due to avoidance of aggregation of drug particles. The particle size distribution of the CNP5 formulation was given in the Fig 2 and the particle size of chitosan nanoparticles (CNP1-CNP5) were given in the table 3.

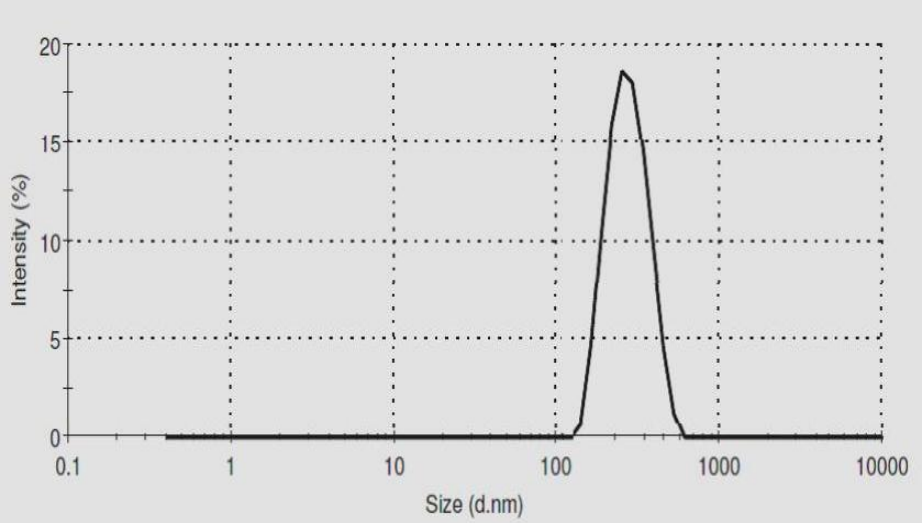

Fig. 2: Particle size distribution of Chitosan nanoparticles (CNP5).

Table 3: Particle Size of chitosan nanoparticles.

S.No	Formulation Code	Particle Size (nm)
1	CNP1	622±42
2	CNP2	604±36
3	CNP3	582±42
4	CNP4	460±42
5	CNP5	360 ±12

n=3 mean±SD

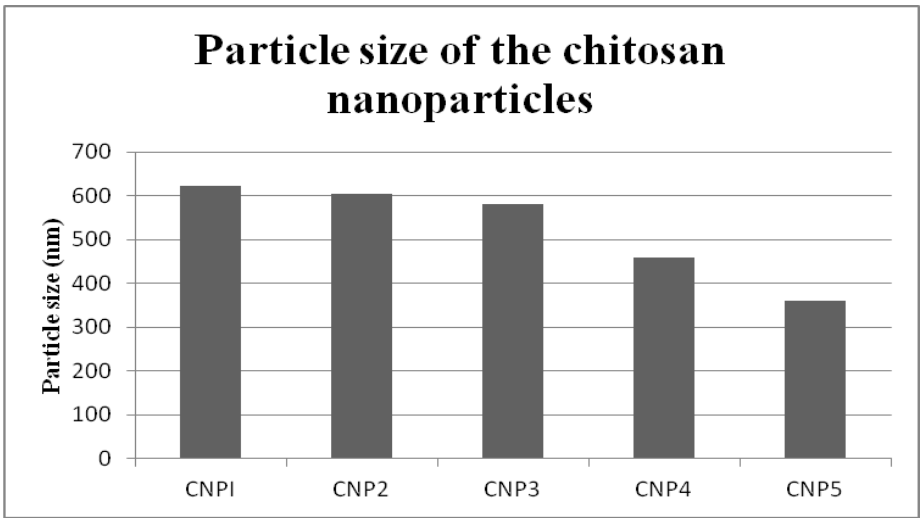

Fig. 3: Particle Size of Chitosan Nanoparticles.

Zeta potential of Chitosan Nanoparticles

The zeta potential values of chitosan nanoparticles were in positive and increased from 11.2±1.2mV to 18.3±0.2mV. The positive value was due to the polar nature (NH_3 group) of chitosan. The CNP5 trial held a value of 18.3±0.2mV. There was significant increase in zeta potential value from CNP1 to CNP5. Hence from these studies, formulation CNP5 (18.3±0.2mV) was considered as optimum trial. The zeta potential of the CNP5 formulation was given in the Fig 4 and the zeta potential of chitosan nanoparticles (CNP1-CNP5) were enlisted in the table 4.

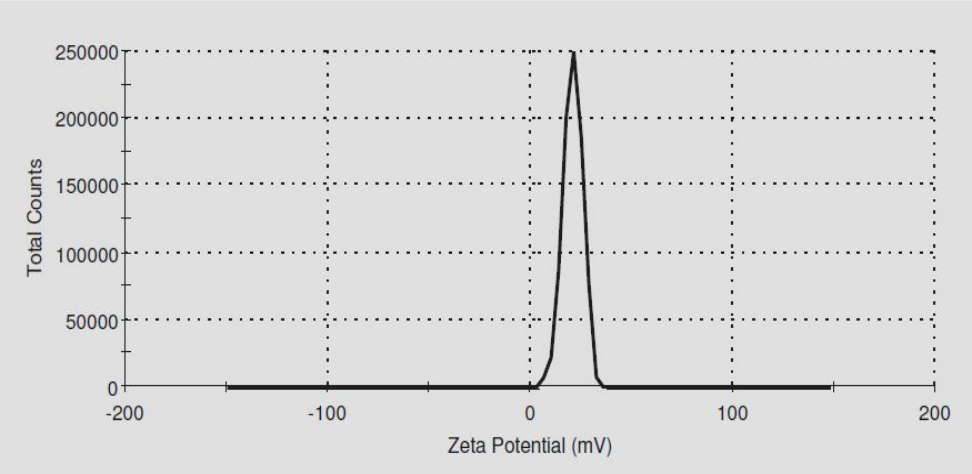

Fig. 4: Zeta potential of Chitosan nanoparticles (CNP5).

Table 4: Zeta potential of chitosan nanoparticles (CNP1-CNP10).

S.No	Formulation Code	Zeta potential(mV)
1	CNP1	11.2±1.2
2	CNP2	12.2±1.3
3	CNP3	13.4±1.4
4	CNP4	14.3±0.6
5	CNP5	18.3±0.2

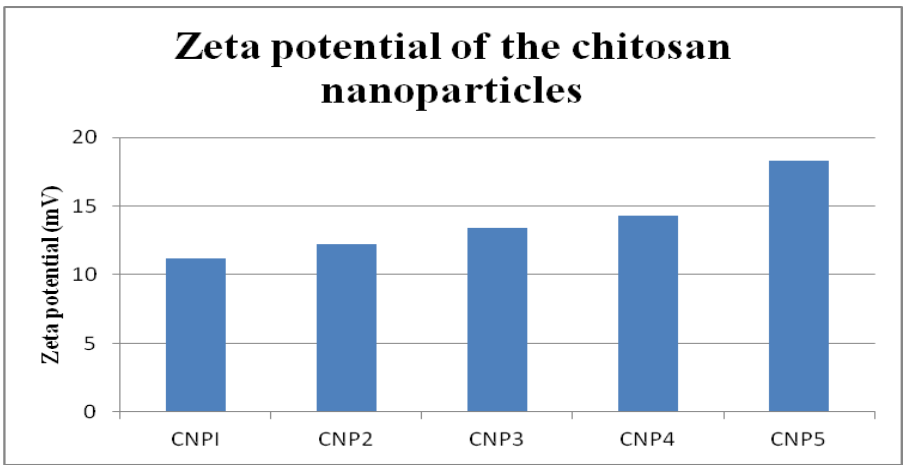

Fig. 5. Zeta Potential of Chitosan Nanoparticles.

***In-Vitro* Release Studies**

The prepared formulations (CNP1–CNP5) were subjected to *in-vitro* release studies. Absolutely, there was no drug release in simulated gastric fluid (acidic pH 1.2) for initial 2 hours. The drug release was found in simulated intestinal fluid (pH 7.4 phosphate buffer) and in colonic medium (pH 6.8 phosphate buffer).

In-vitro release profiles in intestinal/colonic medium were found to have very good controlled efficacy. During dissolution study it was found that, the drug release depends upon the nature of the polymer matrix as well as the pH of the media. In common increase in polymer concentration produced much more time for release of drug for all formulations. The drug release was decreased and sustained with increase in polymer concentration.

Chitosan nanoparticles prepared with 0.5% chitosan (CNP5) showed controlled and sustained drug release for a period of 24 hr. The percentage cumulative drug release of CNP5 at the end of 24 hr was found to be 99.74±0.26%. The results were given in the table 5.

Table 5: In vitro release of chitosan nanoparticles (CNP1 to CNP5).

Time (hrs) (in hr)	% Cumulative drug release				
	CNP1	CNP 2	CNP 3	CNP 4	CNP 5
0	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	0
4	45.83±0.85	33.26±0.17	29.13±0.28	28.58±0.36	17.17±0.22
6	83.77±0.64	68.69±0.34	58.88±0.42	57.82±0.24	35.54±0.09
8	97.36±0.52	90.24±0.49	85.79±0.31	83.37±0.32	70.35±0.35
12	98.33±0.36	97.27±0.67	95.23±0.36	95.58±0.45	85.39±0.27
16	99.14±0.25	99.22±0.71	99.48±0.25	98.15±0.58	91.47±0.46
20	-	-	-	99.24±0.12	95.81±0.38
24	-	-	-	-	99.74±0.26

n = 3; Mean ± S.D

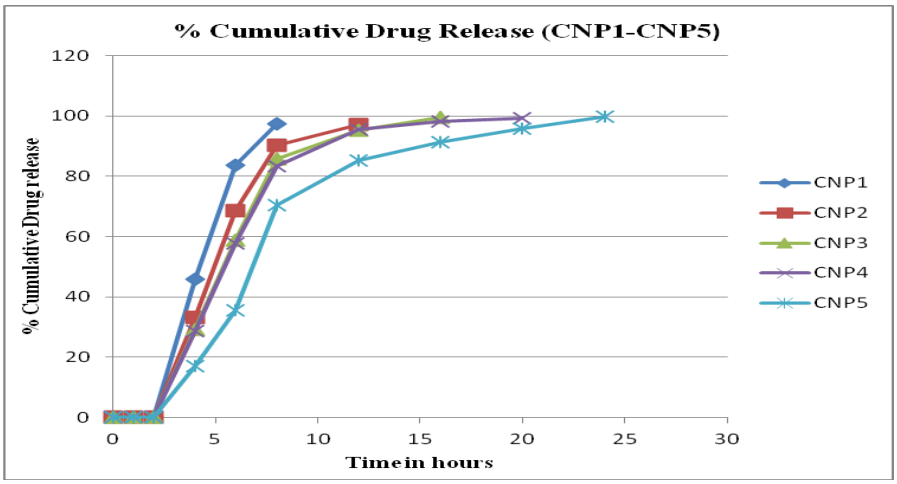

Fig. 6: In vitro release of chitosan nanoparticles (CNP1 to CNP5).

Stability studies

The optimized Chitosan nanoparticles (CNP5) formulation was subjected to stability studies as per ICH guidelines. The samples were kept at three different temperature conditions, i.e 2-8, 25 and 40°C for 6 months. Samples were withdrawn at predetermined time intervals of 0, 1, 2, 3 and 6 months and then evaluated for the drug release. The result showed that there was no considerable changes in the release rate, however minor changes observed in the drug release at elevated temperature conditions. This suggests that the formulations were stable under normal conditions. However the ideal storage of the nanoparticles formulation at temperatures of 25°C or less may be more suitable for stability purposes. Further, none of the nanoparticles formulations indicated any symptoms of agglomeration or colour change during the period of assessment.

From the results it was concluded that the Chitosan nanoparticles were found to be highly stable for a period of 6 months.

Table 6: In vitro release of chitosan nanoparticles CNP5 – Stability studies.

Trials/ Time (hrs) / in months	In vitro release of chitosan nanoparticles-CNP5 formulation				
	0	1	2	3	6
0	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	0
4	17.17±0.22	17.15±0.62	17.14±0.22	17.12±0.20	17.01±0.24
6	35.54±0.09	35.12±0.39	34.76±0.15	34.06±0.40	33.96±0.11
8	70.35±0.35	70.06±0.33	69.89±0.12	69.58±0.32	69.03±0.34
12	85.39±0.27	85.17±0.02	85.06±0.34	84.98±0.25	84.53±0.18
16	91.47±0.46	91.23±0.33	91.11±0.05	90.85±0.37	90.35±0.09
20	95.81±0.38	95.56±0.02	95.37±0.18	95.24±0.54	95.08±0.28
24	99.74±0.26	99.40±0.22	99.24±0.13	99.10±0.07	98.87±0.57

n = 3; Mean ± S.D

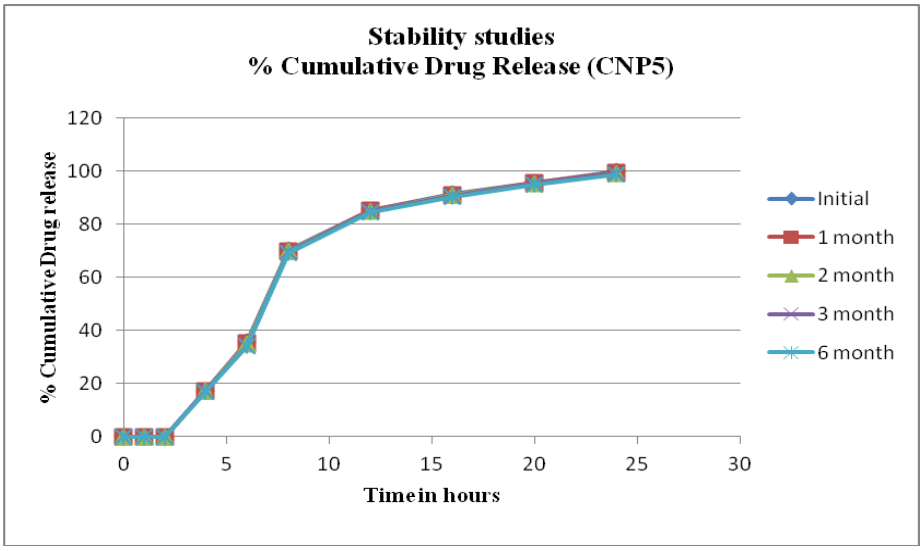

Fig. 7: Invitro release profile of CNP5 (0,1,2,3 and 6 months).

Anti-diabetic activity

Alloxan induced model

Diabetes is a chronic metabolic disorder characterized by altered carbohydrates, fat and protein metabolism, and an increased risk of vascular complications.

Glibenclamide (10mg/kg) showed significant hypoglycemic effect as compared with control group after oral load of glucose in mice. The leaves extract (800mg/kg) exhibits significant reduction in plasma glucose as compared to control. The chitosan nanoparticles of Murva (20mg/kg) produces significant anti-diabetic effect when compared with both glibenclimide and leaves extract. Continued administration of chitosan nanoparticles of Murva (20mg/kg), leaves extract (800mg/kg) and glibenclamide for 7 days in different group of diabetic mice produced significant reduction of plasma glucose level.

Table 7: Anti-diabetic activity in animal model.

Group		Blood glucose level mg/dl (mean ± SE)			
		Base	Day after treatment		
			1	2	3
I	Control	102.43 ± 5.34	105.91± 3.67	103.43 ± 8.12	106.92 ± 6.52
II	Diabetic	202.02 ± 10.89	204.74 ± 7.34	205.45 ± 8.67	208.76 ± 8.57
III	Diabetic + Glibenclamide (20mg/kg)	205.23 ± 12.54	189.70±11.21	181.54 ± 9.54	174.59± 11.37
IV	Diabetic + Test extract 800mg/kg)	203.48 ± 8.46	188.75 ± 7.72	170.49 ±13.94	165.54 ± 12.63
V	Diabetic + Chitosan Nanoparticles (20mg/kg)	192.48 ± 6.68	175.75 ± 5.23	155.49 ±41.38	125.54 ± 26.39

SUMMARY AND CONCLUSION

In the present work, the study was carried out with a view to increase the stability and anti-diabetic effect of murva and also to extend the release rate of murva as in the form of nanoparticles in order to prolong its action and also to achieve desire concentration of drug in blood or tissue which is therapeutically effective and nontoxic for extended period of time.

Chitosan nanoparticles were successfully prepared by ionic gelation method using TPP as a crosslinking agent by varying the concentration of Chitosan. Based on the results of drug content (86.3±0.6%), entrapment efficiency (87.3±0.8%), particle size (360±12 nm), surface charge (18.3±0.2 mV) and *in-vitro* drug release (99.74±0.26%); the formulation CNP5 was selected as an optimized formulation. The formulation CNP5 was further analyzed for stability studies and animal study. The *in-vitro* drug release study showed that the release of the drug from the formulation CNP5 was more sustained release when compared with other formulations. All other formulations (CNP1-CNP4) showed quick release of drug. Stability studies conducted for 6 months at 40ºC ± 2ºC / 75% ± 5% RH confirmed that all the nanoparticles preparations were stable. From the animal study, it was evident that the formulation CNP5 has enhanced anti-diabetic activity than the herbal extract. Hence it can be concluded that the newly developed formulation- nanoparticulate drug delivery system of murva with Chitosan is considered to be potential and effective agent in the management of diabetes mellitus and its related disease conditions.

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