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Development of silver nanoparticle loaded enteric coated microspheres for the treatment of colon cancer

Deepa M K*[1], Suryaprakash T.N.K[2], Pawan Kumar[3]

1 Research Scholar, Department of Pharmacy and Medical Sciences, School of Pharmacy and Medical Sciences, Singhania University, Rajasthan, India.
2 Department of Pharmaceutics, Al Shifa College of Pharmacy, Kizhattur, Poonthavanam Post, Perinthalmanna, Kerala, India.
3 Department of Life Sciences, Singhania University, Rajasthan, India.

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*Corresponding author:

Email : deepa81mk@gmail.com
Tel.: +91 - 9656900511

ABSTRACT

The primary objective of the study was to develop Silver nanoparticles loaded targeted microspheres for colon rectal cancer formulated from bioreduction of silver nitrate solutions using *Ocimum sanctum leaves* extract. Silver nanoparticles have been characterized using UV-Visible spectroscopy, XRD, FTIR analysis and SEM. Microspheres were prepared by ionic gelation method using sodium alginate polymer. Optimized Microspheres were coated with Edragit S 100 in order to make the formulation as colon-targeted delivery system. They were subjected to various parameters such as drug entrapment efficiency, swelling index, and SEM analysis and particle size determination. SEM studies indicate that colon targeted microspheres are spherical in shape. Stability data recorded over 3 month period under room temperature condition. There is no significant variation in drug content indicated that the Formulation to be stable. Five different green synthesized silver nanoparticles loaded microspheres were prepared by orifice-ionic gelation method. The drug release data were subjected to curve fitting various kinetics models like zero order, first order, Higuchi model, and Korsmeyer-Peppas model. The *in vitro* kinetic data is subjected to log time log drug release transformation plot (Korsmeyer Peppa's plot), revealed the fact that the drug release follows super case II transport diffusion. So we conclude that the novel formulation may expect to have a potent effect on colon cancer.

INTRODUCTION

Nowadays cancer has been a research area of particular interest for nano-sized drug carriers due to the enhanced permeability and retention effect which is thought to provide them with significant therapeutic benefit when compared to other small molecule chemotherapy drugs [1-3].

The colon target is the interesting field of research as a site where poorly absorbed drug molecule may have an improved bioavailability. Oral delivery of drugs to the colon is valuable in the treatment of colon diseases like carcinomas, ulcerative colitis, Crohn's disease, and infection.[4].Colorectal cancer is considered as the third most common cause of cancer related death in human being.[5]

Inorganic nanoparticles have received increased attention in recently as potential diagnostic and therapeutic systems in the field of oncology. Inorganic nanoparticles have established successes in imaging and treatment of tumors both *ex vivo and in vivo*, with some promise towards clinical trial.[6-7]

Chemical and physical methods have been developed for the synthesis of silver nanoparticle, but they are found to be expensive as well as the use of a choice of toxic chemicals for their synthesis makes the biological synthesis the more preferred option. Though bacterial, fungal, and plant extract sources can be used for nanosilver synthesis, anyway, the easy availability, the nontoxic nature, the various options available, and the advantage of quicker synthesis make plant extracts the best and an excellent choice for nanosilver synthesis. The uses of silver nanoparticles are varied and many, but the most exploited and desired aspect is their antimicrobial capacity, anti-inflammatory capacity and anticancer can activity.[7-8]

Synthesis of nanoparticles by using plant extract ie green chemistry approach which interconnects nanotechnology and plant biotechnology. The technique for obtaining nanoparticles using naturally occurring reagents such as plant extracts could be considered eco-friendly and attractive for nanotechnology. Plant parts such as leaf, root, latex, seed, and stem are being used for nanoparticle synthesis.

Ocimum sanctum is a widely available common plant in the Indian system, *Ocimum sanctum* (also known as *Ocimumtenui florum*,Tulsi) is popularly used as herbal remedy for various ailments. But the scientific basis for its medicinal use especially in pain and which has been used as antimaterial, anticancer etc [9]

In the present work is an attempt to formulate silver nanoparticles using an aqueous extract of *Ocimum sanctum* plant and develop green synthesized silver nanoparticle loaded colon targeted microspheres for the treatment of colorectal cancer.

MATERIALS AND METHODS

Materials

Silver nitrate, Sodium alginate, de-ionized water and Eudragit S-100 were purchased from Kerala Scientific Company. (Kerala, India) respectively.All polymers and chemicals used were of analytical grade.

Methods

Preparation of Eudragit S-100 coated sodium alginate microspheres involved three steps, *i.e.*, the step I and step II. In step I, silver nanoparticle was synthesis from *Ocimum sanctum* plant extract and the second step which is loaded in sodium alginate microspheres andin 3rd step, optimized formulations from 2nd steps were coated with Eudragit S-100 polymer to prevent the release of drug content in the stomach and small intestine. Procedures of step I , step II and step III were as follows [10-12].Silver nanoparticles loaded colon targeted microspheres was developed by three steps in the first phase silver nanoparticles were formulated from bioreduction of silver nitrate solutions using *Ocimum sanctum* leaves extract and in the second phase the prepared silver nanoparticles were loaded in alginate microspheres and finally, optimized formulation form step II is coated with Eutagit S100.

Collection of plants

The plant *Ocimum sanctum* was collected from local place of Kalpathy, Palakkad and It has been identified and authenticated by Dr. Udyan P.S., Professor, Sree Krishna College, Guruvayur, Thrissur, Kerala, India.

Preparation of *Ocimum sanctum* leaves extract

Ocimum sanctum leaves were washed several times with de-ionized water. 50g of the leaves was finely cut and boiled with 500ml de-ionized water at 100^{0}C for 5minute and filtered to get the extract.

Formulation of silver nanoparticles by using *Ocimum sanctum*

For the preparation of silver nanoparticles, 2ml of aqueous leaf extract was added to the 100ml standard flask containing 98ml of AgNO3 (10^{-3}M) and the mixture was placed at room temperature. As a result, a yellowish-brown solution was formed, indicating the formation of silver nanoparticles. The contents were settled for 15 minutes and the silver nanoparticles were collected.

Formulation and evaluation of silver nanoparticles loaded microsphere

Microspheres were prepared by orifice-ionic gelation method which involved a reaction between sodium alginate and calcium to produce calcium alginate. Sodium alginate was used as mucoadhesive polymers.

Five different green synthesized silver nanoparticles loaded microspheres were prepared (SNF1 to SNF5) by ionic gelation technique with different ratios of the polymer blend, Sodium alginate and silver nanoparticle such as 1:1,1:2,1:3,1:4 and 1:5 respectively was shown in Table[1]. The polymer and silver nanoparticles in various ratios were dispersed in purified water (50ml) to form a homogeneous polymer mixture. The smooth dispersion was added to 4% calcium chloride solution dropwise through needle size #21. The addition was done with continuous stirring. The added droplets were retained in the calcium chloride solution for 15minutes to complete the curing reaction and to produce spherical rigid microspheres. The microspheres were collected by decantation, and the product thus separated washed repeatedly with water to remove excess calcium impurity and air dried.

Preparation of Eudragit S-100 coated microspheres

The optimized batch (SNFE5) of sodium alginate microspheres was coated with Eudragit S-100 according to the composition given in Table 2. Core microspheres were dispersed in a Eudragit S-100 solution (4 %, *m/V*) in acetone and isopropyl alcohol solution at room temperature, followed by emulsification in light liquid paraffin containing Span 80 (2 %, *V/V*) in a beaker with the help of a mechanical stirrer (propeller type) at 500 rpm. The system was agitated for 3 h at room temperature to allow

Table 1 : Formula and composition of green synthesized silver nano microspheres

Sl No	Formulation code	Ratio of silver nanoparticle and sodium alginate	Calcium chloride (%)
1	SNF1	1:1	4
2	SNF2	1:2	4
3	SNF3	1:3	4
4	SNF4	1:4	4
5	SNF5	1:5	4

Table 2 : Composition of Eudragit coated green synthesized silver nanoparticle loaded microspheres

Sl No	Formulation code	Silver nanoparticle polymer ratio	Core: coat ratio
1	SNFE5	1:5	1:10

solvent evaporation.Finally, encapsulated microspheres (SNFE5) were filtered and washed with petroleum ether to remove the traces of oil and dried in a vacuum desiccator for 24 h.

Characterization Of Silver Nanoparticle loaded Microspheres

FT-IR spectroscopy

The FT-IR spectra of silver nanoparticle were recorded (Mode spectrum RX 1, Perkin Elmer, England) using the potassium bromide disk method.

Shape and Surface Morphology

Microspheres were suspended in water a drop was placed on a glass slide, covered with a cover slip and viewed under the optical microscope to examine their shape.The surface morphology of microspheres was observed scanning electron microscopy (SEM). The samples for SEM were prepared by lightly sprinkling the microspheres powder on a double adhesive tape which stuck to an aluminum stub. The stubs were then coated with gold to a thickness of about 300°A using a SEM was used to investigate the morphology as well as the particle size of microspheres.

X-ray diffraction analysis (XRD)

XRD analysis investigates the crystallinity of the silver nanoparticles with an X-ray diffractometer was recorded (XPERT-PRO, PAN analytical, The Netherlands) using the PRS measurement program using Ni-filtered, CuKa radiation with a voltage of 40 kV and a current of 35 mA. The instrument was operated at continuous scanning speed over 2θ range of 20° to 80°.

Surface morphology

Shape and surface morphology of both core and coated microspheres were observed using scanning electron microscopy (JSM 6100 Jeol, Japan). Samples mounted on an aluminum stub were sputter coated with gold under reduced pressure and a thick gold coat was applied using a JFC 1100 (Japan) sputter coater. The sample assembly was placed in the microscope and vacuum was applied. These samples are randomly scanned and photomicrographs were taken, which are shown in Figures 1 and 2.The microspheres were observed under scanning electron microscopy (SEM).

Drug loading and drug loading efficiency

To determine the drug content in microspheres, an accurately weighed quantity of microspheres equivalent to 20 mg of the drug was crushed and dissolved in 100 mL phosphate buffer pH 7.4 in a volumetric flask and stirred for 12 h . After stirring, the solution was filtered through Whatman filter paper, the filtrate was diluted using Phosphate buffer pH 7.4 and absorbance were measured for the determination of un entrapped drug at 272 nm using a UV/Visible spectrophotometer (Systronics, Mumbai, India). calculate drug loading and drug loading efficiency by using the formula [13,14]

In vitro drug release study

Core microspheres

Microspheres equivalent to 2 mg of silver nanoparticle were weighed accurately and suspended in 20 mL of Phosphate buffer pH 7.4 . The mixture was stirred at 37 °C using a magnetic stirrer at a stirring speed of 50 rpm for 3 hr.At specified time intervals, samples were withdrawn (2 mL) and replaced with the same volume of fresh media. The withdrawn samples were centrifuged at 3000 rpm for 10 min and were then filtered and diluted with phosphate buffer pH 7.4. The drug content was measured by taking supernatant absorbance using a UV/Visible spectrophotometer (Systronics, Mumbai, India).

Coated microspheres.

Microspheres equivalent to 100 mg of silvernanoparticle were weighed accurately and suspended in 20 mL of 0.01 mol L1 HCl. The mixture was stirred on a magnetic stirrer at 37 °C at a stirring speed of 50 rpm for 2 h. Samples were withdrawn at specified intervals and an equivalent amount of fresh medium was added. Collected samples were centrifuged, filtered through a membrane filter (0.45 µm) and analyzed for drug content using a UV/Visible spectrophotometer. and stirred magnetically at 50 rpm. The pH of the medium was maintained at pH1.2 for 2 h and then it was replaced by phosphate buffer pH 7.4.Two milliliters of aliquots were withdrawn at predetermined intervals with a replacement of the same volume of fresh medium. The samples were centrifuged, filtered and analyzed for drug content at 272 nm using spectrophotometry.

Drug release kinetics.

The *in vitro* drug release patterns were fitted to various release kinetic models, zero order, first order, Higuchi model and Korsmeyer-Peppas power law equation.

Stability studies

Three different batches of all formulations were subjected to stability studies according to the International Conference on Harmonization (ICH) guidelines. Uncoated and coated microspheres were put into hard gelatin capsules wrapped in aluminum foil laminated on the inside with polyethylene. The samples were kept at room temperature and under accelerated conditions in a stability chamber (Stability Oven, Nirmal Instruments, India). Real time stability studies were performed by periodical testing of the drug content at intervals of 0, 30, 60 and 90 days during 3 months.

$$\text{Drug loading Efficiency} = \frac{\text{Estimated \% Drug Content}}{\text{Theoretical \% Drug Content}} \times 100$$

RESULTS

Table 3 : Composition, particle size and drug loading efficiency of formulations SNF1-SNFE5

Sl No	Formulation code	Particle size(µm)	Drug loading efficient (%)
1	SNF1	150±1.62	74±0.32
2	SNF2	158±1.12	75±0.35
3	SNF3	169±1.42	76±0.26
4	SNF4	179±1.13	79±0.23
5	SNF5	200±1.34	88±0.28

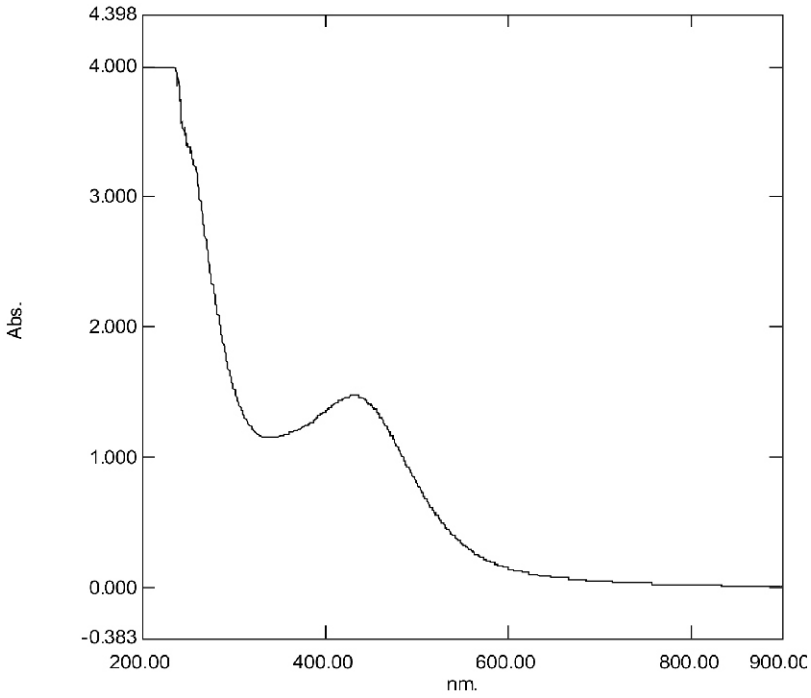

Fig 1 : uv -Visible spectrum of synthesized silver nanoparticles

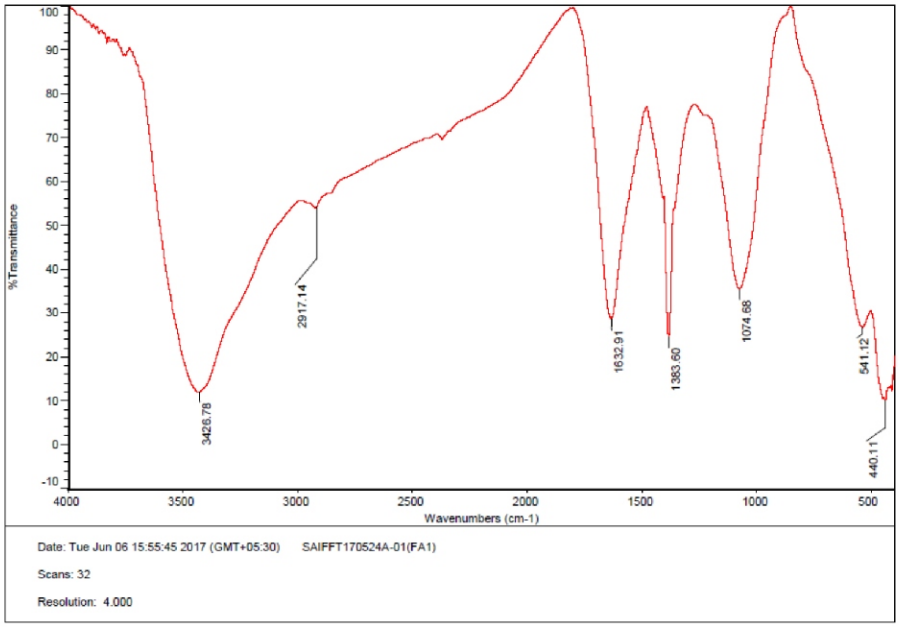

Fig 2 : FTIR spectra of silver nanoparticles

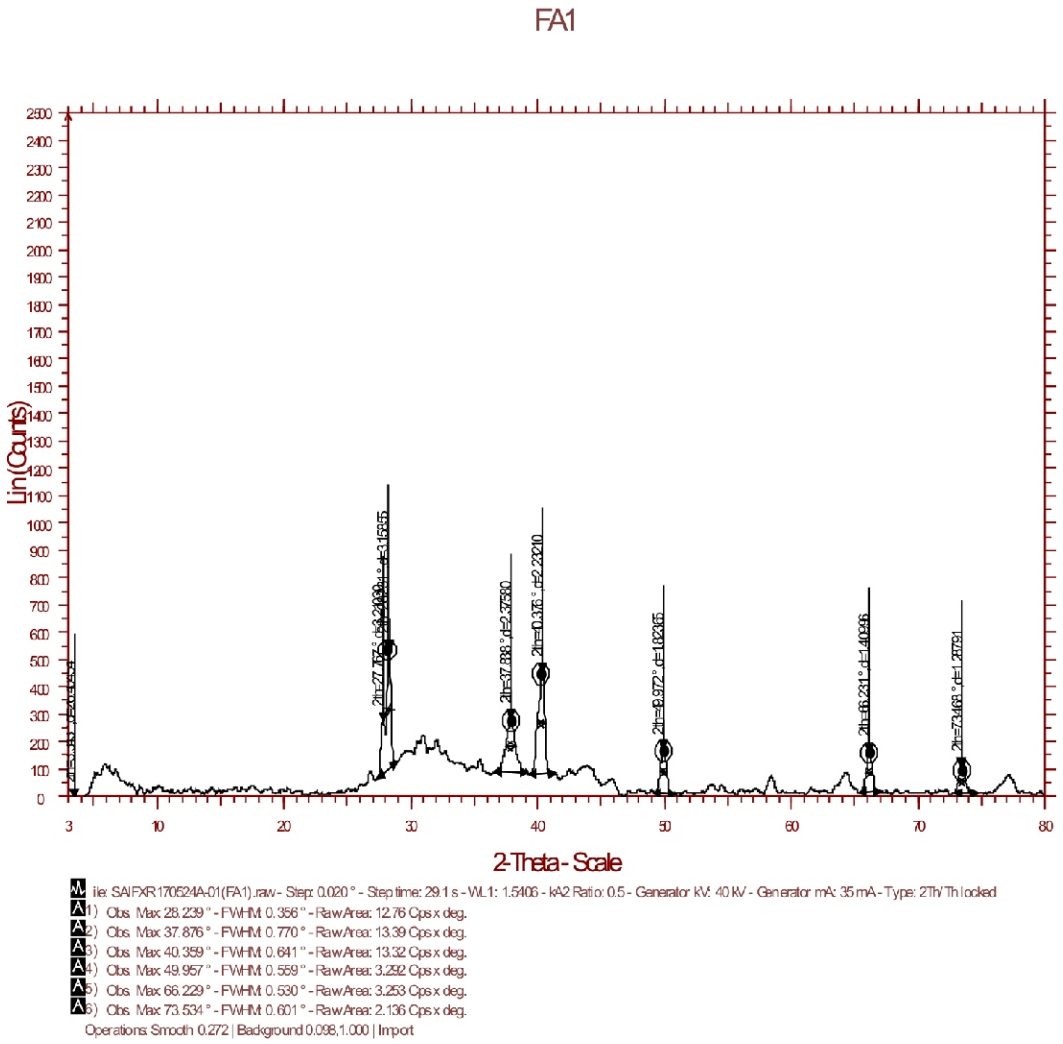

Fig 3 : XRD pattern of synthesized silver nanoparticles.

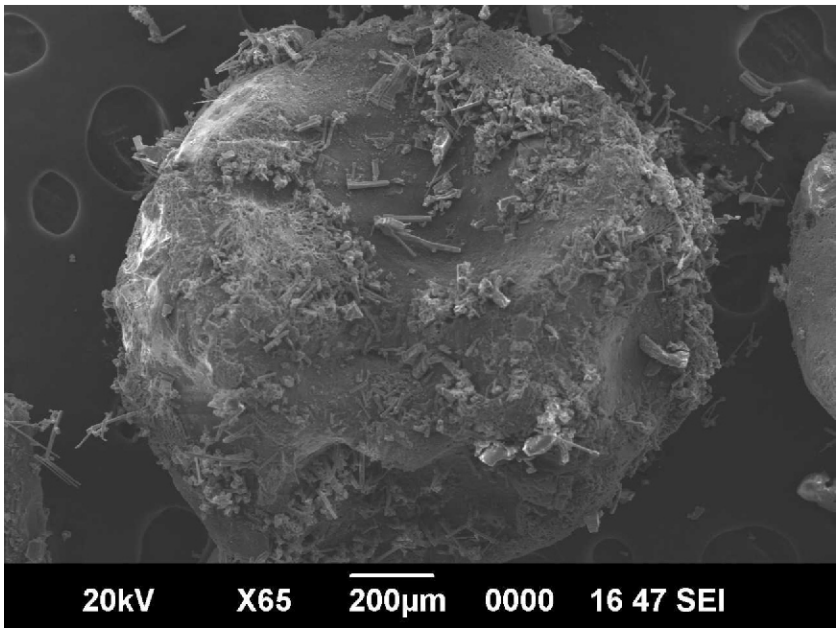

Fig 4 : SEM analysis images

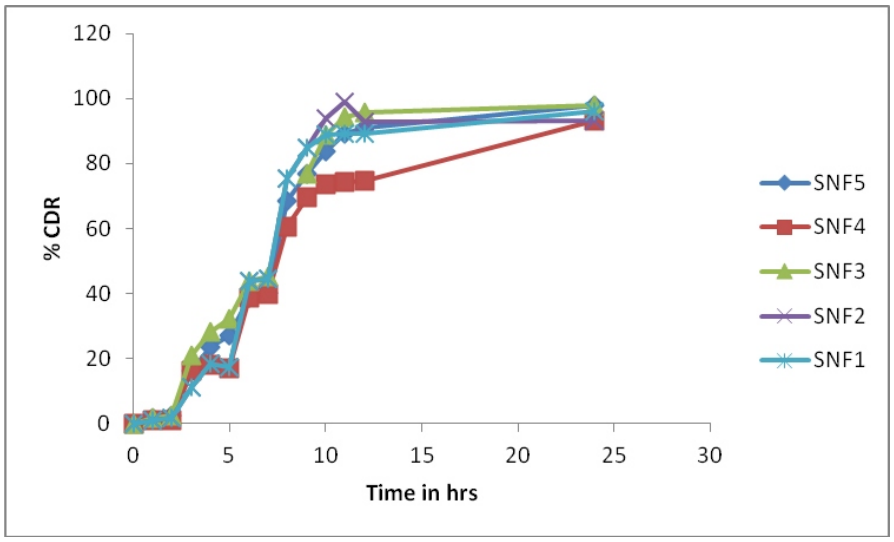

Fig 5 : Drug release profile of silver nanoparticles loaded microspheres SNF1-SNF5

CURVE FITTING ANALYSIS

Zero order kinetics

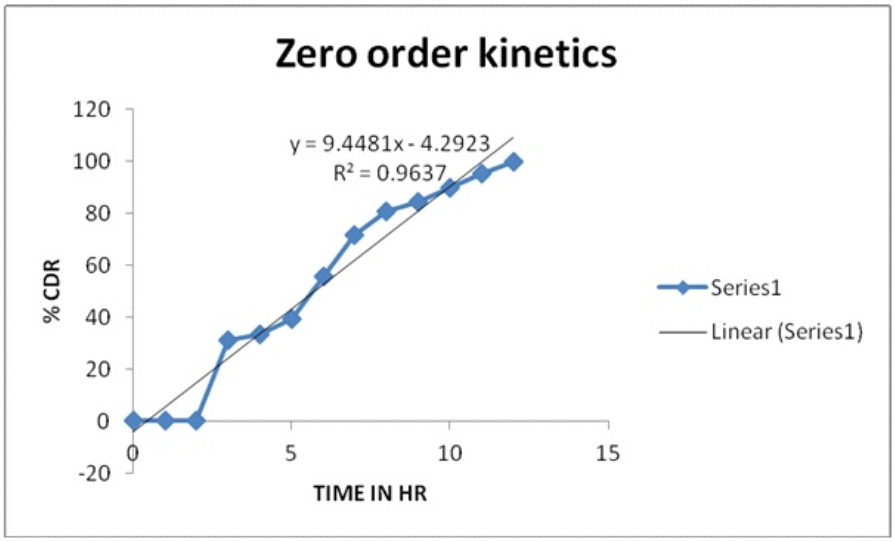

Fig 6 : Graph of zero order kinetics

First order Kinetics

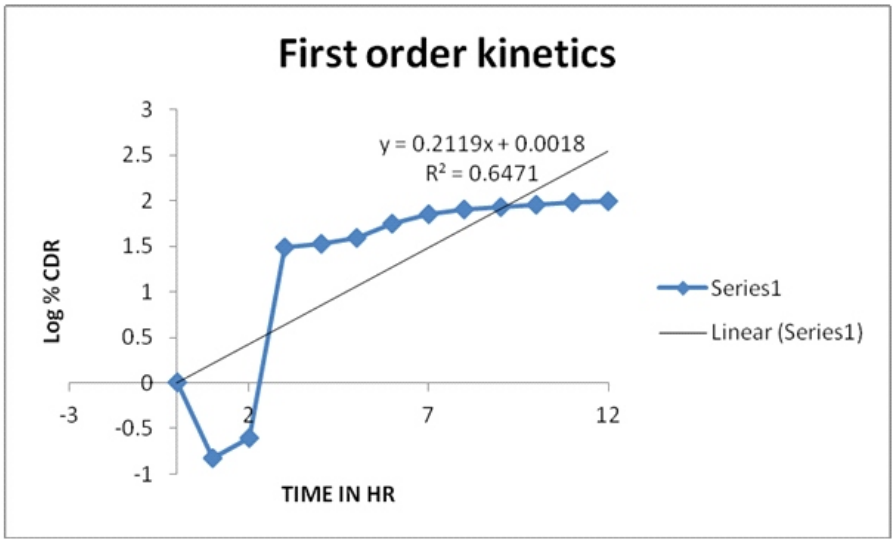

Fig 7 : Graph of First order kinetics

Higuchi kinetic model

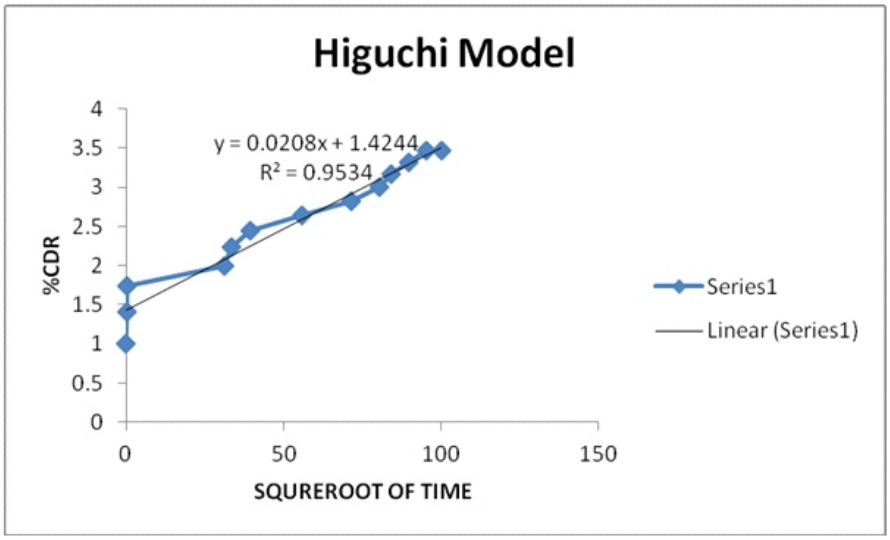

Fig 8 : Graph of Higuchi model

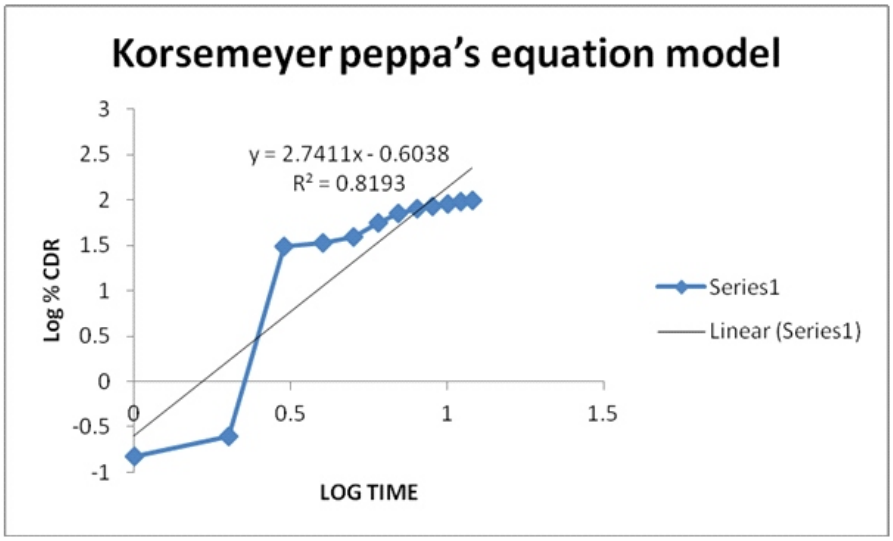

Fig 8 : Graph of Korsmeyer Peppa's equation mode

Table 4 : Correlation coefficient and diffusion exponent in curve fitting analysis of drug release data as per various kinetic models

Optimized Formulation	Zero order		First order		Higuchi model		Korsmeyer Peppa's model	
	R^2 value	n value	R^2 value	n value	R^2 value	n value	R^2 value	n value
SNF5	0.9637	9.4481	.6471	0.2119	.9534	.0208	0.8193	2.7411

Table 5 : Evaluation result of Stability study

Sl No	Formulation code	Month	Drug content
1	SNFE5	0 1 2 3	96.3±0.05* 96.3±0.03* 96.2±0.03* 96.2±0.02*

*SD ± (n=3)

DISCUSSION

Silver nanoparticles were synthesized by leaf extract of *Ocimum sanctum*, it was observed there was a color change from pale yellow to brownish red. This indicates that the reduction of the silver particle in presence of plant extract and the stable product was obtained in dark brown color within 30 mins. Ocimum sanctum leaves have been reported to contain chemical like ursolic acid, rosmaric acid, luteolin, eugenol and oleic acid these water soluble in credits present in the extract are responsible for the reduction of metal ions and efficient stabilization of nanoparticles.

The UV-Visible spectral analysis of the green synthesized nanoparticles was observed a sharp peak at 430 nm indicates the formation of silver nanoparticles as shown in Figure(1). The optical properties of silver nanoparticles change when particles aggregate and the conduction electrons near each particle surface become delocalized and are shared amongst neighboring particles. The surface Plasmon resonance shifts to low energies, causing the absorption and scattering peaks to red-shift to longer wavelengths.

IR measurements were carried out to identify the possible biomolecules responsible for the capping and efficient stabilization of the silver nanoparticles synthesized by the plant extracts. According to Figure (2) the band at 2917.14 cm^{-1} for C-H stretching and the band at 1017 cm^{-1} can be assumed as absorption peak of C-O-C and band at 1383 cm^{-1} in silver nano may attribute to c-o stretching mode which is already reported.[12] FTIR analysis corresponding to vibrational bands such as C=O,-C-O,-C-O-C are derived from compounds such as phenols, flavonoids, and terpenoids present in *Ocimum sanctum* leaves. Hence it may be assumed that phenolic and terpenoid were responsible for capping and efficient stabilization.similar spectra were reported in previous studies [15,16]These biomolecules contribute for the efficient stabilization of silvernanoparticle is suggested by studying the FTIR spectrum.

The crystalline nature of Ag nanoparticles was confirmed by the X-ray diffraction (XRD) analysis. Five diffraction peaks observed at 28.239, 37.876, 49.957, 66.229 and 73.5345 in the 2θ range 20^{o}- 80^{0} can be described to the (111) , (200) , (220), (311) and (222) reflection planes of a face- centered cubic (fcc) structure of Ag phases(JCPDC, file no:04-0783).Similar results were reported by Daizy Philip et.al[15]. XRD pattern of dried powder of gold nanoparticles was shown in the Figure(3)

Five different green synthesized silver nanoparticles loaded microspheres were formulated (SNF1 to SNF5) were prepared by using different ratios of alginate and sivernanoparticle blend with 4% calcium chloride solution as shown in Table (1) As showed in Figure(4) microspheres displayed a spherical shape and no aggregation was observed. No difference was observed in the morphological properties of microspheres due to the presence of the silver nanoparticle as reported by Bigucci etal[13]. The Entrapment efficiency for Formulations SNF1 to SNF5 ranges from 75 to 97% is given Table (2). Drug entrapment efficiency found to be that by increasing the ratio of a silver nanoparticle to sodium alginate, there was an increasing in percentage entrapment of silver nanoparticle. All the formulations The best (SNFA1-SNFA5) subjected to *in-vitro* release study. Dissolution study is carried about in 2 hours in 0.1N HCl (ie gastric pH 1.2) using USP Type II dissolution apparatus (Electrolab TDT-08L) at 50 rpm. At end of second hr dissolution media was changed as phosphate buffer 7.4.study is carried about 12 hrs and the drug release profiles were given in Figure (5).

The optimized batch (SNF5) of sodium alginate microspheres was coated with Eudragit S-100 according to the composition given in Table 2. The optimized Formulation (SNFE5) was further subjected to *invitro* release study. Sustained release of drug from polymer matrix is a combined effect of cross-linked polymer network of sodium alginate. The drug release was retarded up to 12 hours. The invitro performance of silver nanoparticles microspheres showed prolonged and sustained release. from *invitro* dissolution study it was found that 94.72% of the drug was released within 12 hours in alkaline pH, there for the Formulation (SNFE5) may reduce the frequency of dosing of therapy, thereby minimizing the occurrence of side effects, increase residence time in the stomach and increase the effectiveness of a drug.

The drug release data were subjected to curve fitting various kinetics models like zero order, first order, Higuchi model and Korsmeyer-Peppa's model and the graphs were plotted as shown in Figure(6-9).The mechanism of drug released was found to be diffusion controlled by curve fitting analysis of drug release profile data given in Table (4).The *invitro* kinetic data is subjected to log time log drug release transformation plot (Korsmeyer Peppa's plot), slope values were obtained as 2.7411 ($n>1$) revealed the fact that the drug release follows super case II transport diffusion, possibly owing to chain disentanglement and swelling of the hydrophilic polymer.

Optimized formulation (SNF5) was subjected to Stability study as per (ICH) guidelines, observed that there is no significant variation in drug content analysis. i.e. which is significantly stable within the study period. The drug content from the microspheres was compared by statistical analysis using one-way ANOVA, followed by Dunnett's test. A difference was considered statistically significant at a *p*-value less than 0.05 as shown in Table(5).

CONCLUSION

Data obtained from this study demonstrate that sodium alginate microspheres containing Silver nanoparticle were successfully prepared, followed by coating with the pH-sensitive polymer Eudragit S-100. The study also concluded that sodium alginate microspheres (core microspheres) that showed complete drug release within 12h. sodium alginate microspheres were unable to target drug release into the colonic region. Hence, this indicates that there is a need for enteric coating,*i.e.*, Eudragit S100 coating, on sodium alginate microspheres to target drug release to the colon. Coated microspheres showed a longer residence time in the colon after removing the Eudragit S100 coating due to better mucoadhesion properties of sodium alginate. The *in vitro* release profile revealed that microspheres retard drug release in the upper part of GIT due to the pH-sensitive polymer coating. Hence, Eudragit S-100 showed promising drug delivery to the colon.It was concluded that the optimized Formulation (SNF5) could be able to deliver drug for long period of time in intestinal pH. The drug release data were subjected to curve fitting various kinetics models. The *in-vitro* kinetic data is subjected to log time log drug release transformation plot (Korsmeyer Peppa's plot), revealed the fact that the drug release mechanism follows super case II transport diffusion. For sustained release systems, the oral route of drug administration has, by far, received the most attention as it is natural, uncomplicated, convenient and safer route. Oral sustained release site specific drug delivery systems formulated is proved to be useful in the pharmaceuticals for its ease of

formulation, enhanced stability, faster production, avoid degradation by moisture and/or thermal treatment and hydrolytic or oxidative reactions occurred during processing of dosage forms.

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