Chapter-6
Summary and Conclusion
Chitosan is a linear copolymer of N-acetylglucosamine (GLcNAc) and D-glucosamine (GlcN) residues linked via β (1-4) glycosidic bonds. It is derived by deacetylation of naturally occurring biopolymer chitin, while chitin remained as unutilized natural resource for a long time primarily due to its inertness. Interest in chitosan has increased in recent decade due to its better chemical and biological reactivity. Chitosan composed predominantly of glucosamine unites with free amino groups on their second carbon which makes it natural cationic biopolymer. Natural cationic biopolymer is less abundant than anionic polymer; therefore, chitosan attracts attention for application in various fields.

Chitosan finds its applications in vast diverse fields, ranging from food, cosmetics, agriculture, textile, paper and pulp industry, waste water treatment applications etc. Chitosan, being a cationic biopolymer, is favorable material in biomedical and pharmaceutical applications due to its low toxicity, biodegradability and biocompatibility properties. It has been explored in conventional pharmaceutical devices as a potential formulation excipient and in novel drug delivery systems for protein, peptide and vaccine delivery. Chitosan is also investigated as wound healing material, artificial skin and kidney membrane, hypocholesterolemic agent, antimicrobial applications and more.
For biomedical and pharmaceutical applications, chitosan is required to adhere to high quality, purity and well characterization of physicochemical properties.

Commercially available chitosan, at present, is prepared from crustacean shell waste primarily due to low cost availability of large quantity of byproduct generated by sea food processing industries. As per current processing conditions, chitin is first extracted from crab and shrimp shell waste by demineralization, depolymerization and decolorization using harsh acid-alkali treatments and chemical bleaching processes. Chitosan from crustacean chitin are further prepared by deacetylation process by using highly concentrated alkali treatments at elevated temperatures.

The commercially available chitosan from crustacean source have none to limited applicability as a biotechnological material for usage in pharmaceutical and biomedical fields due to their low consistency in quality and purity. Chitosan available at present are not always well characterized, heterogeneous in nature with respect to their physicochemical properties. In addition, chitosan extracted from crustacean sources contains protein impurities which can cause allergic reactions to the individuals allergic to crustacean proteins.

Therefore there is an immense demand of high quality pure chitosan having constant physicochemical properties with well characterization to fulfill the requirements of biomedical and pharmaceutical industries.
In this context, the research work undertaken for the present study is comprehensive, encompasses all the aspects, from isolation of chitosan from fungal source after studying fungal species for optimum chitosan yield, preparation of microspheres for mucosal drug delivery of hydrophobic drug tetrahydrocurcumin and investigation for their protective effect against systemic toxicity of sulfur mustard in mice model.

The specific objectives of the study were as under:

1. Production, purification and characterization of chitosan from five fungal cultures belonging to the class: Zygomycetes for optimum chitosan yield.

2. Optimization of culture conditions of highest chitosan yielding fungal strain with respect to culture media, pH, temperature and agitation for maximizing chitosan production.

3. Preparation of drug tetrahydrocurcumin loaded fungal chitosan microspheres.


5. Evaluation of THC-loaded chitosan microspheres for their protective effect against systemic toxicity of sulfur mustard (as alkylating agent) in mice model.
The chitosan production from *Cunninghamella elegans* (MBT 2186), *Rhizopus oryzae* (MBT 594), *Mucor baineri* (MBT 3951), *Absidia Psudocylindrispora* (MBT 3365) and *Absidia corula* (MBT 601) ranged from 4.90% to 10.5%, and the highest chitosan yield was obtained in their exponential growth phase. In primary screening of fungal cultures, *Mucor baineri* (MBT 3951) showed the highest chitosan production potential. The degree of deacetylation of fungal chitosan ranged from 87% to 90% relatively higher than commercial shrimp chitosan and due to their high degree of deacetylation, fungal chitosan demonstrated excellent solubility in acidic media up to 99%. The crystallinity of fungal chitosan was <0.12, lower than commercial chitosan. The chitosan from fungal source produced medium to low molecular weight chitosan compared to shrimp chitosan, the molecular weight of fungal chitosan ranged from 82.3 kDa to 121.8 kDa. The viscosity of fungal chitosan ranged from 3.7 to 7.0 cP, due to their low molecular weight. The chitosan from fungi was found to be less crystalline than commercial chitosan from shrimp.

The highest chitosan producing fungi *Mucor baineri* (MBT 3951) was investigated on the effect of culture media, pH, agitation and incubation temperature during submerged shake flask fermentation on chitosan production potential. The optimum conditions for cell growth and chitosan production under submerged fermentation of *Mucor biniery* (MBT-3651) were pH 5.0, agitation speed 200 rpm, and an incubation temperature of 30 °C in SSPY
medium. The maximum biomass production was 14.3 g/L and maximum chitosan yield of 13% were obtained under these optimum conditions. The molecular weight of fungal chitosan varied depending upon culture media, whereas, degree of deacetylation, solubility, viscosity and crystallinity of chitosan were not significantly influenced by composition of growth media.

Tetrahydrocurcumin loaded chitosan microspheres were effectively prepared by spray-drying method using fungal chitosan prepared from *Mucor biniery* (MBT-3651). THC-loaded chitosan microspheres prepared by spray-drying method were spherical (spherical geometry) in shape with uniform narrow particle size distribution with mine particle size of 3.88 ± 1.50 µm with positive surface charge of +18.3 mV. The developed microspheres showed relatively low percentage of moisture content and provided long term stability of encapsulated THC. The preparation yields obtained were relatively high with respect to applied method ranged from 45.6 ± 5.8 to 53.3 ± 4.9% for placebo and THC loaded chitosan microspheres, respectively.

The encapsulation efficiency and loading efficiency of THC in chitosan microspheres prepared by spray drying was found to be 80.61 ± 0.35% and 40.30 ± 0.17%, respectively, much higher compared to other reported techniques for microencapsulation. THC was microencapsulated in chitosan microspheres by reaction between keto group of chitosan and amino group of chitosan and by physical
interaction of drug to polymer conformed by FT-IR and DSC analysis. The XRPD results indicated that THC present in chitosan microspheres was in amorphous, disordered-crystalline phase or solid solution state. Easy diffusion of drug molecules can occur through the polymeric matrix, leading to a sustained release of the encapsulated drug.

*In vitro* drug release study of THC-loaded chitosan microspheres in simulated gastric and intestinal fluid indicate similar drug release pattern and the drug was released in slow and sustained control manner from chitosan microspheres which was governed by the diffusion ability and explained by non-Fickian low of diffusion. The drug release data was evaluated by Korsmeyer-Pappas mode. The *in vitro* drug release data fits in first order model and failed to fit in zero-order model and therefore, confirming that THC is released by diffusion from porous chitosan microspheres and the drug release rate is proportional to the amount of drug remaining in microspheres. Therefore the microspheres enable continuous drug release over prolonged period.

Bis(2-chloroethyl)sulphide, commonly known as sulphur mustard (SM) or mustard gas is listed in the Schedule 1 of Chemical Weapons Convention (CWC). The percutaneous exposure of SM (2LD$_{50}$) in mice model induced oxidative stress and resulted in significant depletion in reduced glutathione as well as oxidised glutathione. Also increase in lipid peroxidation was observed.
Significant increase in RBC counts and hemoglobin level were also observed after SM exposure. Progressive decrease in animal body weight was observed after SM exposure. Decrease in Organ to Body Weight Index (OBWI) of liver and spleen were also observed. Treatment with THC-loaded chitosan microspheres showed recovery in body weight loss, biochemical and hematological variables. Histology of liver and spleen were recovered and their weight loss was also recovered by THC-loaded chitosan microsphere but not by THC alone. THC is hydrophobic in nature and due to its poor solubility only slightly absorbed in the gastrointestinal tract. Extensive intestinal and hepatic metabolism and rapid elimination additionally restrains its bioavailability. THC-loaded chitosan microspheres provided better protection than THC alone the due to the release drug from the microspheres which is in slow, sustained and contolled manner at target site. Therefore by slow diffusion process chitosan microspheres, with their mucoadhesive and permeation enhancer property, improved the absorption and bioavailability of THC. High surface to volume ratio provided much more intimate contact with mucosal layer and specific targeting of THC to the absorption site. Transient widening of thigh junctions between epithelial cells resulted in improved uptake of THC and provided better protection then THC alone.

In conclusion, chitosan isolation from fungal source by submerged fermentation of *Mucor biniery* (MBT-3651) showed promising alternative to chitosan from conventional crustacean
sources. Chitosan from fungal source was also observed to have constant high quality and purity with constant physicochemical properties. These fulfill the requirement of pharmaceutical and biomedical industries for low volume high value applications as it can be prepared in economical culture media. THC encapsulated fungal chitosan microspheres were prepared by spray drying method and improved the bioavailability and mucosal absorption of hydrophobic drug. THC-loaded chitosan microspheres improved the efficacy of THC. The research work has high potential for commercial application in pharmaceutical and biomedical industries.