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FORMULATION AND EVALUATION OF QUERCETIN LOADED CHITOSAN-HYDROXY-APATITE BIO COMPOSITE MICROSPHERES FOR PERIODONTITIS

N. M. Munot [*1], K. N. Gujar [2] and Khem Chand Gupta [3]

PAHER's Pacific University [1], Debari, Udaipur - 313024, Rajasthan, India.
Department of Pharmaceutics [2], STES's Sinhgad College of Pharmacy, Vadgaon, Pune - 411041, Maharashtra, India.
Department of Pharmaceutics [3], Pacific College of Pharmacy, PAHER's Pacific University, Debari, Udaipur - 313024, Rajasthan, India.

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Correspondence to Author:
Neha M. Munot

Ph. D Scholar,
PAHER's Pacific University, Debari, Udaipur - 313024, Rajasthan, India.

E-mail: nehamunot@yahoo.com

ABSTRACT: Periodontitis is a group of inflammatory pathological conditions involving periodontal ligaments and supporting alveolar bone which may result in the degeneration of supporting structures of teeth ultimately leading to tooth loss. Present research work is focused on the formulation, optimization and evaluation of chitosan microspheres and chitosan- hydroxyapatite biocomposite microspheres to have controlled release of herbal drug quercetin, of which anti-microbial, anti-inflammatory and wound healing properties are well proven. Mucoadhesive chitosan would retain the drug-loaded microspheres at the affected site and hydroxyapatite used in its combination would improve mechanical strength, osteoconductivity and sustain the release of the drug. Microspheres were formulated by solvent-free precipitation technique using the alkaline solution (NaOH solution). These microspheres were optimized using 3^2 factorial design using design expert software. Optimized chitosan microspheres (QC5) and chitosan-hydroxyapatite microspheres (QCH5) were characterized for surface morphology by SEM and were found to be spherical with a particle size of 5.56 µm and 7.22 µm and entrapment efficiency of 80.74 and 82.97% respectively. The entrapment of drug in microspheres was also confirmed by DSC studies. Change in the zeta potential of QC5 and QCH5 indicated an interaction between chitosan and hydroxyapatite. Antimicrobial activity of microspheres against Gram-positive *S. aureus* and Gram-negative *E. coli* was proved. Developed biocomposite microspheres showed mucoadhesion on porcine buccal mucosa using *in-vitro* wash off test. The introduction of hydroxyapatite sustained the release of drug up to 6 days in contrast to drug release from QC microspheres in 24 h. These results demonstrate that these stable, biocompatible biocomposite microspheres can be successfully utilized for treating periodontitis.

INTRODUCTION: Periodontitis, the most wide-spread infection includes inflammation and gradual destruction of tooth and infection of supporting structures which lose the strength to hold the teeth in the cavity, ultimately leading to tooth loss.

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Periodontal pocket provides ideal conditions for attachment and proliferation of microorganisms.

It is initiated by the colonization of bacteria-causing plaque and biofilms formation leading to gingivitis, if not treated, leads to the detachment of the epithelial tissue, loss of connection of periodontal ligament (PDL) and the loss of alveolar bone, as well as cementum [1] traditional treatment of periodontitis, includes removal of bacteria by mechanical cleaning of plaque and calculus without restoring structure and integrity of surrounding tissues.

The epithelial cells grow rapidly and propagate alongside the root and don't allow the re-establishment of alveolar bone and PDL. Thus, treatment with mechanical means possesses a risk of reinfection leading to recurrent periodontitis. Complete eradication of microbes from actual sites cannot be achieved by surgical and mechanical treatments. Also, the use of systemic antibiotics can induce the development of bacterial resistance [2].

Hence, there is a need to develop a local drug delivery that would be retained in the periodontal pocket and show the desired action at the site of infection for a prolonged period [1, 3]. Quercetin (3, 3, 4, 7-pentahydroxyflavone), a natural flavonoid has antioxidant, anti-inflammatory and anti-microbial activity which provides a necessary microenvironment for periodontal regeneration [4]. It also promotes soft and hard tissue regeneration [5]. Its activity against periodontal pathogens which may be due to several modes of action such as inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function and inhibition of energy metabolism are well proven [6, 7, 8].

In the present study, quercetin loaded microspheres of chitosan and biocomposite microspheres of Chitosan-Hydroxyapatite (HA) were formulated using the solvent-free technique. Microspheres were formulated using chitosan as a polymer due to its properties such as biocompatibility, non-toxicity, biodegradability, mucoadhesion, promotes cell adhesion and migration [9]. Biocomposite microspheres were also formulated using chitosan and Hydroxyapatite (HA) in combination. HA has properties such as osteoconductivity, bio-compatibility, biodegradability and strength, which is required for bone and tissue regeneration in periodontitis treatment. This combination is also suitable to overcome the limitation of one another *i.e.* poor mechanical strength of chitosan and brittleness and poor mucoadhesion of HA alone [10, 11]. These developed microspheres can be incorporated in a carrier such as gels or sensitive hydrogels, or can even be directly injected into the periodontal cavity to achieve effective control of drug release. These could also be converted into /embedded into porous scaffolds for local treatment of periodontal infection as well as further increase their therapeutic potential by supporting periodontal cell proliferation and growth [12].

MATERIALS: Quercetin and hydroxyapatite (HA) were purchased from Sigma Aldrich; Chitosan was obtained from Research Lab Fine Chemical Industries, India. All other chemicals were of analytical reagent grade.

METHODS:

Formulation of Chitosan Microspheres: Chitosan microspheres loaded with quercetin (20 mg) were formulated using solvent-free precipitation method [13]. In this technique, chitosan was dissolved in an aqueous solution of acetic acid and thus cations of chitosan were obtained due to protonated amino groups in chitosan. This solution was then added drop-wise into alkaline solution (NaOH solution) which provided anions under constant stirring. Due to the complexation between oppositely charged species, chitosan underwent ionic gelation and precipitated to form spherical particles.

An accurately measured amount of Quercetin (20 mg) was well dispersed into the clear solution of chitosan (200, 300 and 400 mg) in 10 ml aqueous solution of acetic acid (2% v/v). This dispersion was added dropwise to 30 ml aqueous sodium hydroxide solution (0.25 N, 0.5 N and 0.75 N). This mixture was stirred at 1200 rpm for 15-20 min using a magnetic stirrer (Remi Motors, India). Obtained microspheres were filtered and were washed with distilled water to neutralize excess alkali. These microspheres were placed in ethanol (5 ml) for 20 min for the removal of water. After dehydration, these were again filtered and air-dried for 24 h at room temperature. Details of the formulation are mentioned in **Table 4**.

Formulation and Optimization of Biocomposite Microspheres Containing Chitosan And Hydroxyapatite: In order to improve mechanical strength and osteoconductivity of chitosan microspheres, HA was incorporated along with chitosan to get biocompatible / biocomposite micro-spheres with desired properties. Briefly, to the dispersion of chitosan and HA in acetic acid, quercetin (20 mg) was added and well dispersed. This dispersion was heated for 30 min at 40 ºC in order to have homogeneous dispersion. The above dispersion was added dropwise to 30 ml aqueous NaOH solution (0.5 N) as an external phase, which was stirred for 15-20 min. Obtained microspheres filtered & washed with water (to neutralize excess

alkali) and kept in ethanol (20 ml) for 20 min for removal of water. These were refiltered & kept for drying (air dry) for 24 h at room temperature. Response surface methodology (RSM) was used to optimize process and formulation parameters and to determine optimum conditions for the preparation of microspheres. To study the effect of variables on microsphere formation, different formulations were prepared by varying independent variables such as the concentration of chitosan, concentration of HA. The responses, entrapment efficiency and mucoadhesion were selected as dependent variables. Experimental trials were performed at 3 possible combinations higher, lower, and middle. The resulting data were fitted into Design Expert 11 software and analyzed statistically using Analysis of Variance (ANOVA). Values of all variables and responses are shown in **Table 1, 2** and **3** and corresponding response surface curves were obtained.

TABLE 1: CODED FORMULATIONS FOR QUERCETIN LOADED CHITOSAN-HA MICROSPHERES

Variables	QCH1	QCH2	QCH3	QCH4	QCH5	QCH6	QCH7	QCH8	QCH9
X_1	-1	-1	-1	0	0	0	1	1	1
X_2	-1	0	1	-1	0	1	-1	0	1

TABLE 2: CODED LEVELS FOR QUERCETIN LOADED CHITOSAN-HA MICROSPHERES

Coded levels	-1	0	1
X_1 (Amount of chitosan (mg)	200	300	400
X_2 (Amount of HA (mg)	50	100	150

TABLE 3: OPTIMIZATION OF QUERCETIN LOADED CHITOSAN-HA BIOCOMPOSITE MICROSPHERES

Ingredients (mg)	Formulations								
	QCH1	QCH2	QCH3	QCH4	QCH5	QCH6	QCH7	QCH8	QCH9
Quercetin	20	20	20	20	20	20	20	20	20
Chitosan	200	200	200	300	300	300	400	400	400
Hydroxyapetite	50	100	150	50	100	150	50	100	150
NaOH (N)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

Evaluation of Quercetin Loaded Microspheres:

Percentage Yield: The % yield of prepared microspheres of various batches (QC1 to QC9) and (QCH1 to QCH9) was calculated using the weight of the final product after drying with respect to the initial total weight of the drug and the polymers used. The yield was calculated as per the formula:

% Yield = Weight of dried microspheres / Weight of drug + Weight of polymer × 100

Entrapment Efficiency: To determine the amount of quercetin entrapped in the prepared microspheres, accurately weighed quercetin loaded microspheres (equivalent to quercetin content of 20 mg) were dispersed in methanol to dissolve polymer completely and extract out the drug. The sample was ultrasonicated for 3 consecutive periods of 5 min each, with a resting period of 5 min. This suspension was filtered. Aliquots were taken from polymer-drug solution and analyzed for quercetin content by UV Spectrophotometer at 369 nm. The entrapment efficiency was calculated as follows:

Percent Drug Entrapment = Measured amount of drug / Theoretical amount of drug × 100

Particle Size Analysis: Particle size of microspheres was obtained by suspending them in water and observing under an optical microscope (magnification of 45X). The eyepiece was calibrated using a stage micrometer. Each determination was carried out on a minimum of 100 particles. The average particle size was expressed as the projected diameter. Particle size and particle size distribution analysis of the optimized batches of microspheres were studied by Malvern particle size analyzer. The instrument measures the vesicle size and its distribution based on the dynamic light scattering theory. The apparatus consists of a He-Ne laser beam of 632.8 nm focused with a minimum power of 10 mW using scattering angle 90 °C to the sample cell of 10 × 10 mm^2 and calculates particle size by 3D cross-correlation technique.

Mucoadhesion Test for Quercetin Loaded Bio-composite Microspheres: Mucoadhesive properties of various formulations of quercetin loaded biocomposite microspheres (QCH1 to QCH9) were evaluated by *in-vitro* wash-off method [14]. Freshly excised pieces (2 cm × 2 cm) of porcine buccal

mucosa were mounted on a glass slide using thread. About 100 microspheres were spread out on each piece of mucosa and allowed to adhere to the mucosa firmly. The slide was then hung from the arm of the USP tablet disintegration test apparatus (Electrolab). The tissue specimen was given a regular up and down movement in a vessel containing 900 ml of phosphate buffer (pH 6.8) maintained at 37 ± 0.5 °C. The adherence of microspheres was regularly observed and those remained adhered to the mucosa were counted at the end of 8 h. Mucoadhesion (%) of microspheres was calculated according to the following formula:

% Mucoadhesion = Number of microspheres remaining adhered to the mucosa / Initial number of microspheres applied to the mucosa × 100

Thermal Analysis Differential Scanning Calorimetry: Thermal analysis of quercetin and optimized formulation of quercetin loaded microspheres was performed using Differential Scanning Calorimeter with thermal analyzer (Perkin Elmer 4000) with Pyris Manager Software. The prepared samples of drug or formulation (in sealed aluminum pans) were placed in the equipment. This was heated at a scanning rate of 100 °C/min between 30 and 300 °C under 20 ml/min of nitrogen flow. For reference, an empty aluminium pan was used.

Shape and Surface Morphology: The shape and surface morphology of optimized formulations of quercetin loaded microspheres was examined by Scanning Electron Microscope (JEOL, JSM-6360, Japan, 20 KV) and Motic BA 210, Digital). Scanning Electron Microscope (SEM) images of microsphere were taken by coating the microspheres with gold to make them conductive and placed on a copper stub.

Zeta Potential: To determine the zeta potential of optimized formulation, microspheres were dispersed in deionized water at pH 6 and the surface charge (zeta potential) was measured by Laser Doppler Anemometry using a Zetasizer (Malvern Instruments).

Antimicrobial Activity of Quercetin Loaded Microspheres: To assess the potency the optimized formulations against microorganisms implicated in infection of the periodontal region, it was essential to determine antimicrobial activity. The antimicrobial assay was performed using agar well diffusion method Cup plate technique [15].

Briefly, 0.5 ml of the culture of *S. aureus* - ATCC-29213 and *E. coli* – ATCC-2592 was spread on sterile nutrient agar containing plates separately using a glass spreader. Optimized formulations, QC5 and QCH5 (containing quercetin equivalent to 20 mg) were placed in wells punched in agar plates using a sterile cork borer. The petriplates were placed in a refrigerator for the first two hours (for the diffusion of drug in the agar) and then incubated for 48 h at 35-37 °C in an incubator (Pathak Electrical Works). The diameter of the zone of inhibition around each cavity was measured. All microbiological studies were performed in the aseptic area. The study was replicated (n=3 times).

***In-vitro* Drug Release Studies:** Microspheres containing quercetin equivalent to 20 mg were suspended in 2 mL of release medium *i.e.* pH 6.8 buffer which was taken in the dialysis tube (mw co 12,000 D, 16 mm diameter, HiMedia, Mumbai, India). This dialysis tube was placed in 100 ml (including 2 ml used for suspending quercetin microspheres) of release medium maintained at 37 °C, with constant stirring at 100 rpm in an incubator shaker (Chromus). Aliquots were withdrawn at time intervals 0, 1, 2, 3, 4, 5, 6, 7, 8, 24, 48, 72, 96, 120, 144, 168 h and to maintain sink conditions, they were replaced by the same volume of fresh medium. Drug release from microspheres was determined using UV Spectrophotometer at a wavelength of 369 nm (λ_{max} of quercetin)

RESULTS AND DISCUSSION:

Formulation and Evaluation of Chitosan Microspheres Loaded with Quercetin: Simple and safe method was used for the formulation of chitosan microspheres. Due to alkaline pH (because of NaOH) in the external phase, chitosan precipitated leading to the formation of microspheres. Hence, the effect of concentration of NaOH and chitosan played an important role and affected entrapment efficiency (EE) and particle size. As seen in **Table 4**, the EE of quercetin loaded chitosan microspheres was found to be within the range of 60.6 to 87.42%. It can be seen that upon an increase in the concentration of

chitosan, the EE increased. This can be explained through the fact that the amount of polymer per unit weight of the drug is high at a higher drug: polymer ratio. Thus, more amount of polymer is available to coat drug particles. But above a certain limit, upon increasing the concentration of polymer, the solution becomes too viscous and leads to aggregation of microspheres. The second factor studied was the concentration of NaOH as it plays a crucial role in the formation of chitosan microspheres by precipitation technique. The optimum concentration of NaOH will result in the interaction of protonated chitosan and negatively charged hydroxyl group of NaOH leading to the formation of spherical microspheres with maximum entrapment efficiency. Further increase in the concentration of aqueous NaOH above 0.75 N, formed agglomerates because there may be complete neutralization of charge on microspheres. The particle size of quercetin loaded chitosan microspheres was found to be within the range of 990 to 7043 nm. It can be seen that upon an increase in the concentration of polymer, the particle size of microspheres also increased. An increase in chitosan concentration above 300 mg, leads to a viscous solution and enlarged particles. The concentration of NaOH did not have a significant effect on particle size. It helped to maintain spherical shape and prevented agglomeration of microspheres. Thus, batch QC5 with the amount of chitosan of 300 mg and concentration of NaOH of 0.5N was considered optimum in terms of entrapment efficiency and particle size.

TABLE 4: FORMULATION AND EVALUATION OF CHITOSAN MICROSPHERES LOADED WITH QUERCETIN

Ingredients (mg)	Formulations								
	QC1	QC2	QC3	QC4	QC5	QC6	QC7	QC8	QC9
Quercetin	20	20	20	20	20	20	20	20	20
Chitosan	200	200	200	300	300	300	400	400	400
NaOH (N)	0.25	0.5	0.75	0.25	0.5	0.75	0.25	0.5	0.75
Particle Size	990	1021	3487	4379	5500	5782	6024	6783	7043
(nm)	±0.23	±0.82	±0.46	±0.26	±0.19	±0.36	±0.20	±0.16	±0.03
Entrapment	60.6	63.28	69.37	75.24	80.74	77.97	84.28	87.42	83.64
Efficiency (%)	±0.86	±0.21	±0.45	±0.39	±0.28	±0.62	±0.23	±0.91	±0.23

Formulation and Optimization of Chitosan–Hydroxyapatite Biocomposite Microspheres: The demand of site of action for periodontitis treatment is that the dosage form should be retained at the site (periodontal pocket) for prolonged duration and should not be washed off due to continuous action of saliva or crevicular fluid secreted in the oral cavity. This can be attributed to the amount of chitosan in the formulation. Also, these should possess sufficient mechanical strength which can be attributed to the presence of HA. The presence of HA also increased the entrapment efficiency of the prepared microspheres. Hence, biocomposite, biocompatible and biodegradable microspheres were formulated using chitosan and hydroxyapatite.

TABLE 5: OPTIMIZATION DATA ANALYSIS FOR QUERCETIN LOADED CHITOSAN - HA MICROSPHERES

Ingredients (mg)	Formulations								
	QCH1	QCH2	QCH3	QCH4	QCH5	QCH6	QCH7	QCH8	QCH9
Quercetin	20	20	20	20	20	20	20	20	20
Chitosan(X1)	200	200	200	300	300	300	400	400	400
HA (X2)	50	100	150	50	100	150	50	100	150
NaOH (N)	0.5 N	0.5 N	0.5 N	0.5 N	0.5 N	0.5 N	0.5 N	0.5 N	0.5 N
%	75.92	77.02	79.99	81.34	83.59	84.05	84.90	85.04	85.94
yield	±0.45	±0.49	±0.26	±0.39	±0.24	±0.96	±0.29	±0.18	±0.73
Particle size	1.05	3.91	4.99	5.76	7.22	8.02	8.43	9.08	9.56
(µ)	±0.87	±0.76	±0.38	±0.28	±0.54	±0.58	±0.39	±0.20	±0.19
Entrapment	60.99	68.72	72.34	74.23	82.97	85.76	79.2	76.28	71.97
Efficiency (%)	±0.81	±0.34	±0.12	±0.78	±0.72	±0.65	±0.98	±0.41	±0.25
Mucoadhesion (%)	52±0.8	47±0.62	42±0.54	75±0.9	72±0.23	64±0.66	60±0.38	57±0.52	49±0.07

Optimization of Quercetin Loaded Biocomposite Microspheres: Prepared microspheres were optimized to get optimum mucoadhesion and entrapment of drug to have improved efficacy. Effect of independent variables - the amount of chitosan (X1) and amount of HA (X2) on

dependent variables *i.e.* Entrapment efficiency and mucoadhesion (%) was studied using 3^2 factorial design using Design-Expert software 11. The results are mentioned in **Table 5**.

Response 1- Entrapment Efficiency (Ee): Effect of amount of Chitosan (X_1) and Hydroxyapatite (X_2) were studied on entrapment efficiency of microspheres and coefficient observed for EE fitted in the equation.

Final Equation in Terms of Coded Factors

$$R1\text{-}EE\ (\%) = +82.258+4.23X_1+5.76X_2-4.65X_1X_2$$

Final Equation in Terms of Actual Factors

R1-EE (%) = + 82.25889 + 4.2333 Chitosan + 5.76500HA-4.64500Chitosan*HA

As seen from the above equation, positive correlation exists between the amount of chitosan and HA for the entrapment efficacy of microspheres. The entrapment efficiency of quercetin loaded chitosan-HA bio-composite microspheres was found to be within the range of 60.99 to 85.76%. It can be seen that upon an increase in the concentration of chitosan and HA, the EE (%) increased. This can be attributed to the amount of polymers available to coat the same amount of drug quercetin (20 mg). This trend is observed until the concentration of chitosan and HA is 300 mg and 150 mg.

Further increase in the concentration of the polymers leads to a decrease in EE (%) and an increase in size. This may be attributed to increase in the viscosity of the medium containing drug and polymer due to which uniform dispersion of HA and drug did not take place, hence larger precipitates were formed ultimately forming larger microspheres. Thus, batch QCH5 with a concentration of Chitosan of 300 mg and HA of 100 mg was considered an optimized batch. Data were analyzed statistically by one-way analysis of variance (ANOVA) using Design Expert11 software. As seen in **Table 6**,

The Model F-value of 85.25 implies the model is significant. There is only a 1.16% chance that an F-value this large could occur due to noise. Values of "Probability > F" less than 0.0500 indicate model terms are significant.

TABLE 6: ANOVA FOR RESPONSE SURFACE MODEL FOR ENTRAPMENT EFFICIENCY OF QUERCETIN LOADED BIOCOMPOSITE MICROSPHERES

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	448.67	6	74.78	85.25	**0.0116**	Significant
A-X1-CS	107.53	1	107.53	122.59	0.0081	
B-X2-HA	66.47	1	66.47	75.78	0.0129	
AB	86.30	1	86.30	98.39	0.0100	
A^2	176.85	1	176.85	201.61	0.0049	
B^2	7.28	1	7.28	8.30	0.1023	
A^2B	29.89	1	29.89	34.08	0.0281	
Residual	1.75	2	0.8772			
Cor Total	450.43	8				

Factor coding is coded. Sum of squares is Type III – Partial

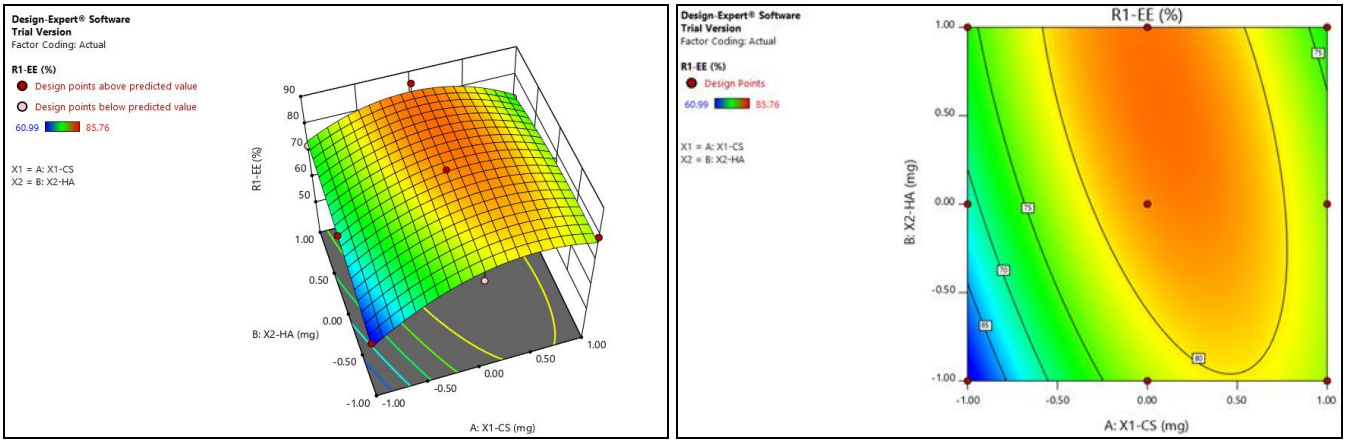


FIG. 1: 3D RESPONSE SURFACE GRAPH AND CONTOUR PLOT FOR EFFECT OF AMOUNT OF CHITOSAN AND HA ON ENTRAPMENT EFFICIENCY OF QUERCETIN MICROSPHERES

TABLE 7: MODEL FIT SUMMARY FOR ENTRAPMENT EFFICIENCY OF QUERCETIN LOADED CHITOSAN-HA BIOCOMPOSITE MICROSPHERES

Std. Dev.	0.9366	R^2	0.9961
Mean	74.72	Adjusted R^2	0.9844
C.V. %	1.25	Predicted R^2	0.8817
		Adeq Precision	30.3601

As seen in **Table 7**, the predicted R^2 of 0.8817 is in reasonable agreement with the adjusted R^2 of 0.9844; *i.e.* the difference is less than 0.2. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Hence, the ratio of 30.360 indicates an adequate signal. This model can be used to navigate the design space. The 3D Response Surface **Fig. 1** predicts optimum entrapment at chitosan concentration of 300 mg and HA concentration of 100 mg, hence showing QCH5 batch as an optimized batch.

Response 2: Effect on Mucoadhesion: Effect of amount of Chitosan (X1) and Hydroxyapatite (X2) were studied on mucoadhesion (%) of microspheres and coefficient observed for EE fitted in the equation.

Final Equation in Terms of Coded Factors

$$R2 - MA (\%) = +71.44+4.17X_1-5.33\ X_2-0.2500X_1X_2-19.17X_1^2-1.67X_2^2$$

Final Equation in Terms of Actual Factors

R2 - MA (%) = +71.444 + 4.16667 Chitosan - 5.333HA - 0.250 Chitosan *HA-19.166 Chitosan ² -1.666 HA²

As seen from the above equation, positive correlation exists between the amount of chitosan and mucoadhesion of microspheres whereas the presence of HA has a negative impact on the mucoadhesion of microspheres. Chitosan, being a mucoadhesive polymer, as the concentration of chitosan goes on increasing, mucoadhesion also is increased. This may be due to the availability of more polymer chains for entanglement with the mucus. But above a certain concentration (300 mg), the mucoadhesiveness of microspheres goes on decreasing. This may be due to the coiling of chitosan molecules at higher concentrations, reducing the flexibility of the polymeric chain and reducing mucoadhesion strength [16].

Hydroxyapatite on the other hand is bioceramic and does not possess mucoadhesion. It also crosslinks chitosan, hence as the amount of HA increases, mucoadhesion is decreased due to a reduction in a number of polymer chains available for entanglement with the mucus, but microspheres possess mechanical strength due to HA, which is desirable.

From the above results, batch QCH5 was considered an optimized batch. Data were analyzed statistically by one-way analysis of variance (ANOVA) using Design Expert 11 software and is represented in **Table 8**.

TABLE 8: ANOVA FOR RESPONSE SURFACE MODEL FOR MUCOADHESION OF QUERCETIN LOADED CHITOSAN –HA BIOCOMPOSITE MICROSPHERES

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1015.36	5	203.07	212.93	**0.0005**	Significant
A-X1-CS	104.17	1	104.17	109.22	0.0019	
B-X2-HA	170.67	1	170.67	178.95	0.0009	
AB	0.2500	1	0.2500	0.2621	0.6440	
A^2	734.72	1	734.72	770.39	0.0001	
B^2	5.56	1	5.56	5.83	0.0947	
Residual	2.86	3	0.9537			
Cor Total	1018.22	8				

TABLE 9: MODEL FIT SUMMARY FOR MUCO-ADHESION OF QUERCETIN LOADED CHITOSAN-HA BIOCOMPOSITE MICROSPHERES

Std. Dev.	0.9766	R^2	0.9972
Mean	57.56	Adjusted R^2	0.9925
C.V. %	1.70	Predicted R^2	0.9690
		Adeq Precision	42.3265

The Model F-value of 212.93 implies the model is significant. There is only a 0.05% chance that an F-value this large could occur due to noise. As seen in **Table 9**, the Model F-value of 212.93 implies the model is significant. There is only a 0.0005% chance that an F-value this large could occur due to

noise. Values of "Probability > F" less than 0.0500 indicate model terms are significant. The Predicted R^2 of 0.9690 is in reasonable agreement with the Adjusted R^2 of 0.9925; *i.e.* the difference is less than 0.2. Adequate Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 42.327 indicates an adequate signal. This model can be used to navigate the design space for optimization. The 3D response surface **Fig. 2** predicts optimum entrapment at chitosan concentration of 300 mg and HA concentration of 100 mg, hence showing QCH5 batch as an optimized batch. Observed responses were fitted to Design Expert Software version 11, the dependent variables demonstrated that the model was significant for both response variables (EE and mucoadhesion). Comparison between the experimental and predicted values for the most probable optimal formulation QCH5 is reported in **Table 10** which shows the desirability of 0.962. From the above responses, it can be concluded that as predicted values agree with experimental values, it demonstrates the feasibility of the model in the development of quercetin loaded Chitosan –HA biocomposite microspheres.

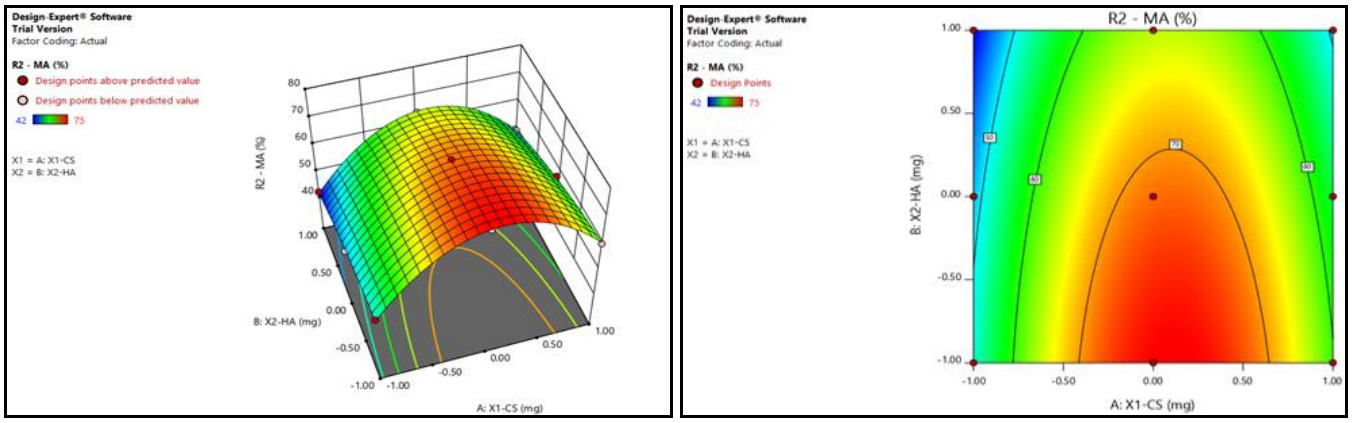


FIG. 2: 3D RESPONSE SURFACE GRAPH AND CONTOUR PLOT FOR EFFECT OF AMOUNT OF CHITOSAN AND HA ON MUCOADHESION OF QUERCETIN MICROSPHERES

TABLE 10: COMPARISON BETWEEN EXPERIMENTAL AND PREDICTED VALUES FOR OPTIMIZED FORMULATION QC5

Response (Dependent variables)	Predicted	Experimental
EE (%)	83	82.97 ± 0.72
Mucoadhesion (%)	71.2	72 ± 0.23
Desirability	0.962	

Evaluation of Optimized Microspheres:

DSC Analysis of Optimized Microspheres: As seen from DSC analysis of physical mixture **Fig. 3A**, three endotherms are seen at 114.62 °C, 190.41 °C and 313.83 °C corresponding to HA, chitosan and quercetin respectively indicating drug-excipient compatibility. **Fig. 3B** represents the DSC thermogram of QCH5 microspheres where endo-therm corresponding to quercetin is not seen, hence it can be said that the drug is entrapped into the microspheres. There was no peak corres-ponding to HA and chitosan but instead, a new endotherm was seen at 264.16 °C indicating desirable interaction between chitosan and HA in the final microspheres formulation.

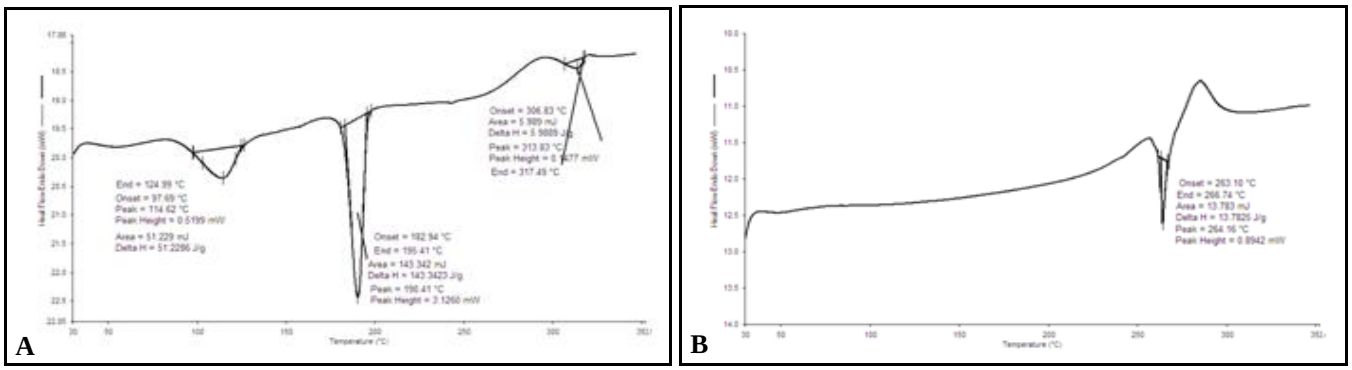


FIG. 3: DCS THERMOGRAM OF (A) PHYSICAL MIXTURE HA, CHITOSAN AND QUERCETIN AND (B) DCS THERMOGRAM OF QCH5 MICROSPHERES

Particle Size and Surface Morphology: The mean particle size of formulation QC5 and QCH5 was found to be 5560 nm and 7220 nm respectively with polydispersibility index (PDI) of 0.112 and 0.146 respectively indicating uniform size distribution of microspheres. Incorporation of HA increased the particle size of microspheres as shown in **Fig. 4**. As seen from SEM studies **Fig. 5** the microspheres obtained solely from Chitosan (QCH5) were spherical with a smooth surface and smaller size as microspheres obtained with a combination of Chitosan and HA (QCH5). Hence, the total surface area of the composite microsphere can be said to be more according to Rusu VM *et al.* [17]

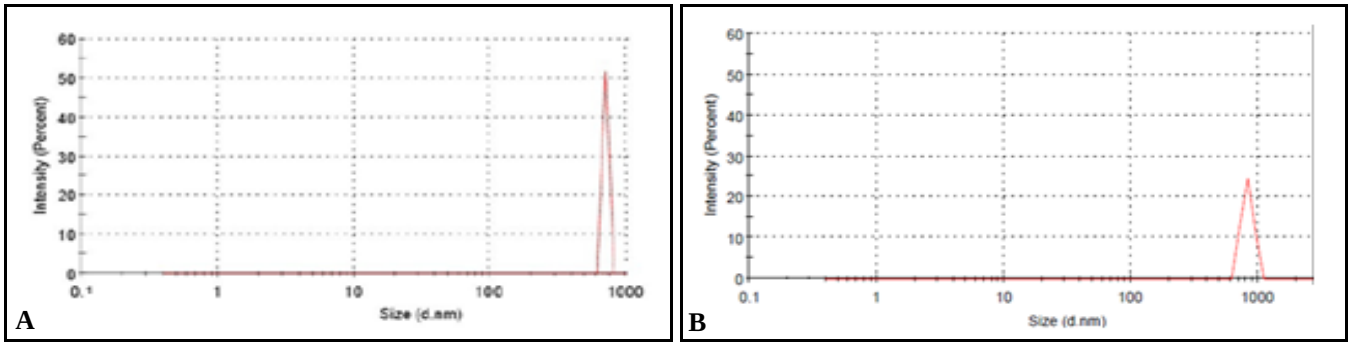


FIG. 4: PARTICLE SIZE ANALYSIS OF QC5 (A) AND QCH5 (B) MICROSPHERES

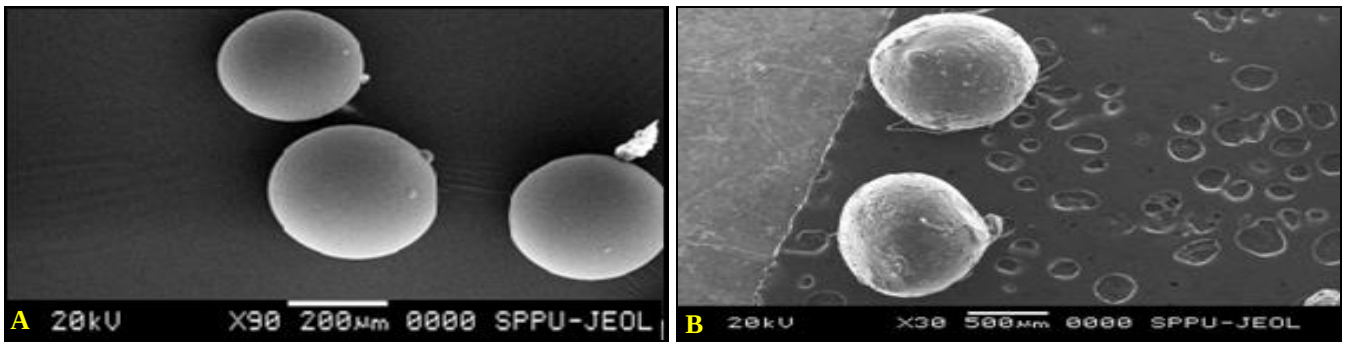
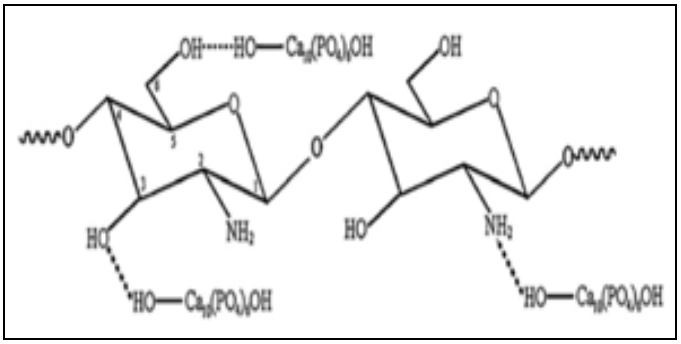


FIG. 5: SEM ANALYSIS OF MICROSPHERES QC5 (A) AND QCH5 (B)



HYDROGEN BONDS BETWEEN CHITOSAN AND HYDROXYAPATITE10

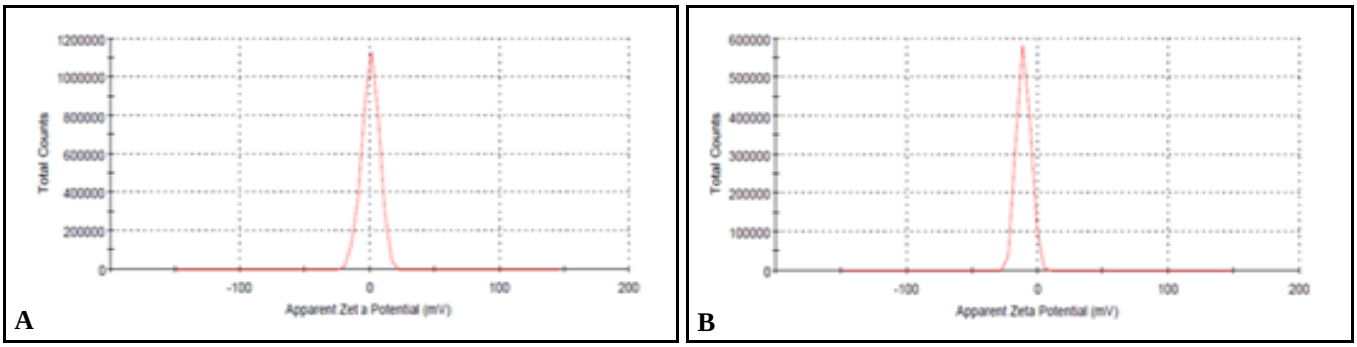


FIG. 6: ZETA POTENTIAL OF DRUG LOADED MICROSPHERE QC5 (A) AND QCH5 (B)

Zeta Potential: The zeta potential has a substantial influence on the stability of the formulation. As seen in **Fig. 6A**, chitosan microspheres were charged positively, although hydroxide ion (NaOH solution) was used as a precipitant. This indicates that only part of the amino group is neutralized during microsphere formation. The residual amino group would be responsible for the positive zeta potential which would prevent the aggregation of microspheres and thus leads to a stable formulation. As seen in **Fig. 6B**, chitosan is positively charged and the phosphate group in HA has a negative charge. This indicates that the amino group in chitosan is neutralized during composite microspheres formation. The residual phosphate group would be responsible for the negative zeta potential. This would prevent the aggregation of microsphere and thus leads to the stable formulation. It also indicates the interaction between Calcium Phosphate (in HA) and chitosan which is desirable.

Antimicrobial Activity of Quercetin Loaded Microspheres: Antimicrobial activity of quercetin against periodontal pathogens is already reported. Antimicrobial activity of the drug-loaded formulations was also studied. The results of the antimicrobial activity of the optimized formulation indicated that the drug release from the microspheres inhibited the growth of *E. coli* and *S. aureus*. This study was carried out for 48 h. It was found that the amount of drug released throughout the 48 shows the better zone of inhibition. From QCH5 microspheres, fewer drugs were released as compared to the QC5 because of HA. Thus, QC5 showed better antimicrobial activity in this 48 h study but as drug release from QCH5 is more prolonged than QC5 it will show more prolong antimicrobial action in the long run. The results are represented in **Table 11**.

TABLE 11: ANTIMICROBIAL ASSAY OF QUERCETIN FORMULATIONS

Formulation	*E. coli* (zone of inhibition in mm)	*S. aureus* (zone of inhibition in mm)
QC5	10 ± 0.54	13 ± 0.47
QCH5	5 ± 0.87	8 ± 0.96

***In-vitro* Release of Quercetin from Chitosan-HA (QCH) Microspheres:** Drug release from chitosan-hydroxyapatite microspheres was studied. Presence of hydroxyapatite retarded the release of quercetin from microspheres. As seen in **Fig. 7**, formulation QCH1 to QCH9 the release of the drug decreased as the concentration of hydroxyapatite increased. According to the disease which is targeted in this study *i.e.* periodontitis, it is important to have the bulk release of drug in the initial 24 h to kill the microbes already present in the periodontal pocket. At the same time, the delivery of drug should be such that it will prevent the re-infection at the same time, which is possible by a slow and gradual release of drugs for a prolonged duration. Thus, from the results obtained, batch QCH5 was considered ideal amongst other batches as it showed a dual-release mechanism.

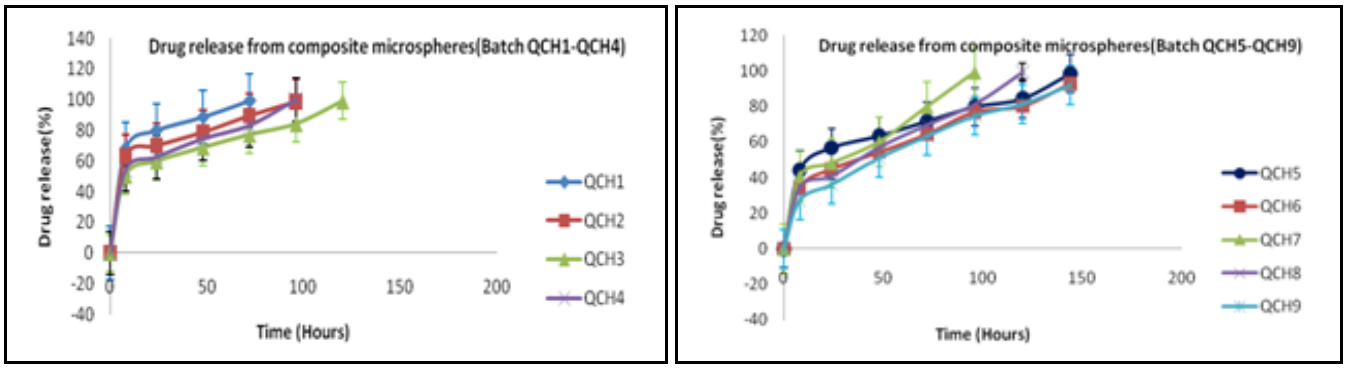


FIG. 7: *IN-VITRO* RELEASE OF QUERCETIN FROM CHITOSAN-HA BIOCOMPOSITE MICROSPHERES

***In-vitro* Release of Quercetin from Chitosan (QC) and Chitosan-HA Biocomposite Microspheres: Fig. 8** demonstrates the *in-vitro* release profile of quercetin from optimized chitosan microspheres in (formulation QC5). As seen in the plot, initially there was burst release of the drug may be due to unentrapped drug adsorbed onto the surface of microspheres. In the first 4 h, 45.82% drug was released while in the first 8 h 72.07% and 97.25% drug was released in 24 h. Microspheres formulated only with chitosan could sustain the release of the drug only for 24 h.

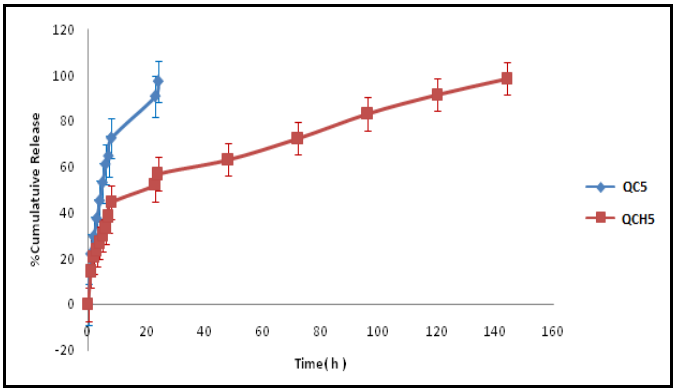


FIG. 8: DRUG RELEASE PROFILE OF QC5 AND QCH5 MICROSPHERES

But this short term treatment may not be satisfactory for treating periodontitis. **Fig. 8** also demonstrates the *in-vitro* release profile of quercetin from optimized chitosan-HA composite microspheres (formulation QCH5). As seen in the plot, the drug release was slow and gradual for a prolonged period of time. In the first 8 h, 44.40% drug was released 56.92% drug released in 24 h while 63.58% drug was released in 48 h and 98.58% drug released in 144 h. The controlled release of drug from microspheres would help to avoid infection for 6 days. The drug would release in the affected area for a prolonged period of time. The ability of chitosan-HA microspheres to sustain the release of drugs up to approximately 6 days was because of the addition of HA which increased interaction of chitosan with PO4 group leading to cross-linking. Also after interaction with the phosphate buffer solution, HA forms a stiff layer around microspheres, thus increasing the diffusion path length that the drug has to traverse, leading to the slow release of drugs for a prolonged period of 6 days. These mucoadhesive, biocomposite chitosan-hydroxyapatite microspheres loaded with quercetin would sustain the release of drugs and thus prevent reinfection throughout treatment as well as promote cell adhesion and in growth and would be retained at the affected site showing faster healing and improved efficacy.

CONCLUSION: The biocomposite combination of chitosan with hydroxyapatite provides a novel drug delivery having antimicrobial property and also fasten the reparative process by enhancing periodontal regeneration for treating periodontitis. The method used for the preparation of microspheres is simple and effective which does not make use of any hazardous chemical for cross-linking chitosan. Thus the developed microspheres in the present study are site-specific dosage forms, which would provide local delivery of drugs in a sustained manner, which may reduce systemic side effects of drugs by preventing their systemic absorption. Systemic absorption may eliminate bacteria from nondental sites, the risk for bacterial resistance development, dilution of the drug before it reaches the site of infection, super-infections, bacterial regrowth and ultimately patient non-compliance.

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CONFLICTS OF INTEREST: Nil

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