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Chitosan-Coated Alginate Microbeads Improve the Anti-inflammatory Potential of Etodolac: Optimization Using Box-Behnken Design, *In-vitro* Drug Release and *In-vivo* Study

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Abstract

ETODOLAC is a non-steroidal anti-inflammatory drug used for treatment of Rheumatoid arthritis and Osteoarthritis. Its therapeutic effect results from inhibition of both isoforms of cyclo-oxygenase enzyme COX-1 and COX-2. This decreases the synthesis of peripheral prostaglandins (PG) involved in mediating inflammation and this results in a number of undesirable side effects such as GIT complaints. To overcome these side effects and to control the rate of its release, alginate microbeads and chitosan coated alginate microbeads prepared. A Box-Behnken experimental design was employed to produce controlled release Etodolac microbeads by Extrusion/External gelation technique. A three-factors, three levels design within 15 runs was suitable for this research. The independent variables were the drug-polymer ratio, concentration of the cross linker and speed intensity. The influence of these formulation factors was evaluated for entrapment efficiency and *in-vitro* release of Etodolac from the microbeads at the end of two hours and at the end of eight hours. The microbeads were characterized for their shape and size. Alginate microbeads showed rough surface whilst chitosan coated alginate microbeads showed smooth surface. The microbeads exhibited entrapment efficiency from 61.31% to 97.11% and particle size in the range of 700µm to 900 µm. The release results indicated that the chitosan-coated microbeads displayed a more controlled release than the uncoated microbeads. Selected formulae exhibited a significant anti-inflammatory effect on incited rat paw edema after four hours.

Keywords

Box-Behnken design; Controlled release; Etodolac; Microencapsulation; anti-inflammatory.

1. Introduction

In recent years, new drug delivery system based on polymeric micro/nanospheres and beads have attracted much attention due to their excellent biocompatibility and biodegradability properties and successful controlled release characteristics. Such formulations have several advantages compared to the conventional type of dosage formulations. These advantages are: reduced toxicity, minimized drug dose, extended drug administration range and improved patient compliance [1]. In addition, in the encapsulation process, the system must protect the core material from the acidity of gastric juice while allow its consequent release in the basic environment of intestinal fluid to exert its function in an appropriate form [2]. Also, drug side effects may occur when administered in large quantities and controlled release formulations might be a suitable way to decrease drug complications due to its high concentration and increased patient compliance [3].

Ionotropic gelation can be divided into external and internal gelation. The main difference between these two methods is the type of calcium salt used. In external gelation, nanoparticles are produced by dropping a drug loaded alginate solution into the aqueous solution of a soluble salt of calcium, such as calcium chloride. On the other hand, in Internal gelation calcium ions are released in a controlled way from an insoluble calcium source, such as calcium carbonate, within the alginate solution [4]. Hence, the formation of the particles can occur either by external or internal gelation. The method differ in the way the crosslinking ions are introduced to the alginate polymer [5].

Extrusion technique or external gelation is the simplest and widest technique used in drug loaded alginate microbeads fabrication by ionic gelation of alginate which involves a simple diffusion and crosslinking reaction by Ca^{+2} [6,7].

In extrusion technique, the gelation of alginate is highly dependent on the concentration of $CaCl_2$ due to that Ca^{+2} ions are capable of binding to the carboxylic groups of alginate leading to the formation of a thermostable gel. In the contrary, the internal gelation has weak gelation, so the particles are often soft and tend to have high agglomeration leading to lower encapsulation efficiency and faster release profile.

In Extrusion technique, the alginate chitosan beads can be produced by two different methods: A-One- step simultaneous chitosan coating and Ca^{+2} crosslinking process leads to competition of Ca^{+2} and chitosan for alginate binding site, and

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increase the amount of chitosan on alginate microbeads.In this process, the droplets of alginate solution fall into an aqueous solution containing both the gelling agent for alginate (e.g. Ca^{+2} ions) and chitosan.

Having both Ca^{+2} and chitosan in the collection bath increases the amount of chitosan that is incorporated into the alginate beads and is reported to form a thicker chitosan coating.chitosan is introduced in the collection bath, it acts as a cross linker in addition to the Ca^{+2} and increases the overall rate of crosslinking and therefore retention of microbeads. [8,9].

A-two-step procedure: Ca alginate gel beads are produced by dropping a solution of alginate into a gelling bath containing calcium ions. The resulting beads are then transferred into a chitosan solution to form the membrane on their surface.

The two-step coating was ineffective in producing a sufficient chitosan layer and provided no additional simulated gastric fluid protection. The failure of the two-step process to make sufficient protective chitosan coating was speculated due to the loss of the electrostatic driving force. The guluronic acid (G) residues on the alginate chain were already saturated with calcium, which is an ionic cross linker that allows the alginate to gel.

After calcium cross linking, the alginate beads are submerged in a pH 5.5 chitosan solution. The pH of the solution is near the pka of the chitosan, which means that a lower percentage of the chitosan amine groups are protonated. This, coupled with the calcium saturated carboxylate groups on alginate, leads to little or no chitosan coating on the alginate microbeads .

Gastric protection is not seen in the two step coating and this was speculated due to the high G content of the alginate used , as Ca^{+2} would have saturated the electrostatic binding sites, forming a tight interfacial membrane on the alginate that prevent chitosan from interacting with and penetrating the crosslinked alginate bead. [8,10].

Sodium alginate is a natural, non-toxic and water-soluble anionic polymers with biodegradable, biocompatible and mucoadhesive properties which favors its utility in pharmaceutical and biomedical applications. It is obtained from marine brown algae, formed of alternating units of 1-4 linked α-L- guluronic acid (G) and β-D- mannuronic (M) acid residues. The success of SA in drug delivery is established on its competence of interactions with cationic divalent ions such as Ca^{+2} [11]. Ca^{+2} has strong affinity for carboxylic acid moieties of G and M blocks of SA. These cationic anionic interactions form "egg- box-like- structure" whereby carboxylate units of SA molecules (box) are coordinated by divalent cations (eggs). Ca-alginate networks had shown entrapment of large range of products like lipid droplets or lipophilic drugs [12]. The calcium alginate beads prepared by the ionotropic gelation technique using $CaCl_2$ as a cross linking agent show high entrapment efficiency for controlled oral drug delivery [13].

After the drug release, the alginate is degraded into water soluble oligomers and further metabolized and eliminated from the body due to its biodegradability [7].

Chitosan, a biopolymer obtained from the shells of crustaceans, is widely used as a carrier for delivering drugs in the gastrointestinal tract. Chitosan with amino groups is soluble at low pH and insoluble at high pH. Also, Chitosan is nontoxic, biodegradable and biocompatible polymer with excellent absorption, controlled release and bioadhesive properties [14].

Alginate- Chitosan polyelectrolyte complex is formed through the ionic interaction between the carboxylic group of alginate and amino group of chitosan by ionic gelation. The electrostatic interaction reduce the complex porosity, protecting the loaded drug and slowing the drug release [15].

The chitosan- alginate gel beads, prepared by dropping a solution of alginate into chitosan solution, is an eco-friendly physical cross linking process that reduces possible toxicity associated with chemical cross linking [16].

Non-steroidal anti-inflammatory drugs (NSAIDs) are a heterogeneous group of compounds that are used for the treatment of various inflammatory conditions, pain and fever. The principal mechanism of action of NSAIDs involves the inhibition of cyclooxygenase (COX) enzyme. COX is the enzyme that catalyzes the synthesis of prostanoids (Thromboxane and Prostaglandins) from Arachidonic acid. COX inhibitors are believed to act as an analgesic, anti-inflammatory and antipyretic by decreasing prostaglandin synthesis. This decrease in prostaglandin synthesis is associated with the occurrence of several unwanted effects accompanied with the use of NSAIDs, especially gastrointestinal (GI) irritation and ulceration. Additionally, several NSAIDs have a free carboxylic acid group; therefore, oral administration is linked with the side effects on the gastric system, which are due to direct GI irritation [17].

Etodolac (EDL) belongs to the NSAIDs, which possess anti-inflammatory, analgesic and antipyretic activities. It is used mainly to relief Rheumatoid arthritis and Osteoarthritis [18]. EDL is a derivative of pyrano-indole acetic acid. The GI problems associated with EDL necessitate the preparation of oral controlled release dosage form of EDL.

This study tends to develop alginate beads and chitosan- alginate beads containing EDL through ionotropic/external gelation technique. The prepared microbeads (MBs) were used to study the drug encapsulation efficiency and *in-vitro* drug release in both acidic (pH 1.2) (0.1N HCl) and alkaline medium (pH 7.4) (phosphate buffer saline) using different kinetic models. Also, the microbeads were evaluated for their size and shape. In addition, selected microbeads were tested *in-vivo*.

2. Materials and Methods

2.1. Materials

Etodolac (EDL) was kindly provided by Pharco Co. for pharmaceuticals (Alexandria, Egypt). Sodium alginate (NaA) and low molecular weight chitosan (LMW) were bought from Sigma chemicals Co., St. Louis (USA)

Calcium chloride ($CaCl_2$), glacial acetic acid and potassium dihydrogen orthophosphate were bought from Adwia, El-Nasr pharm. Chem. Co. (Egypt).

Other materials and solvents of analytical grade and they were used without further purification.

2.2. Experimental Design

Design of the experiments has been widely used in pharmaceutical industry for the optimization of formulations. Response surface methodology (RSM) is a method to optimize the experimental data, which can be defined as an empirical statistical technique to solve multivariate equations at the same time. In this study, the Box-Behnken design was applied to optimize microbead formulations and the aim was to achieve the best one by the minimal numbers of experiments using Minitab 17.[3,19].

A three factors three levels design within 15 runs was suitable for this research. The independent factors were speed (X_1), drug:

polymer ratio (D:P) (X_2) and $CaCl_2$ concentration (X_3). The three levels of the speed were 1100 rpm, 1300 rpm and 1500 rpm which denoted the values -1, 0 and +1 respectively.
Drug: Polymer ratio was varied to be (2:1), (1:1) and (1:2) which denoted the values -1, 0 and +1 respectively. Finally, $CaCl_2$ concentration was chosen to be 0.1M, 0.25M and 0.4M which denoted the values -1, 0 and +1 respectively.
The dependent variables to be tested for the prepared EDL microbeads were the drug release at the end of two hours (Y_1), the drug release at the end of eight hours (Y_2) and the loading efficiency (Y_3).

2.3. Preparation of the alginate microbeads

Sodium alginate was dissolved in 10 ml distilled water (2.5% w/v) in a small beaker with heating at 100°C, then EDL was dispersed in this solution with stirring for 10 minutes.
The alginate drug suspension was added drop wise to the gelling bath containing the specified concentration of $CaCl_2$ in 100 ml distilled water, (1 ml/minute) with stirring for 20 minutes at the three different stirring speed at ambient temperature.
The formed microbeads were separated by filtration, washed with $CaCl_2$ solution in distilled water (the same concentration of $CaCl_2$ used in preparing the formula), then dried for 48 hours at ambient conditions and stored in a desiccator for the following experiments[19].

2.4. Preparation of Chitosan-coated alginate microbeads

The microbeads were prepared by the one step method. Briefly, sodium alginate was dissolved in 10 ml distilled water (2.5% w/v) in a small beaker with heating at 100°C on a hot plate, then EDL was dispersed in this solution with stirring for 10 minutes.
Chitosan solution was prepared by the addition of predetermined amounts chitosan to 100 ml distilled water containing 1% (v/v) acetic acid and left for maceration overnight. The required amount of $CaCl_2$ was added to chitosan solution (table 1).
Using a 10 ml syringe, the alginate drug suspension was added drop wise to the previous solution at a rate of 1 ml/minute with stirring for 20 minutes at the three different stirring speed at ambient temperature.

The formed microbeads were separated by filtration, washed with $CaCl_2$ solution in distilled water contains the same concentration of $CaCl_2$ used in preparing the formula, dried for 48 hours at ambient conditions and stored in a desiccator until starting the experiments[16].

2.5. Production yield determination

The dry beads were weighed and the % bead yield was calculated using the equation 1(eq.1) below [20]:
Bead yield % = (Actual weight of dried beads/ Total Theoretical weight) * 100 (eq.1)

2.6. Morphology and Particle size of the microbeads

Analysis of the surface of the microbeads was performed by Scanning Electron microscopy (Fei Quanta 400 FEG ESEM/EDAX Pegasus X4M) for the formula had the best rank order. Microbeads were spread on a carbon double-adhesive layer on a metal holder and gold-coated using an Ion-Sputtering device (Jeol Fine-Coat JFC 1100E, Joel Ltd., Tokyo, Japan) [1].

The dried microbeads were weighed and sized using USP standard sieve (Rx-86-1, Cole-Palmer Instrument Co., USA) [27].

Table (I): Composition of different formulae of EDL microbeads**.**

Formula No.	EDL (gm)	Chitosan (gm)	Alginate (gm)	$CaCl_2$ (M)	Speed (rpm)	Total Weight (gm)
F1	0.5	-------	0.25	0.25	1100	0.750
F2	0.5	-------	0.25	0.1	1300	0.750
F3	0.5	-------	0.25	0.4	1300	0.750
F4	0.5	-------	0.25	0.25	1500	0.750
F5	0.5	0.25	0.25	0.1	1100	1
F6	0.5	0.25	0.25	0.4	1100	1
F7	0.5	0.25	0.25	0.25	1300	1
F8	0.5	0.25	0.25	0.25	1300	1
F9	0.5	0.25	0.25	0.25	1300	1
F10	0.5	0.25	0.25	0.1	1500	1
F11	0.5	0.25	0.25	0.4	1500	1
F12	0.5	0.75	0.25	0.25	1100	1.5
F13	0.5	0.75	0.25	0.1	1300	1.5
F14	0.5	0.75	0.25	0.4	1300	1.5
F15	0.5	0.75	0.25	0.25	1500	1.5

2.7. Determination of drug content

The drug content of the prepared microbeads was determined by the Digestion method as follows, 20 mg of the microbeads were crushed carefully in a dry clean glass morter and transferred to a 500 ml volumetric flask then the volume was completed to 500 ml with phosphate buffer pH 7.4 and the flask left overnight.
The withdrawn samples were filtered and the drug concentration was determined spectrophotometrically at 277 nm[1].

2.8. In-vitro release of EDL microbeads

Dissolution studies were conducted to determine the *in-vitro* release pattern of the drug from the product formulations. Accurately weighed quantities of EDL microbeads, equivalent to 100 mg drug, were placed in simulated gastric fluid (200 mL, 0.1 N HCl, pH 1.2) for the first 2 hours, followed by addition of 5.7 ml of 7M potassium dihydrogen orthophosphate containing 16.75%(w/v) NaOH in order to change the pH of the medium to 7.4 to be alkaline as the intestinal fluid and the experiment was continued for another six hours. The dissolution test was performed with the rotating basket apparatus according to USP 24 apparatus 1 (SR 11 6 flask, Hanson Co., USA) with a speed of 50 rpm, at 37 ± 0.5°C. At predetermined time intervals, an aliquot of 5 mL samples of the dissolution fluid was collected from the dissolution medium and immediately was replaced with the same volume of fresh media to maintain the sink conditions. These samples were filtered, diluted, and then measured spectrophotometrically using a UV visible spectrophotometer at 277 nm. All experiments were performed in triplicate. Percent cumulative drug release was calculated [13,21].

2.9. Kinetics of the *in-vitro* release of EDL microbeads

The kinetic parameters for the *in-vitro* release of EDL were determined and then analyzed in order to find the proper order of the drug release. Zero and first order kinetics, as well as Higuchi diffusion model

Zero order : $Q_t = Q_\circ + K_\circ t$

First order: $\log Q_t = \log Q_\circ - k_1 . t / 2.303$

Higuchi diffusion: $Q_t = k_H . t^{1/2}$

Where Q_t is the amount of drug released at time t, $Q_\circ$ is the initial amount of the drug in the MBs., $k_\circ$, k_1 and k_H are the release rate constant for zero order, first order and Higuchi diffusion respectively [22,23].

2.10. Statistical analysis

In this study, experiments were designed using Box-Behnken design to perform a quadratic model consisting of 15 trials formulations. By applying multiple regression analysis to the experimental data, the input variables speed (X_1), drug-polymer ratio (X_2) and $CaCl_2$ concentration (X_3) were fitted to the polynomial equations [24].

The mathematical model was expressed as follows (eq.2):

$$\mathbf{Y = b_\circ + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_1X_2 + b_5X_2X_3 + b_6X_1X_3 + b_7X_1^2 + b_8X_2^2 + b_9X_3^2} \quad \text{(eq.2)}$$

where Y is the response, $b_\circ$ is the intercept and b_1-b_9 are the regression coefficients. X_1, X_2 and X_3 are the individual effects. X_1X_2, X_1X_3 and X_2X_3 are the interaction effect and X_1^2, X_2^2 and X_3^2 are polynomial terms of individual effects. Multiple regression analysis procedures were used to evaluate the coefficients of the models.

The coefficients of these equations describing Rel_2 (Y_1), Rel_8(Y_2) and %EE (Y_3). The values of the coefficients reflect the influence of each factor on the overall responses, while the sign denotes the nature of the influence of each factor (+ increase, - decrease).

One way ANOVA has been used to compare the results to determine the statistically highly significant, significant and non-significant interpretation at 95% confidence level interval and p-value < 0.05 was considered highly significant [25].

2.11. Anti-inflammatory effect of formulated EDL microbeads

The anti-inflammatory effect of EDL in the chosen formulae F1, F5 and F15, which had the best rank order among the prepared formulae, the commercial tablets and Etodolac crude drug was studied on rats using paw edema method. The paw edema induced by injection of carrageenan in the paw was used as a model for evaluation of the anti-inflammatory potential of the formulated EDL microbeads [26]The experimental procedure was reviewed and approved according to Sohag University Ethical Guidelines for Animal care and Use in scientific research (Sohag-IACUC approved protocol No. 12/1/2022/2).

Rats (150-200 gm) were divided into six groups; each group consists of six animals. Each group of animals received the specified drug product through a special gastric intubation into the esophagus in a dose equivalent to 2 mg/kg. The selected formulations, the commercial product as well as EDL crude drug were suspended in 1% Carboxy Methyl Cellulose solution in distilled water. Inflammation was induced by subcutaneous injection of 0.1 ml of 1% carrageenan solution in distilled water into the sub-plantar tissue of one hind paw. the percentage of reduction of edema size was measured after 0.5, 1, 2, 3, 4 and 5 hours.

Groups were divided into:

(Control group) : control group received carrageenan injection without treatment.

Group I: rats with oral administration of ETODOLAC crude drug

Group II: rats with oral administration of Etodolac commercial tablets (Etodolac-Elite Pharma ® (Etodolac 600 mg))

Group III: rats with oral administration of alginate coated microbeads (F1) of 2:1 drug to polymer ratio using 2.5% Sodium alginate and 0.25M $CaCl_2$,

Group IV: rats with oral administration of Chitosan-coated alginate microbeads of (F5) 1:1 drug to polymer ratio using 250 mg chitosan, 2.5% sodium alginate and 0.1M $CaCl_2$,

Group V: rats with oral administration of Chitosan-coated alginate microbeads (F15) of 1:2 drug to polymer ratio using 750 mg chitosan, 2.5% sodium alginate and 0.25M $CaCl_2$

3. Results and Discussion

3.1. characterization of EDL microbeads

The microbeads were successfully prepared having spherical shape.The prepared MBs were examined using the Scanning Electron microscope (figure 1). It can be observed that the Chitosan coated alginate MBs were completely spherical, and their surface was smooth due to presence of Chitosan. Similar results were obtained by Bulut 2019 [1].

The average size of the prepared MBs varied between 700 µm and 900µm. The mean particle size values (±SD) for formulations number 1 to 15 were: 870.8±35.1, 849.9±41.5, 800.2±39.7, 790.2±39.8, 809.9±42.6, 899.8±32.9, 791.3±45.2, 766.8±47.9, 729.9±43.1, 770.1±39.2, 717.4±37.9, 878.3±45.2, 806.8±47.9, 792.3±45.2, 702.8±47.9 µm respectively. In general, the size of the MBs increased with increasing the drug-polymer ratio. Alginate MBs have a lower mean particle size compared to chitosan-coated alginate MBs. The increased MBs size with increasing the drug-polymer ratio could be attributed to the electrostatic interactions between alginate and chitosan led to formation of a polyelectrolyte membrane on the surface of the MBs and increasing the particle size [16,34]. In addition, it was observed that increasing the concentration of $CaCl_2$ from 0.1 M in F 10 to 0.4 M in F11has decreased the mean particle size from 770.1±39.2 µm.to 717.4±37.9 µm, respectively. Similar results were obtained by Umaredkar 2018 [16] who proved that the concentration of $CaCl_2$ imparts a negative effect on the particle size of chitosan alginate beads loaded with Clinidipine and Bulut 2019 [1] who reported the decreased particle size of Chitosan coated microspheres of sodium carboxymethyl cellulose/polyvinyl alcohol crosslinked by ferric ions loaded with Flurbiprofen. with increasing $FeCl_3$ concentration. This may be attributed to that increasing the concentration of $CaCl_2$ results in shrinkage of polymeric gel due to the higher degree of the crosslinking with the high concentration of the crosslinker [24] so the particle size is decreased.

In addition, higher stirring speed (1500 rpm) produced MBs with small particle size while low stirring speed (1100 rpm) produced MBs with large particle size. It was observed that increasing the speed from 1100 rpm in F12 to 1500 rpm in F15 has decreased the particle size from 878.3±45.2µm to 702.8±47.9µm respectively. Similar results were obtained by previous studies with Mahmoud 2020 [27], Rai 2016 [28] and Li 2017 [29] that

revealed that as the stirring speed increases the particle size decreases.

The percentage of the production yield are listed in table II. The values range between 109.38%±3.02 (the maximum) for F3 and 45.08%±4.84 (the minimum) for F13.

3.2. Encapsulation Efficiency percent (%EE)

The EE% of EDL-MBs is listed in table II. The equation (eq.3) which studies the effect of the three independent variables on %EE is:

$Y_3 = 84.12 + 3.08\ X_1 + 8.57X_2 - 6.74\ X_3 + 6.10\ X_1^2 - 5.45\ X_2^2 - 2.21X_3^2 + 0.94\ X_1X_2 + 3.07\ X_1X_3 + 0.36\ X_2X_3$. (eq.3)

Since the p-value in the ANOVA table concerning %EE versus D:P ratio is less than 0.05 (= 0.001), hence D:P ratio has a highly significant effect on %EE at the 95% confidence level (table III, figure 2). These results are in consistence with previous studies with Bulut 2019, Sinha 2018 and Ghosal 2020 that revealed the positive effect of D:P ratio on the %EE [1,30,31]. The increased %EE with the increased drug-polymer ratio may be attributed to the more polymer matrix available for creating more molecular sites for interaction with the drug leading to enhanced entrapment [1, 31]and the increased size of the vesicles containing more polymer. In addition, combination of chitosan and sodium alginate mixtures significantly improves the %EE and this due to the fact that higher chitosan concentration results in the formation of a denser matrix structure that probably decreases the loss of drug to the curring medium [30].

The p-value of %EE versus speed was found to be less than 0.05 (0.004) (table 3) indicating the highly significant effect of speed on %EE at the 95% confidence level. Table III shows increased %EE from 89.33%±3.2 to 95.54%±3.1 by increasing the speed during formulation from 1100 rpm in F 12 to 1500 rpm in F 15. Increasing %EE with the increase in the speed may be due to that larger amount of drug get dissolved and incorporated in the polymer matrix with higher speed of rotation [27,29]**.**

On the other hand, Shukla 2014***;*** Rai 2016 and Mouffok 2016 proved that as the stirring speed increased, the %EE decreased and this may be attributed to that higher stirring speed produce small size of droplet with increased surface area, such that diffusion of drug from such microspheres will be fast resulting in the loss of drug with consequent lowering in entrapment efficiency [35, 28, 36].

Table II: Production yield%, Theoretical drug content%, Actual drug content% and %EE of the prepared MBs.

Formula No.	% Yield	Theoretical Content %	Actual Content %	% EE
F1	99.6±3.11	66.66	50.57±2.3	75.87
F2	89.6±4.23	66.66	48.42±3.2	72.64
F3	109.38±1.02	66.66	40.87±2.7	61.31
F4	93.33±5.08	66.66	52.21±3.1	78.32
F5	72.9±4.32	50	47.62±1.8	95.25
F6	106.97±3.98	50	36.38±1.9	72.76
F7	96.1±4.51	50	39.56±2.3	79.13
F8	96.47±4.11	50	44.11±2.4	88.23
F9	96.2±5.44	50	42.49±2.7	84.99
F10	67±4.51	50	48.55±3.1	97.11
F11	74.1±3.24	50	43.45±2.8	86.91
F12	60.23±4.43	33.33	29.77±3.2	89.33
F13	45.08±4.84	33.33	30.29±2.1	90.89
F14	81.83±5.84	33.33	26.99±4.1	80.98
F15	60±4.44	33.33	31.84±3.1	95.54

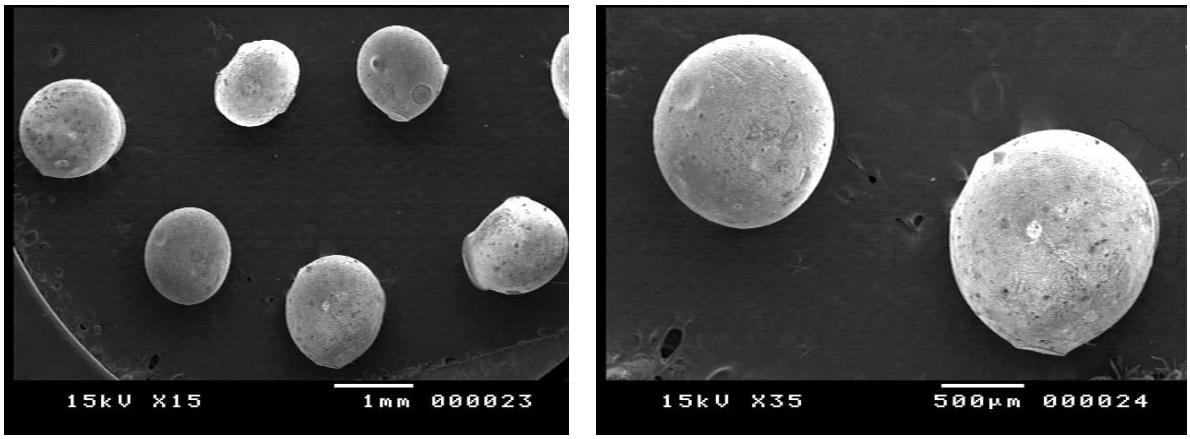

Figure (1): SEM images of the bead surface of F15 indicating a smooth surface

Table III: Factors combinations and responses parameters of EDL MBs prepared with Box-Behnken design.

Formula No.	Variable coded level			% Cumulative release		% EE
	X_1 Speed	X_2 D:P ratio	X_3 $CaCl_2$ Conc.	Rel_2	Rel_8	
F1	-1	-1	0	14.46±2.21	92.72±2.3	75.87
F2	0	-1	-1	18.42±2.02	91.08±1.2	72.64
F3	0	-1	+1	24.75±1.33	100	61.31
F4	+1	-1	0	21.47±2.12	89.2±3.4	78.32
F5	-1	0	-1	11.67±2.06	71.19±2.21	95.25
F6	-1	0	+1	16.43±2.81	83.54±2.8	72.76
F7	0	0	0	18.9±2.05	97.87±2.33	79.13
F8	0	0	0	14.5±2.33	89.15±1.42	88.23
F9	0	0	0	14.89±2.61	91.16±2.81	84.99
F10	+1	0	-1	18.17±1.7	89.98±1.05	97.11
F11	+1	0	+1	24.68±1.12	99.09±0.31	86.91
F12	-1	+1	0	9.01±1.08	81.34±2.61	89.33
F13	0	+1	-1	10.97±2.31	75.23±1.33	90.89
F14	0	+1	+1	15.59±2.01	86.05±2.07	80.98
F15	+1	+1	0	13.17±1.8	74.35±2.81	95.54

Rel_2 = release of EDL in acidic mediumRel_8 = the release of EDL in alkaline medium
EE= Encapsulation Efficiency; Higher level(+1), Medium level (0), Lower level (-1)

Li, 2017[29] and Mahmoud, 2020 [27] have reported the positive effect of rotation speed during formulation on the EE%. Table (3). Shows a strong correlation between %EE and $CaCl_2$ concentration with p-value less than 0.1 (0.07), hence $CaCl_2$ concentration has a significant effect on %EE at the 90% confidence level. It is obvious that increasing $CaCl_2$ concentration causes a decrease in % EE. That was in consistence with (Rai, 2016) who revealed the negative correlation between $CaCl_2$ and % EE [28]. Similar results were obtained by Rai 2018 who reported that as the volume of crosslinking agent increased, %EE decreased, and this may be attributed to that higher degree of crosslinking since microspheres are denser and free volume space within the matrix would decrease resulted in reduced EE [28].

On the other hand, Benfattoum 2018 reported that the increase in $CaCl_2$ concentration led to a decrease of the drug leaching out from the matrix beads. That, drug leaching out may occur through the large pores that is due to insufficient crosslinking agent which may result in a lower encapsulation [13]. Also, Baimark 2014 reported a rise in EE with the increase in $CaCl_2$ concentration and explicated that by the higher Ca^{+2} concentration inducing faster hardening of AMs which hinder the diffusion of drug out of the alginate droplets during crosslinking process [37]. In addition, Hu 2017 who studied the effect of $CaCl_2$ concentration on curcumin loaded alginate beads by extrusion method, proved that high $CaCl_2$ concentration has a positive effect on the rise of EE, and this may be due to the presence of more crosslinked network [19]. Also, Bulut 2020 proved that an increase in the crosslinker concentration resulted in an increase in the DEE [33].

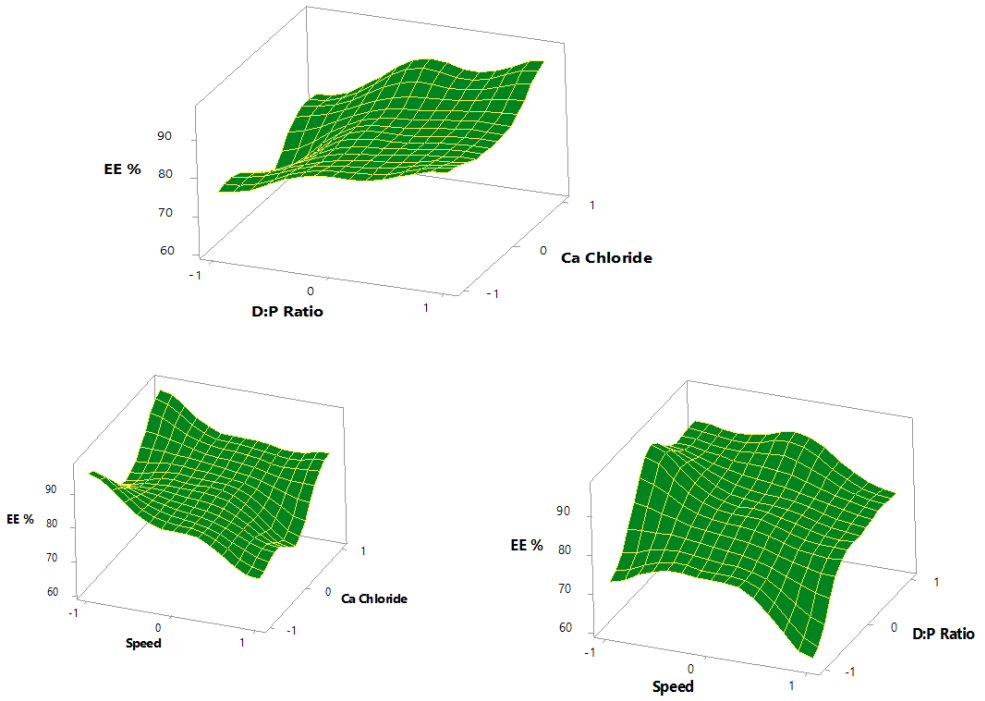

Figure (2): Three dimensional contour plot for the effect of the speed (X_1),drug-polymer ratio (X_2) and $CaCl_2$ concentration (X_3) on the percent encapsulation efficiency (Y_3).

3.3. In-vitro release of EDL microbeads

To gain more insight into the factors affecting the release of EDL from formulated microbeads, release study was conducted. The release profile of DEL formulations was added as a supplementary figure (S1). The equation (eq.4) which studies the effect of the three independent variables on the drug release after two hours was developed as:

Y_1 (Rel_2) = 16.097 + 3.24 X_1 – 3.795 X_2 + 2.778 X_3 – 0.632 X_1^2 -0.937 X_2^2 + 2.273 X_3^2- 0.713 X_1X_2 + 0.437 X_1X_3 – 0.427 X_2X_3. (eq.4)

it is obvious that p-value concerning release versus D:P ratio in the ANOVA table is less than 0.05 (0.001), hence D:P ratio has a highly significant effect on Rel_2 (table (1), figure (3). results show negative correlation between release and D:P where increasing D:P ratio causes a decrease in release. It was observed that increasing the drug-polymer ratio causes a decrease in drug release after both two and eight hours. Similar results were obtained by Bulut 2019 [1]. Benfattoum 2018 explained that the decrease in the drug release with the increase in the polymer content that the MBs containing higher polymer content, the more hydrophilic properties of the polymers enhance water uptake to form a viscous gel structure which might block the pores on the surface of the beads and delay the release of the drug from the ionotropically gelled beads [24].

It was observed that in the acidic gastric pH, the released amount of the drug was minor. This could be due to the shrinkage of alginate gel at an acidic pH. The trace amount of drug release could be due to presence of drug crystals into particle bead surface. Once the particle beads introduced in the alkaline intestinal pH after two hours, the drug release gradually increases [24] and [38]. Also, the low release of the drug in acidic medium may arise from barrier properties of chitosan coating the alginate porous matrix. Whilst, in PBS higher release was obtained as a consequence of greater pH values during which deprotonates chitosan and weaken the integrity of chitosan-alginate matrix [39].

On the other hand, results show a positive correlation between release of EDL and the speed of rotation (figure 3). The p-value in the ANOVA table was less than 0.05 (0.006), hence the speed has a highly significant effect on Rel_2. Similar results were obtained by Mouffok 2016 and Mahmoud 2020 who proved that the level of the speed was found to have a positive influence on the drug release [36, 27]. This may be due to that the increase in drug release was due to the decrease in the particle size of the prepared MBs which increased the surface area available for drug diffusion and consequently increased the permeability of the polymers [28].

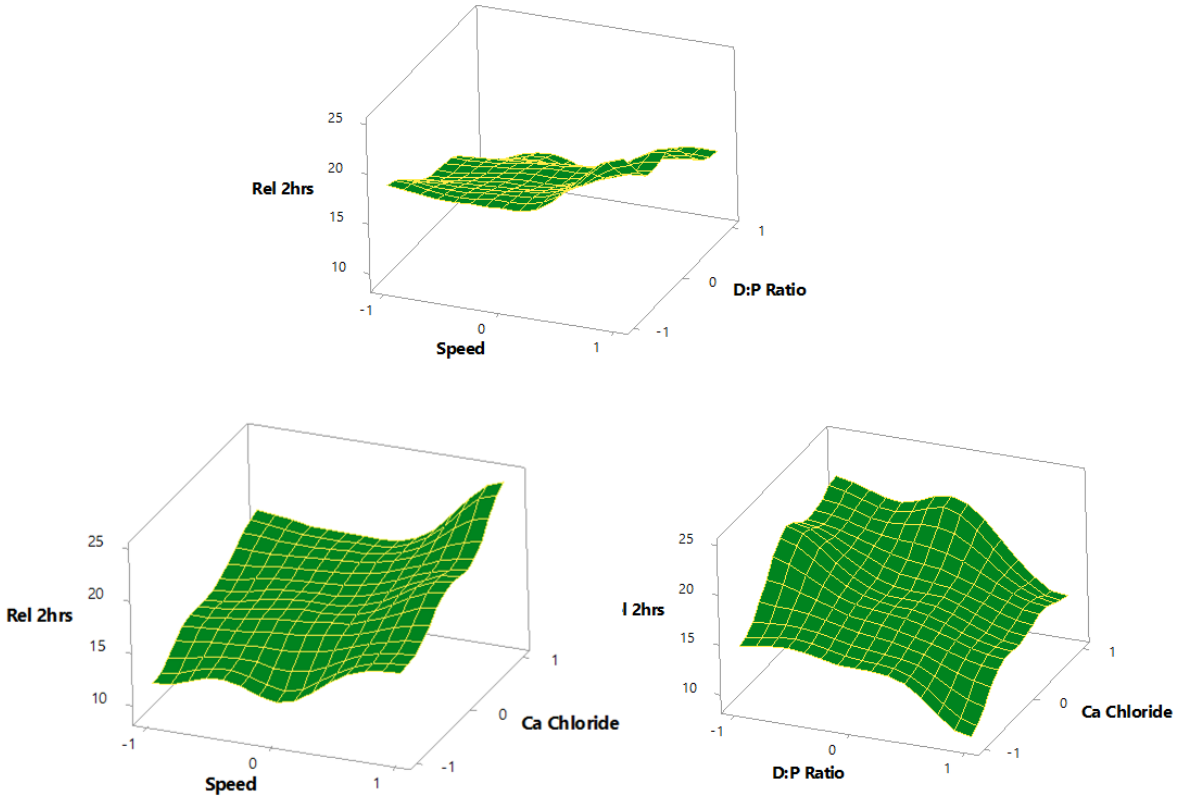

Figure (3): Three-dimensional contour plot for the effect of speed (X_1), drug-polymer ratio (X_2) and $CaCl_2$ concentration (X_3) on the cumulative percent release after two hours (Y_1)

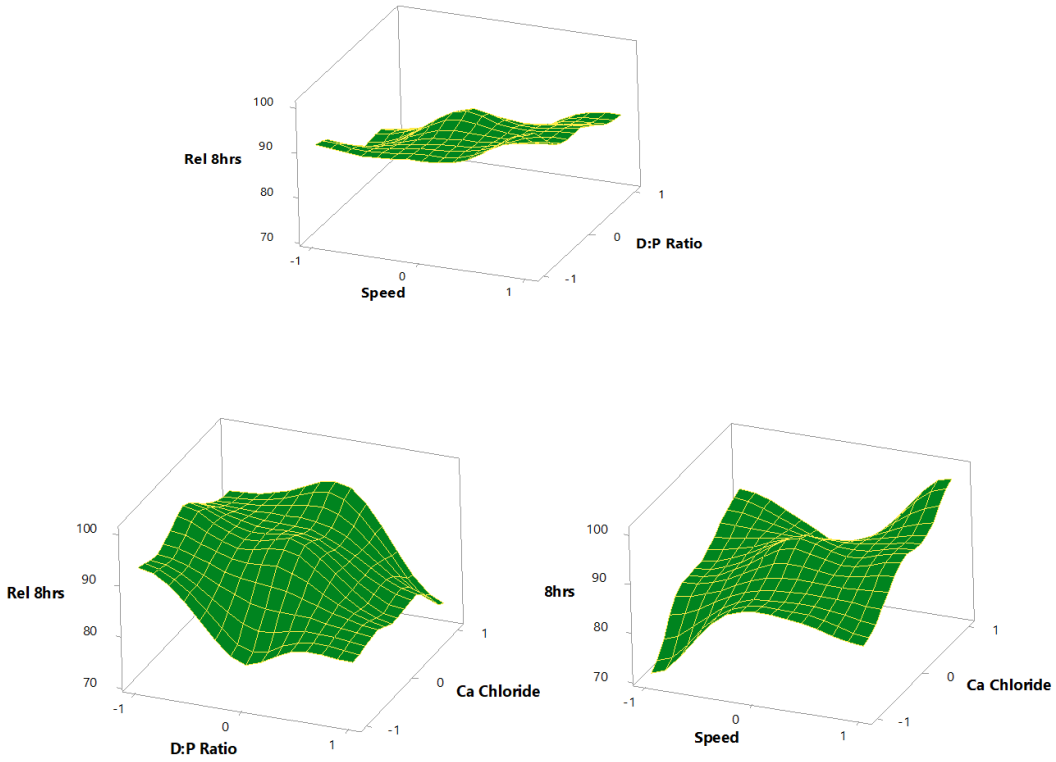

Figure (4): Three-dimensional contour plot for the effect of speed (X_1), drug-polymer ratio (X_2) and $CaCl_2$ concentration (X_3) on the cumulative percent release after eight hours (Y_2)

Table (1) and figure (3). Show significant correlation between $CaCl_2$ concentration and cumulative release with p value less than 0.05 (0.003). Increasing $CaCl_2$ concentration has significantly increased Rel_2.

These results indicate that the drug release (Rel_2) could be minimized by maintaining low level of speed of rotation , decreased $CaCl_2$ concentration and high polymer level.

The equation (eq.5) which studies the effect of the three independent variables on the drug release after eight hours (Y_2) is developed as:

$$Rel_8\ (Y_2) = 92.73 + 2.98X_1 - 7.00X_2 + 5.15X_3 - 5.23X_1^2 - 3.09\ X_2^2 - 1.54\ X_3^2 - 0.87\ X_1X_2 - 0.81\ X_1X_3 + 0.47\ X_2X_3. \text{ (eq.5)}$$

Results show that D:P ratio has a highly significant effect on cumulative release of EDL (P value is 0.05) (table 2). A negative correlation between Release of the drug in both acidic and alkaline medium and D:P ratio is shown in table III.

On the other hand, the effect of $CaCl_2$ concentration on Rel_8 was insignificant (The p-value was higher than 0.1 (0.323))., hence $CaCl_2$ concentration has a non-significant effect on Rel_8. From table III, it can be observed a positive correlation between the drug release in both acidic (Rel_2) and alkaline (Rel_8) medium and $CaCl_2$ concentration. Similar results were obtained by Bensouiki 2020 who attributed that due to the swelling of Na-alginate cross linking by $CaCl_2$ due to the ion exchange between the Ca^{+2} ions which are binding with COO^- groups with Na^+ ions present in the external solution, as a result of the electrostatic repulsion among COO^- groups increases which ultimately causes the chain relaxation and enhance the drug release and Bulut 2020 who attributed that due to the crosslinking agent solution which was $FeCl_3$ was used to cross link Na-alginate and MC polymer blend, but Fe^{+3} ions cross link only Na-Alg but not MC in the mixture. Therefore, the release may have occurred faster in the loosely crosslinked polymer blend where the MC amount was high [32, 33].

Similarly, rotational speed had insignificant effect on the release of EDL in alkaline medium (P value higher than 0.1 (0.116) table 2), the speed has both a positive and a negative correlation with Rel_8.

From the above-mentioned results, the D:P ratio emerged as the only factor which exerts a significant effect on Rel_8, and the drug release after eight hours can be minimized by using a high level of D:P ratio.

3.4. Kinetics

By using various mathematical models, the *in-vitro* drug release data from different EDL MBs were evaluated kinetically. On comparing the correlation coefficients of the models, the drug release from the beads was found not follows a specific order or system but follow different kinetic orders and systems. That may be attributed to the different factors affecting release from the polymer matrix [40]. Among the factors that may affect release in this case is swelling and diffusion and they are affected by hydrophilicity, degree of swelling, and density of polymer chains [41]. Swelling of the polymer matrix occurs by a non-Fickian diffusion process, where the active substance is delivered concurrently via erosion and diffusion. The relaxation constant affects the matrix swelling device with slab, spherical and cylindrical geometries. The more significant the value of the relaxation constant, the slower the drug is released from the

matrix. The Weibull model seems most applicable to describe the release process. May be further studies are quired to determine the exact mechanism of release kinetics from EDL microbeads.

3.5. In-vivo study

The anti-inflammatory effect of the selected EDL MBs, EDL crude drug and the commercial tablets on the size of edema induced by injection of carrageenan in the right hind paw of rats was studied .The values (±SD) of the percentage reductionin rat paw edema thickness for treatment with (EDL crude drug) were 32.65±2.48%, 35.76±5.14%, 40.23±3.11%, 55.17±4.11%, 54.72±5.09% and 42.14±3.11% after the time intervals of 0.5, 1, 2, 3, 4 and 5 hours, respectively.

For commercial tablets, the maximum anti-inflammatory activity was maintained along the time intervals (3 and 4 hours). The percentage reductionin rat paw edema thickness were 65.31±3.53% and 65.73±4.24% after 3 and 4 hours, respectively.

For treatment with (F1), the values of the percentage reductionin rat paw edema thickness were 57.21±3.12% and 67.33±4.44% after 3 and 4 hours, respectively.

For treatment number four (F5), the highest percentage reductionin rat paw edema thickness was 68.33±3.55% and was reached after four hours.

The value of percentage reductionin rat paw edema thickness for treatment number five (F15) were 67.2±3.01% and 69.5±4.7% after 3 and 4 hours, respectively.

The selection of the three formulae for the in-vivo study was based on the rank order. F1 to F4 have the same drug-polymer ratio 2:1 and F1 was the one which had the best rank order between those four formulae. F5 to F11, F5 was the one of choice between the formulae had the drug-polymer ratio 1:1. Regarding F12 to F15, F15 was the one which had the best rank order between those four formulae.

The rank order was calculated according to the formula had the lowest release of the drug in both acidic and alkaline medium and the maximum % encapsulation efficiency of the drug inside the microbeads.

Initially, both of F1 and F15 showed maximum % edema reduction after 0.5hr. and 1hr. in comparison with the other treatments but over the time, F15 showed the maximum % edema reduction throughout the experiment and the maximum percent was reached after 4hrs. then gradually decreased.

From table III, it can be observed that F1 shows 75.87%±2.3 EE and this formula shows 57.89% of the in-vitro drug release after 4hrs., and F15 shows 95.54%±3.1 EE and shows 52.36% of the in-vitro drug release after 4 hrs. Thus, the lower % EE of F1 compared to F15 explains the lower % edema reduction of F1 than that of F15 despite the similarity of the in-vitro release results after 4 hrs. Although F5 shows % EE near to that of F15 (95.25% ±1.8 for F5 and 95.54% ±3.1 for F15) and the in-vitro release of the drug from F5 after 4 hrs. was (61.6%) higher than that of F15 (52.36%) but the in-vivo results show that F15 had higher % edema reduction than F5, and this may be explained due to that in F5 a low level of speed was used (1100 rpm) which produced microbeads with a large size (809.9±42.6 µm) compared to F15 where a high level of speed was used (1500 rpm) which produced microbeads with a small size (702.8±47.9 µm) which increases the surface area available for drug diffusion and consequently increases the permeability of alginate- chitosan polymers. In addition, intestinal enzymes

Table IV: The calculated Correlation Coefficient for the in-vitro release of EDL microbeads employing different kinetic orders and systems.

Formula No.	Zero order	First order	Higuchi diffusion model
F1	0.961	0.938	0.955
F2	0.859	0.925	0.908
F3	0.264	0.952	0.153
F4	0.84	0.9	0.893
F5	0.974	0.961	0.96
F6	0.989	0.989	0.986
F7	0.98	0.955	0.969
F8	0.987	0.982	0.978
F9	0.971	0.967	0.965
F10	0.948	0.988	0.972
F11	0.932	0.979	0.961
F12	0.992	0.978	0.973
F13	0.975	0.986	0.971
F14	0.970	0.891	0.922
F15	0.988	0.987	0.975

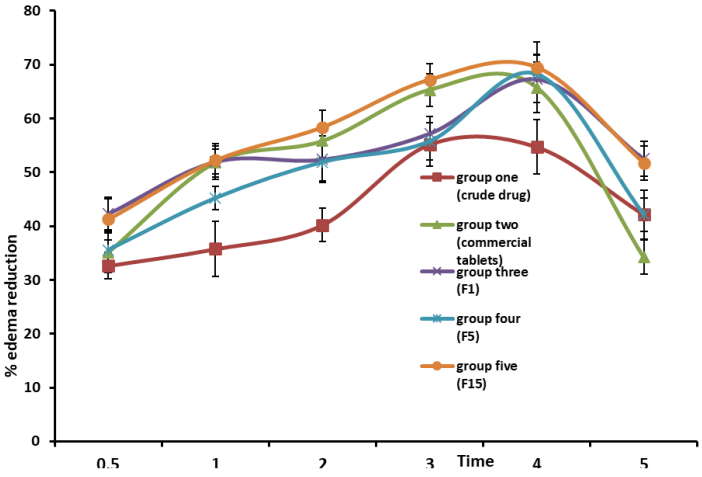

Figure (5): Effect of the selected EDL MBs. on the reduction of rat hind paw edema induced by carrageenan.

Statistical analysis of the anti-inflammatory data was done by applying the one way ANOVA test at the time intervals of the maximum percentage reduction in rat paw edema thickness (3 and 4 hours period), and it was observed a p-value < 0.05 which proves the presence of a significant difference.

must be considered. Similar results were obtained by (Mahmoud, 2020) who proved that the anti-inflammatory activity of the prepared Tolmetin Sodium ALG-CH microspheres showed highest inhibitory effect after the injection of carrageenan compared to the commercial tablets [27]. Also, (Abdellatif, 2016) proved that the anti-edema effect of beads loaded with Flurbiprufen was higher than the plain Flurbiprofen after 5 hrs. post carrageenan injection [42].

4. Conclusion

In this study, Alg. and Ch. coated Alg. MBs for oral delivery of EDL were successfully prepared by Extrusion/ External gelation technique in the presence of $CaCl_2$ as a cross linking agent. SEM images showed that Ch. coated Alg. MBs were completely spherical and showed smooth surface. The coating of Alg. MBs with Ch. improved the drug loading efficiency, delayed the drug release in the gastric environment and released the drug in a controlled manner in the intestinal environment. In addition, MBs diameter increased with the increase in the polymer. Speed of rotation has a positive influence on the drug loading efficiency and the drug release while the crosslinking agent concentration has a positive influence on the drug release but a negative influence on the drug loading. Different kinetic orders and systems were studied, and it was found that the drug release follows different kinetic orders and systems and not a specific order or system. In-vivo studies revealed that the selected formulae were effective in inhibiting rat paw edema. The behavior of the MBs is of great interest for the delivery of a NSAID into the intestine.

Table (1): ANOVA test for quadratic model for cumulative percent drug release after two hours.

Source	Sum of squares	Df	Mean squares	F-ratio	p-value
A: speed	61.716	1	61.716	21.28	0.006
B: polymer ratio	115.216	1	115.216	39.73	0.001
C: $CaCl_2$	83.981	1	83.981	28.96	0.003
AA	19.075	1	19.075	6.58	0.05
AB	0.731	1	0.731	0.25	0.637
AC	0.766	1	0.766	0.26	0.629
BB	3.242	1	3.242	1.12	0.339
BC	2.031	1	2.031	0.7	0.441
CC	1.475	1	1.475	0.51	0.508
Total error	14.501	5			
Total correlation	304.585	14			

Table (2): ANOVA test for quadratic model for cumulative percent release after eight hours.

Source	Sum of squares	Df	Mean square	F-ratio	p-value
A: speed	212.18	1	212.18	3.6	0.116
B: polymer ratio	392.42	1	392.42	6.65	0.05
C: $CaCl_2$	70.98	1	70.98	1.2	0.323
AA	8.81	1	8.809	0.15	0.715
AB	0.9	1	0.9	0.02	0.906
AC	2.62	1	2.62	0.04	0.841
BB	35.3	1	35.3	0.6	0.474
BC	3.01	1	3.01	0.05	0.830
CC	101.08	1	101.08	1.71	0.248
Total error	295.1	5			
Total correlation	1108.54	14			

Table (3): ANOVA test for quadratic model for percent encapsulation efficiency

Source	Sum of squares	Df	Mean square	F-ratio	p-value
A: speed	363.56	1	363.556	25.16	0.004
B: polymer ratio	588.24	1	588.245	40.71	0.001
C: $CaCl_2$	76.08	1	76.076	5.27	0.07
AA	18.03	1	18.027	1.25	0.315
AB	0.5	1	0.504	0.03	0.859
AC	37.76	1	37.761	2.61	0.167
BB	109.75	1	109.755	7.6	0.04
BC	3.53	1	3.534	0.24	0.642
CC	137.41	1	137.41	9.51	0.027
Total error	72.24	5			
Total correlation	1428.56	14			

Table (4): ANOVA test of % reduction of rat paw edema of the different Etodolac formulations of three hours period

Source of variation	Df	Sum of squares	Mean squares	F ratio
Between regimens	5	1379.82	271.17	3.20
Within regimens	30	2477.61	82.33	
Total	35	3857.43		

Table (5): ANOVA test of % reduction of rat paw edema of the different Etodolac formulations of four hours period

Source of variation	Df	Sum of squared	Mean squared	F ratio
Between regimens	5	1593.23	308.81	6.4
Within regimens	30	1388.28	48.22	
Total	35	2981.51		

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