



## Comparative Study for *In-vitro* Evaluation of Metronidazole Prepared Using Natural Chitosan from Oyster Shells of *Egeria radiata*

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### Authors' contributions

This work was carried out in collaboration between both authors. Author SOM designed the study, wrote the protocol and wrote the first draft of the manuscript. Author EAEA managed the literature searches, analyses of the study, performed the spectroscopy analysis and the experimental process. Author SOM identified the species of plant. Both authors read and approved the final manuscript

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### ABSTRACT

**Objective:** The present study investigates the capability of preparing metronidazole loaded microspheres employing a natural chitosan extracted from oyster shells of *Egeria radiata*.

**Methods:** Microspheres were prepared by external gelation technique using tripolyphosphate (TPP) as the cross-linker. The drug to polymer ratio was selected at various levels of polymer-drug quantities with varying amount of TPP. The prepared microspheres were characterized for % yield, drug entrapment efficiency, surface morphology and drug release profile.

**Results:** Almost spherical, rough and porous microspheres were obtained. % yield and drug entrapment efficiency were found to be in the range of 63.5 – 70.8% and 63.4 – 77.5% respectively; and about 80% of the drug was released in about 6 hours.

**Conclusion:** The results revealed that formulation of metronidazole loaded microspheres in natural chitosan from an oyster shell can be used as a potential oral drug delivery system.

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**Keywords:** Metronidazole; *Egeria radiata*; chitosan; microspheres; external gelation technique.

## 1. INTRODUCTION

Chitosan is considered one of the most important polymers for biomedical and pharmaceutical applications due to its biodegradability, biocompatibility, anti-microbial, non-toxicity, and anti-tumor properties. Nanoparticles, microspheres, hydrogels, films, fibres are typical chitosan based forms. Examples of such application include nasal, oral, ocular, parenteral, and transdermal drug delivery [1].

The pH-dependant solubility of chitosan is a function of the amino groups in the molecule and is a drawback for oral delivery in that chitosan microspheres formed by electrostatic interaction between a polyion and counterions become unstable in gastric fluid. This problem can be countered by irreversible chemical crosslinking with a dialdehyde such as glutaraldehyde [2].

Metronidazole is a chemotherapeutic and antibiotic, synthetic drug that is derivative of nitroimidazole with an adult dosage of 400mg three times daily hence the problem of compliance may arise among patients taking the drug. It is active against anaerobic bacteria and protozoa. It is useful in the eradication of *Helicobacter pylori* which stays mainly in the gastric mucosa and is an etiologic factor in the development of gastric ulcer, gastric carcinoma, and gastritis [3]. The sustained release ability of metronidazole microspheres will reduce the frequency of dosing and thus, enhance compliance. Metronidazole is also used to treat trichomoniasis which is a common non-viral sexually transmitted disease that affects about 170 million people annually and the causative protozoan is *Trichomonas vaginalis* [4].

*Egeria radiata* (Larmack) Fig. 1, Family-Donacidae, a mollusk in the class of bivalvia, of the order Veneroida is found in large fresh rivers of western Africa including Cross River and Akwa-Ibom state of Nigeria [5]; Local name: Nkop, sanaga (Cameroon) (Etim and Brey 1994).

This study aims to attempt to formulate metronidazole microspheres orally using chitosan obtained from oyster shell and evaluating the effect of natural chitosan on the sustained release of the drug by comparing it with standard chitosan. This is the first time metronidazole is being formulated into microspheres using a natural polymer from an

oyster shell and percent yield, drug entrapment efficiency and percent drug release as assessment parameters using standard chitosan for comparison.



**Fig. 1. Shells of *Egeria radiata***

## 2. MATERIALS AND METHODS

Metronidazole powder was a gift from May and Baker, Ikeja, Lagos; extracted chitosan from oyster shells of *Egeria radiata* as previously extracted by Majekodunmi et al. [5]; standard chitosan was obtained from Sigma Aldrich (St Louis, MO, USA). All other reagents and materials are of analytical grades.

### 2.1 Methods

#### 2.1.1 Extraction of chitosan

The shells of *Egeria radiata* were washed and oven-dried for three consecutive days at 50°C. Size was reduced into powder by an industrial blender and later made to pass through Endecott sieves to obtain a 250 micron size. Extraction of chitosan from *Egeria radiata* was carried out via three procedures namely: deproteination, demineralization and deacetylation processes. Powdered shells (500 g) were treated with 4% w/v NaOH at room temperature for 24 hours. The alkali was drained from the shell and washed with distilled water repeatedly until the pH dropped to neutral. After this process of deproteination, demineralization was carried out by treatment with 4% v/v HCl at room temperature for 12 hours to yield chitin. The acid was drained off from and the chitin was washed with distilled water and dried at room temperature on a white tile. This process was repeated with 2% w/v NaOH and then with 1% v/v HCl. Further

decolourization was achieved by soaking chitin in 1% w/v potassium permanganate for 30 min followed by 1% w/v oxalic acid for 30 min. The decolourized chitin was deacetylated to form chitosan by treating with 65% w/v NaOH for 3 days at room temperature. The alkali was drained and the chitosan was washed repeatedly with distilled water until the pH was lowered. The resulting chitosan was dried at room temperature and then stored in a bottle until needed [5]. The quality control method of the chitosan has been given elsewhere [5].

### **2.1.2 Formulation of microspheres**

The microspheres were prepared by external gelatin technique. The characteristic feature of this technique includes ease of preparation, higher drug entrapment, less frequency of administration, required higher amount of polymer, ensures extended drug release, large particle size than those prepared by other methods. The process variables selected for the present study are drug and polymer ratio and concentration of cross linking agent.

### **2.1.3 Formulation design**

The drug: Polymer ratio and amount of extracted chitosan and triphosphate present in the 120 various batches of the microspheres while the concentration of the drug remained constant are shown in Table 3.

### **2.1.4 Preparation of microspheres**

Metronidazole powder (50 mg) was dissolved in 10 ml 2% acetic acid. The polymer was added to the solution with gentle stirring. The solution was

left for 15 to 20 minutes to make it bubble free. The triphosphate solution (TPP) was prepared in 50 ml of water by heating a stoichiometric mixture of disodium hydrogen phosphate  $\text{Na}_2\text{HPO}_4$  and monosodium phosphate under carefully controlled 140 conditions. The drug-polymer solution was slowly dropped into the tripolyphosphate solution with the help of 22 gauge syringe. The microspheres were left in TPP solution until it settled to the bottom of the container, allowed for 15 minutes for sufficient cross linking. The TPP solution was decanted carefully and washed with 10 ml acetone twice. The acetone solution was decanted and the microspheres were air dried.

## **2.2 Evaluation of Microsphere**

### **2.2.1 Percentage yield**

This was calculated as the total weight of microspheres (practical value) to the total raw materials used (theoretical amount)

$$\text{Percentage yield} = \frac{\text{practical value}}{\text{theoretical value}} \times 100\%$$

### **2.2.2 Drug entrapment efficiency**

Chitosan microspheres loaded with metronidazole (50 mg) were placed in 400 ml 0.1 N HCL for 10 minutes. The solution was filtered and assayed spectrophotometrically at 277 nm to quantify its drug content.

Drug entrapment =

$$\frac{\text{amount of drug in known amount of sphere}}{\text{initial drug load}} \times 100\%$$

**Table 1. Formulation design**

<b>Batch number</b>	<b>Chitosan (mg)</b>	<b>Drug (mg)</b>	<b>Drug: Polymer</b>	<b>Triphosphate (g)</b>
B1	100	50	1:2	2
B2	200	50	1:4	3
B3	250	50	1:5	4
B4	300	50	1:6	5
B5	150	50	1:3	3
B6	50	50	1:1	3
B7	350	50	1:7	6
B8	200	50	1:4	2
B9 (Standard)	350	50	1:7	6
B10 (Standard)	50	50	1:1	3
B11 (Standard)	300	50	1:6	5

### **2.2.3 Drug release study**

Accurately weighed microspheres (50 mg) were used to study drug release profile using a RCZ-6C<sub>3</sub> dissolution instrument. Dissolution flask was filled with 400 ml of 6.8 phosphate buffer and rpm of the paddle stirrer was adjusted to 50. At frequent time intervals, 1 ml aliquot was withdrawn and after sufficient dilution, it was analyzed spectrophotometrically at 258 nm wavelength. The sample withdrawn was replaced by an equal quantity of fresh phosphate buffer. Metronidazole release data were analyzed according to zero order kinetic, first order kinetic and Higuchi model to characterize mechanism of drug release.

### **2.2.4 Physicochemical characterization of microspheres**

#### *2.2.4.1 Bulk density*

This was determined by placing a small quantity of microspheres into 10 ml graduated cylinder without compacting. The unsettled apparent volume was read to the nearest graduated unit. The bulk density was calculated using the formula:

$$\text{Bulk density} = \frac{\text{mass of microspheres}}{\text{Bulk volume}} \text{ gm/cm}^3$$

#### *2.2.4.2 Tapped density*

This was determined by taking a small quantity of microsphere sample carefully introduced into 10ml graduated cylinder. The cylinder was dropped at 2 seconds interval on hard wood surface 100 times from height 1 inch. Tapped density of each sample was obtained by dividing the weight of each sample by final tapped volume of the sample present in the cylinder. The tapped density was calculated using the formula

$$\text{Tapped density} = \frac{\text{mass of microspheres}}{\text{volume of microspheres after tapping}} \text{ gm/cm}^3$$

#### *2.2.4.3 Hausner ratio*

It is a ratio which is correlated to the flowability of a powder or granule. It is named after the engineer Henry H. Hausner (1900-1995). The Hausner ratio is calculated by the formula:

$$\text{Hausner ratio} = \frac{\text{tapped density}}{\text{bulk density}}$$

#### *2.2.4.4 Carr's index*

This is also known as Carr's compressibility index. It is an indication of the compressibility of a powder. It is named after pharmacologist Charles Jelleff Carr (1910-2005). It can be calculated using the formula:

$$\text{Carr's index} = \frac{\text{tapped density} - \text{bulk density}}{\text{tapped density}} \times 100\%$$

### **2.2.5 Microsphere morphology and surface characteristics**

Microsphere shape and morphology were analyzed by scanning electron microscopy (Model SEM PROX, Phenomworld, Eindhoven, Netherlands) for selected batches.

### **2.3 Statistical Analysis**

Percentage yield and percentage drug entrapment and other parameters were calculated using Graph Pad Prism 4.

## **3. RESULTS**

### **3.1 Characteristics of Metronidazole Loaded Microspheres**

The characteristic features of microspheres prepared by external gelation technique include ease of preparation, high drug entrapment, less frequency of administration, higher amount of polymer ensures extended drug release. The impact of drug-polymer ratio, and cross-linking agent was studied. It was observed that about 1 hour was sufficient for proper cross-linking of the microspheres. When the microspheres were properly cross-linked, they settled to the bottom of the container. Over-night cross-linking led to ruptured microspheres.

### **3.2 Percentage Yield, Drug Entrapment Efficiency, Bulk and Tapped Density, Hausner's Ratio and Carr's Index**

The results of the bulk and tapped density, Hausner ratio, Carr's index and effect of formulation variables on percentage yield and drug entrapment efficiency are shown in Table 2.

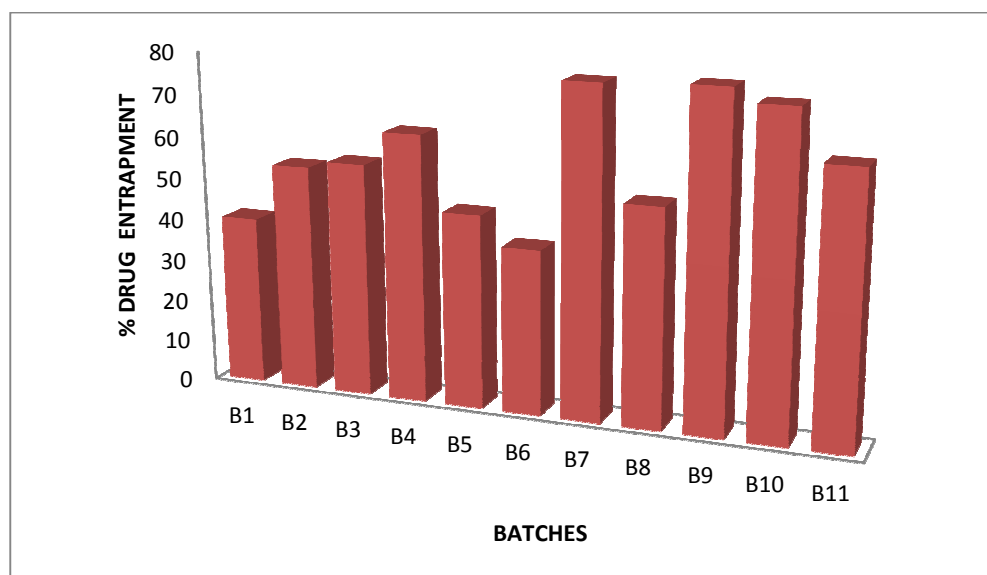
### **3.3 Determination of Drug Release**

The cumulative % release of metronidazole from microspheres is shown in Table 3 while the representative plots of percentage release of metronidazole versus time are shown in Fig. 6.

**Table 2. Bulk and Tapped density, Hausner ratio, percentage yield and drug entrapment efficiency of metronidazole loaded microspheres**

Formulation code	Drug: Polymer ratio	Bulk density	Tapped density	Hausner ratio	Carr's index	% Percentage yield	% Drug entrapment
B1	1:2	0.37	0.4	1.08	7.5	10.8± 0.12	40.0± 0.58
B2	1:4	1.36	1.5	1.10	9.3	26.5± 0.12	53.7± 0.06
B3	1:5	0.39	0.4	1.03	2.5	29.9± 0.06	55.4± 0.12
B4	1:6	0.5	0.5	1.09	8.0	32.6± 0.12	63.6± 0.12
B5	1:3	1.1	1.2	1.09	8.3	20.0± 1.12	45.8± 0.12
B6	1:1	1.3	1.8	1.38	27.7	11.4± 0.12	39.0± 0.58
B7	1:7	1.5	1.7	1.13	11.8	35.2± 0.12	77.6± 0.06
B8	1:4	1.1	1.4	1.27	21.4	23.0± 0.58	51.4± 0.12
B9(standard)	1:7	1.6	1.8	1.13	11	70.8± 0.12	78.4± 0.12
B10(standard)	1:1	0.7	0.8	1.14	12.5	68.8± 0.06	77.5± 0.01
B11(standard)	1:6	1.8	1.9	1.06	5.3	63.5± 0.06	63.4± 0.12

All data are presented as mean ± SD with triplicate experiments



**Fig. 2. Column representation for percentage drug entrapment of metronidazole loaded microspheres for different batches**

**Table 3. *In vitro* release of metronidazole loaded microspheres**

Formulation code	Drug-polymer ratio	Cumulative % drug release											
		Time (Min)											
		30	60	90	120	150	180	210	240	270	300	360	400
B1	1:2	7.2	12.8	16.4	20.9	25.0	30.0	50.1	66.4	74.7	80.1	85.0	86.0
B2	1:4	7.6	11.1	14.6	19.0	27.9	32.4	48.0	63.6	75.7	81.2	85.6	87.1
B3	1:5	6.6	10.5	14.4	18.4	26.0	31.2	46.9	62.6	74.0	81.0	84.8	89.0
B4	1:6	7.2	10.5	13.8	18.4	30.0	31.6	47.4	62.4	75.0	78.8	85.0	89.6
B5	1:3	8.4	11.9	15.4	20.1	30.0	33.4	49.2	66.0	74.3	80.1	85.8	87.4
B6	1:1	6.4	12.1	17.8	22.0	30.3	34.4	50.6	66.8	72.0	80.3	85.0	85.0
B7	1:7	10.2	11.8	13.5	17.7	26.1	30.9	46.1	65.6	75.0	80.2	85.3	84.3
B8	1:4	7.8	11.3	14.8	19.4	28.5	33.0	48.7	64.4	70.2	80.0	85.0	90.0
B9	1:7	13.0	14.8	16.6	20.1	29.0	33.5	49.2	64.9	70.0	80.2	87.3	86.1
B10	1:1	9.3	14.9	20.4	24.8	33.1	37.1	53.2	64.1	72.3	80.1	87.9	87.0
B11	1:6	10.0	13.4	16.3	21.1	25.4	34.7	54.3	65.2	75.1	83.2	90.8	89.3

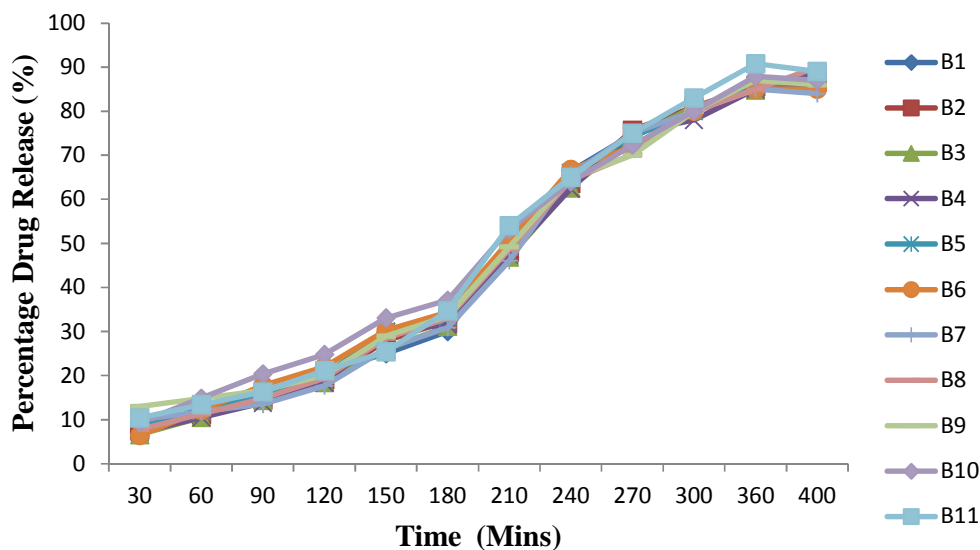


Fig. 3. % Drug release of different metronidazole loaded microspheres batches (B1-B11)

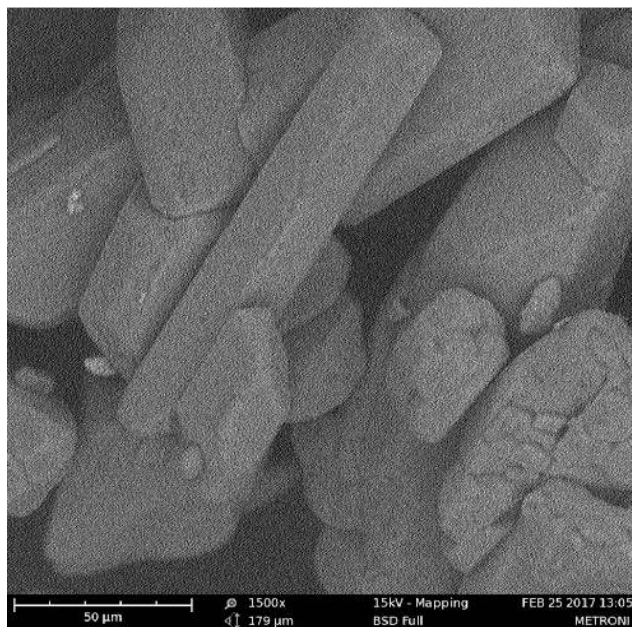


Fig. 5. SEM of metronidazole

### 3.3.1 Beer- Lambert's plot of determination

Calibration curve of pure drug metronidazole was carried out at a wavelength of 277 nm in phosphate buffer at a pH of 6.8 using a UV spectrophotometer (Labomed Inc). The graph obeyed Beer Lambert's law which states that the amount of energy absorbed or transmitted by a solution is proportional to the solution's molar

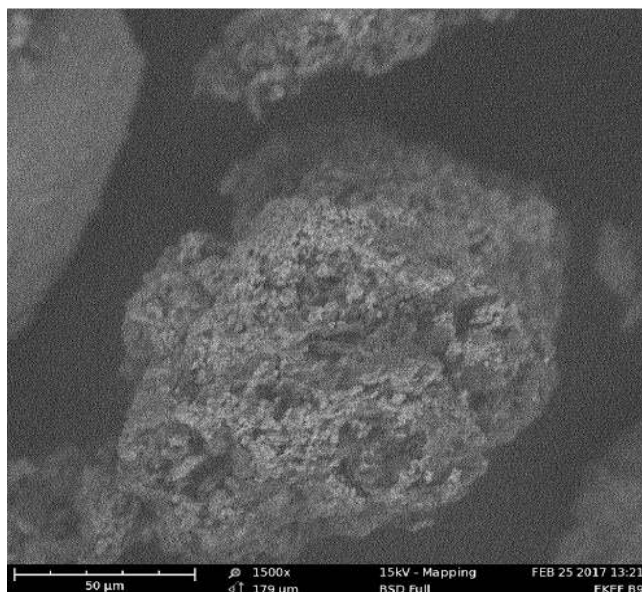
absorptivity and concentration of the solute.

## 3.4 Surface Morphology

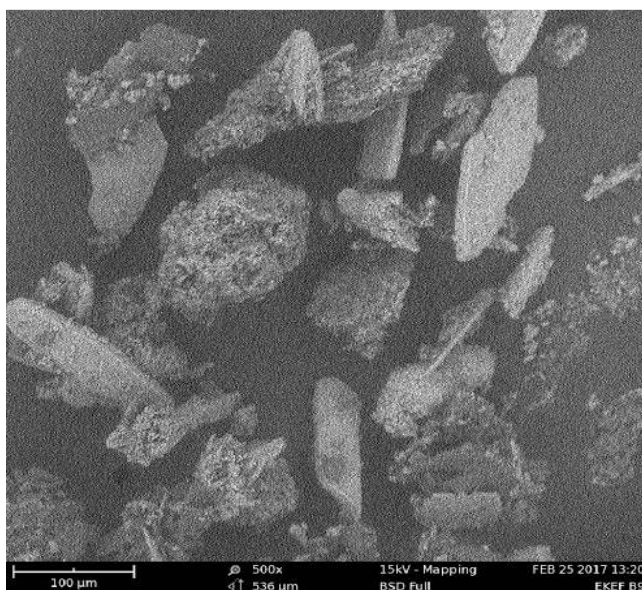
### 3.4.1 Scanning electron microscope (SEM)

The SEM of pure metronidazole is shown in Fig. 5 while SEM of metronidazole loaded microsphere is shown in Fig. 6.





**Fig. 6. SEM of B9 formulated with drug- polymer (standard chitosan) ratio of 1:7 i. e. 350 mg chitosan and 50 mg drug**



**Fig. 7. SEM of B7 formulated with a drug-polymer (extracted chitosan) ratio of 1:7 i. e. 350 mg of chitosan and 50 mg of the drug**

#### 4. DISCUSSION

##### 4.1 Percentage Yield, Drug Entrapment Efficiency, Bulk and Tap Density, Hausner's Ratio and Carr's Index

Percentage yield determines whether the preparation procedure used for incorporating a

drug into the polymer is efficient and important. The raw materials, amount of active material, polymers and other process parameters are deciding factors for the percentage yield of the product at the time of microsphere preparation. From the results obtained, the percentage yield increases as the drug- polymer ratio increases while the amount of drug remains constant. The

percentage yields of B1-B8 were relatively poor. Similar research carried out by Namdev, [6] recorded a percentage yield of about 57.15% - 94.0%. The large variation could be as a result of loss occurred during washing and drying. But formulation B9-B11 which consisted of standard chitosan had a better yield ranging from 63.5% - 70.8%. This could be as a result of the high viscosity of the standard chitosan resulting in increased drug polymer incorporation. Loss as a result of sticking could be minimized by using an apparatus made of plastic or polyethylene.

The ranking of percentage yield is: B9 > B10 > B11 > B7 > B4 > B3 > B2 > B8 > B5 > B6 > B1.

#### 4.2 Drug Entrapment Efficiency

Drug entrapment efficiency refers to the capability of a drug to be entrapped being the ratio of weight of drug entrapped into a carrier system to the total drug added. Keeping the quantity of drug constant, the effect of changing the quantity of polymer was studied. The entrapment efficiency of microspheres containing drug- polymer in various ratios of 1:2, 1:4, 1:5, 1:6, 1:3, 1:1, 1:7, and 1:4 was found to be 40%, 53.7%, 55.4%, 63.4%, 45.8%, 39%, 77.5%, and 51.4% respectively. Entrapment efficiency carried out with standard chitosan with drug-polymer ratio of 1:6, 1:1, 1:7 was found to be 78.4%, 75.12%, 63.4% respectively. There was a steady increase in the entrapment efficiency on increasing the polymer concentration in the formulation. The formulation B7 (possessed the highest entrapment of 77.5%. The batch with formulation code B 7 (drug: natural polymer ratio of 1:7) showed maximum drug entrapment efficiency of 77.5 which was comparable with B9 (drug: polymer standard chitosan 1:7) with maximum drug entrapment efficiency of 78.4 as shown in Table 2. The batch with formulation code B4 (drug: natural polymer ratio 1:6) showed drug entrapment efficiency of 63.4 which was also comparable with B11 (standard) with drug: standard polymer ratio 1: 6). It was observed that as the concentration of the polymer increased, the encapsulation efficiency increased from 39.0 to 78.4%. The results suggest that for higher encapsulation of metronidazole a higher amount of the extracted polymer is desirable. Due to greater gel strength formed at higher TPP levels, entrapment efficiency of the microsphere also increased. Lower drug entrapment efficiency observed with the metronidazole loaded chitosan

microsphere could be due to lower microsphere matrix density [7]. The ranking of percentage drug entrapment is: B9 > B7 > B10 > B4 > B11 > B3 > B2 > B8 > B5 > B1 > B6.

#### 4.3 Determination of Drug Release

Drug release refers to the process in which drug solutes migrate from the initial position in the polymeric system to the polymer's outer surface and then to the release medium [8]. In general, solute diffusion, polymeric matrix swelling, and material degradation are suggested to be the main driving force for solute transport from drug containing polymeric matrixes [9]. A phosphate buffer of pH 6.8 was selected for dissolution study. Stirring speed had a positive effect on drug release at lower polymer concentration. A sustained drug release was observed from all formulations during the 5 hours dissolution studies. Formulation B6 which consists of extracted chitosan showed maximum drug release after 5 hours, which might be due to its lower level of drug-polymer. While formulation B4 which consists of extracted chitosan showed the minimum drug release which might be due to its high drug-polymer, formulation B9-B11 which consist of standard chitosan possessed a higher drug release when compared to the formulation with extracted chitosan at similar drug polymer ratios. The rate of drug release was decreased with an increase in drug polymer concentration. The drug and polymer mixture was physically observed at different intervals. Cumulative percentage drug released for B1-B11 were found to be 86.0%, 87.1%, 89.0%, 89.6%, 87.0%, 85.0%, 84.3%, 90.0%, 86.1%, 87.0%, 89.3% respectively as shown in Table 3. It was apparent that *in vitro* release of metronidazole showed an initial burst and then followed by a sustained drug release. About 80% of the drug was released in about 6 hours. An initial fast release suggests that some drugs were localized on the surface of the microspheres. *In vitro* release profile showed that a decrease in drug polymer concentration increases the rate of drug release from microspheres. This can be as a result of increased amount of drug present close to the surface, and the amount of uncoated drug increases with decreased polymer concentration and greater binding of the drug with the polymer. As the concentration of the polymer increases, larger amounts of drug got bound to the polymer matrix. It could also be as a result of sufficient entanglement of the drug within the polymeric matrix of the microspheres composed of higher





**Fig. 8. SEM of B6 formulated with drug-polymer ratio (extracted chitosan) ratio of 1:1 i. e. 50 mg of chitosan and 50 mg of the drug**

levels of coat material. Release of metronidazole from microsphere also depends on the type of polymeric matrix and its rigidity. The release of the active ingredient from the matrix involves initial swelling followed by diffusion of the drug [10] which resulted in burst effect [11]. A denser matrix of the microsphere might exhibit slower release rates of the drug. Probably, the drug was located at the outer layer of the microsphere. From the data obtained, it can be concluded that the controlled release of the drugs from the microspheres was almost uniform. Overall, the result of dissolution studies showed that drug release from the microspheres can be efficiently tailored by altering the formulation and process parameters. The ranking of the % drug release is: B8 > B4 > B11.

#### **4.4 Release Kinetics**

Batches B1-B11 followed zero order kinetics via Fickian diffusion. This implies that these batches are ideal for sustained release formulation. The release of the drug from this formulation is independent of initial drug dose or concentration. First order is dependent on initial concentration. The log of percentage drug remaining is directly proportional to time. Since all the batches follow zero-order kinetics,

they are ideal for sustained release of metronidazole

### **4.5 Physico-chemical Properties of Metronidazole Loaded Microspheres**

#### **4.5.1 Hausner ratio**

Hausner ratio is related to inter-particle friction. Hausner ratio is an indirect measure of bulk density, size, and shape, surface area, moisture content and cohesiveness of the particles. A higher Hausner ratio and more fine particles indicate greater cohesion between particles while a low range of Hausner ratio indicates good flowability. The desirable value of Hausner ratio is 1.25 for very good flow of particles. The Hausner ratio of different formulations was determined and found to be in the range of 1.03-1.38. The results suggest that the formulation exhibited good flow property.

#### **4.5.2 Carr's index**

A high Carr's index is indicative of the tendency to form bridges between particles. The smaller the Carr's index value, the better the flow properties. A value of 5-15 indicates excellent, 12-18 good, 19-21 fair, 22-35 poor, 36-40 very poor and > 40 extremely poor flow. The results show that the formulation B4, B5, B7, B2, B1 has

excellent flow property. On the other hand, B6, B8 exhibited poor flow property.

#### **4.5.3 Bulk and tapped density**

Bulk density is the weight of a unit volume of loose material to the same volume of water. It is expressed in kilogram per cubic meter ( $\text{kg}/\text{cm}^3$ ) or pounds per cubic foot. Tapped density is the bulk density of a material after a specified compaction process, usually involving vibration of the container. The tapped density is an increased bulk density attained after mechanically tapping a container containing the powder samples. A slight difference in the bulk and tapped density indicates that the change in volume is very small even after 50 tapings, which indicates similar particle size range and reproducibility of drug content.

#### **4.6 Surface Morphology**

Scanning electron microscope (SEM) is a very powerful tool to study the crystal growth morphology and assist the micro- and nanofabrication. The SEM of metronidazole loaded microspheres appeared to be little rough, heterogenous and porous surface which is important for bioadhesion [12]. Therefore, the rough, coarse structure may have led to bioadhesion.

### **5. CONCLUSION AND RECOMMENDATIONS**

#### **5.1 Conclusion**

Natural chitosan is suitable for the formulation of microspheres. It is cheap and environmentally friendly. However, the % drug release of microspheres produced from standard chitosan is comparable to the natural polymer. Natural chitosan produced almost the same results as the standard. Batches B3 consisting of standard chitosan with drug-polymer ratio 1:5, B4 with drug-polymer ratio 1:6, B8 with drug-polymer ratio 1:4 and B11 produced the best results. An increase in drug-polymer ratio resulted in increased percentage yield and entrapment efficiency as observed with B9 and B11 formulations. A decrease in drug-polymer concentration increased the rate of drug release from microspheres as was observed with B6 and B7 formulations. Hence, these batches B7, B8, B9, B10 and B11 are suitable for the formulation of microspheres. From the investigation, it can be

concluded that the microspheres can be produced by natural chitosan as polymer obtained from oyster shells of *E. radiata* by simple and reproducible method, in which the use of complex apparatus and special precautions were avoided. The ranking of the cumulative percentage drug release is: B8 > B11 > B4 > B3 > B5 > B10 > B9 > B1 > B2 > B6 > B7.

#### **5.2 Recommendation**

*In vivo* studies should be carried out using animal model to investigate inside the biological system the sustained release profile of metronidazole loaded microspheres produced by natural chitosan.

#### **CONSENT**

It is not applicable.

#### **ETHICAL APPROVAL**

It is not applicable.

#### **COMPETING INTERESTS**

Authors have declared that no competing interests exist.

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