The Pharma Innovation

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Synthesis and evaluation of chitosan-alginate microspheres loaded with various combinations of enrofloxacin and selected phytochemicals against pathogenic bacteria

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Abstract
Enrofloxacin in combination with phytochemicals such as Curcumin (CUR), Piperin (PIP), Cinnamic acid (CIA), Caffeic acid (CAA) and Syringic acid (SYA) exhibits notable synergism against pathogenic bacteria. Chitosan-alginate encapsulated microspheres containing enrofloxacin and phytochemicals prepared and evaluated for their synergistic effect and reduction in individual agent's disadvantages. CS-ALG microspheres were prepared by impregnating enrofloxacin (CS-ALG-EN) alone and in combination with respective phytochemicals such as CS-ALG-EN-CUR, CS-ALG-EN-PIP, CS-ALG-EN-CIA, CS-ALG-EN-CAA & CS-ALG-EN-SYA and evaluated for shape, size, loading efficacy, release kinetics of enrofloxacin and MIC of enrofloxacin along with various phytochemicals against MTCC and clinical isolate bacteria. Microspheres were spherical. When combined with phytochemicals the enrofloxacin loading efficacy decreased variably with respective phytochemicals. The % cumulative release of enrofloxacin from all microspheres was maximum at pH 1.2 and further increased at pH 6.8. CAA and SYA improved the release and CIA, CUR and PIP decreased the release of enrofloxacin from respective microspheres compared to CS-ALG-EN. The dissolution efficacy increased by addition of SYA, CAA while PIP, CIA and CUR decreased. The mean dissolution time is same in PIP, SYA, CAA while CIA showed lowest and CUR highest when compared with enrofloxacin alone loaded microspheres. The release of enrofloxacin followed korsmeyer-peppas model by following Fickian diffusion/Quasi-Fickian diffusion from spheres. The MIC of enrofloxacin significantly lowered in combination with CUR, PIP, CIA, CAA on both MTCC and clinical isolates of pathogenic bacteria. In conclusion chitosan-alginate encapsulation improved the bioavailability of enrofloxacin and phytochemicals and combination showed synergistic antibacterial effect.

Keywords: Chitosan-alginate microspheres, enrofloxacin, phytochemicals

Introduction
The advent of antimicrobial resistance has necessitated look out for novel agents effective against resistant organisms and therefore search for new classes of antibacterial substances, especially from natural sources is gaining momentum. Certain phytochemicals have been reported to be effective and economical as alternatives to antimicrobial agents not only in the treatment of infection but also to counter bacterial resistance [1]. It has been well documented that several plant species like turmeric and pepper possess microbiostatic and microbiocidal activities against a range of pathogens [2] due to the presence of various phyto-chemicals [3]. Various mechanism of actions of antimicrobial activity for phytochemicals such as curcumin and piperine were postulated such as binding and complex formation with adhesion to cell wall of bacteria and inactivating enzymes of bacteria through reaction of SH groups (CAA and CIA), disrupting cell membrane of bacteria and intercalating into the cell wall and causing breaks in the continuity of cell wall leading to leakage of cell contents [4].
Further, the addition of phyto-constituents to synthetic and semi synthetic antimicrobial agents has produced notable synergistic effects during the therapy of infectious diseases [5]. Enrofloxacin, an antimicrobial fluroquinolone exclusively available for veterinary use and has wide spectrum of antimicrobial activity with good bioavailability even at very low concentrations [6]. Enrofloxacin was also reported to possess significant post antibiotic effect against both Gram-negative and Gram-positive bacteria.
In recent past, there were reports that E. coli and other microorganisms have developed

resistance to enrofloxacin. Hence, combining enrofloxacin with a phytochemical can result in a novel preparation capable of countering resistant organisms. However, the low water solubility of enrofloxacin and erratic absorption of phytochemicals from GIT is a major challenge in the development of novel therapeutic preparation [7]. Encapsulation of various therapeutic agents using bio-polymers can overcome these problems. Chitosan, a biodegradable polymer improves the transport of drug across biological membranes through adhesion and increases paracellular permeation and absorption of drugs *in vitro* and *in vivo* [8, 9, 10]. Chitosan microspheres are used as drug carriers to deliver drug to the areas of interest and to slowly release the encapsulated drug over a desired period of time to maintain an effective local drug concentration [11].

Keeping all these facts as backdrop, the present study was carried out to synthesize, characterize and evaluate chitosan-alginate encapsulated microspheres containing enrofloxacin in combination with phytochemicals (PC) such as Curcumin (CUR), Piperine (PIP), Cinnamic acid (CIA), Caffeic acid (CAA) and Syringic acid (SYA) for their antibacterial activity against standard MTCC cultures and clinical isolates.

Materials and Methods
Drugs, chemicals and medias
All the chemicals and microbial culture media are analytical grade and obtained from the commercial sources like Sigma-Aldrich Chemicals, Pvt, Lobachemie, Thermo fisher Scientific (India), Hi-Media Ltd. Pure enrofloxacin (99%) was supplied as gratis from INTAS Pharma, Pvt, Ltd, Mumbai.

MTCC reference microbial cultures
Enterococcus faecalis, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Streptococcus pyogenes* and *Staphylococcus aureus* were procured from MTCC, Institute of Microbial Technology (IMTECH), Chandigarh, India.

Clinical isolates
E. coli, *Klebsiella* spp., *Pseudomonas* spp., *Salmonella* spp. and *Staphylococcus aureus* were supplied by the department of Veterinary Microbiology, NTR College of Veterinary Science, Gannavaram.

Preparation of chitosan microspheres with enrofloxacin
Chitosan microspheres impregnated with enrofloxacin were prepared as described with slight modified method Srinatha *et al*, 2008 [12]. In this process 0.4 g of Enrofloxacin was added to 20 mL of 2.5% sodium alginate. This solution was added (30 mL/h) drop wise under constant stirring to 100 mL of 0.1% chitosan in 2% acetic acid and 1.5% calcium chloride solution (pH adjusted to 5.5 using 10% NaOH). The stirring was continued for an hour for the polymerization of chitosan and alginate. The microspheres formed were filtered and washed thrice with distilled water. Finally, acetone was added for drying the microspheres. The drying of microspheres was confirmed by achieving constant weight on consecutive days. The microspheres were stored in a cool and dry place at 4 ℃ and were designated as CS-ALG-EN.

Preparation of chitosan microspheres with enrofloxacin and various phytochemicals
Microspheres were prepared as per the procedure mentioned above, in this 10 mg of respective phytochemicals such as CUR, PIP, CIA, CAA and SYA was added along with 0.4 g of enrofloxacin to 20 mL of 2.5% sodium alginate to prepare respective phytochemical and EN microspheres. The microspheres were stored in a cool and dry place at 4℃ and were designated as CS-ALG-EN-CUR (Curcumin), CS-ALG-EN-PIP (Piperine), CS-ALG-EN-CIA (Cinnamic acid), CS-ALG-EN-CAA (Caffeic acid) and CS-ALG-EN-SYA (Syringic acid).

Shape and size of microspheres
The shape and size of the microspheres were evaluated using optical microscope and micrometry. As the microspheres were irregular after drying, the longest diameter was measured for fifty microspheres to arrive at an average diameter.

Loading efficacy of enrofloxacin and phytochemical microspheres
Twenty five mg of the microspheres were crushed into powder and treated with 47.5 mL of 0.1N HCl and 2.5 mL of methanol. The resulting mixture was stirred at 250 rpm. The temperature was maintained at 37±0.2℃. At the end of two hours, the solution was filtered and analyzed using UV-vis spectrophotometer at 271 nm for enrofloxacin, 428 nm for CUR, 342.5 nm for PIP, 290 nm for CIA, 327 nm for CAA and 327 nm for SYA. The concentrations of enrofloxacin and respective phytochemicals were determined by comparing with standard curves of respective drugs and expresses in mg%.

In vitro enrofloxacin release under simulated gastrointestinal conditions
Enrofloxacin release characteristics from microspheres were evaluated *in vitro* by incubating 10 mg of microspheres in elution medium pH 1.2 for two hours afterwards the elution medium of pH 6.8 was replaced and incubated upto 26 hours and then maintained at 37.0±0.2℃ under stirring (50 rpm) [13]. Elution medium of pH 1.2 was prepared by adding 0.1N HCl to 10 ml of 2.5% methanol in 0.1N HCl to make up the final volume of 100 ml and pH adjusted to 1.2 by using either 0.1N HCl or 0.1N NaOH. The elution medium with pH 6.8 was prepared by adding 0.1N NaOH to 49 ml of 2.5% methanol in 0.1N HCl to make the final volume of 100 ml and pH adjusted to 6.8 using 0.1N HCl or 0.1N NaOH. Samples from elution medium were collected at 30 min interval during initial two hours and then at hourly interval up to six hours and subsequently the 8th, 12th and 24th hour interval. The samples were analyzed at 271 nm using UV-vis spectrophotometer after suitable dilution. The concentration of enrofloxacin was determined by comparing with standard curve obtained using enrofloxacin.

Mathematical modeling of enrofloxacin release
In order to investigate the mechanism of release of enrofloxacin from the microspheres, the release data was fitted to various release kinetic models using Kinetic DS software 3.0 version. The data was fitted to zero order, first order, korsmeyer-peppas model and Higuchi model. The best fit model was determined by R^2 value.

Minimum inhibitory concentration by microdilution method
The minimum inhibitory concentration (MIC) of enrofloxacin

and enrofloxacin along with various phytochemicals loaded microspheres was evaluated as per Clinical and Laboratory Standards Institute [14]. Pure enrofloxacin was dissolved in 0.1N sodium hydroxide to obtain 2 mgmL^{-1} stock solution. Ten mg of microspheres of respective phytochemical was eluted in 10 mL of 0.1N sodium hydroxide for 24h to obtain stock solution. A two fold dilution of these solutions was made in 100μL of Mueller-Hinton (MH) broth in a microplate. To each well, 50μL of 1:10 diluted 0.5 McFarland units of bacterial suspension was added to provide a final concentration of 5×10^5 cfu/mL per well. Positive and negative controls for culture and broth and 0.1N sodium hydroxide controls were also maintained. The plates were incubated at 37 ºC for 18h. One hour before the completion of incubation, 50μL of Nitro Blue Tetrazolium chloride (NBT) (2 mg/mL in distilled water) [15] was added to each well and the plates were incubated at 37 ºC for another hour. The minimum inhibition concentration was defined as the minimum concentration of the compound, which inhibited visible growth of bacteria, evidenced by lack of development of any colour.

Statistical analysis
The data of microsphere's diameters was represented as mean±S.E and one way ANOVA followed by Duncan's *post-hoc* test was used to compare the mean diameters of the microspheres. The release of enrofloxacin from various microspheres was represented as % cumulative release. Kinetic DS software 3.0 version was used for fitting mathematic models for the release data. The antibacterial activity on the MTCC Cultures and their clinical isolates was represented by determination of Minimum Inhibitory Concentration (MIC) with 95% confidence limits. Statistical package for social sciences (IBM SPSS 19.0 version) was used for the statistical analysis.

Results
Characterization of chitosan-alginate drug complexes
Shape and size
The microspheres synthesized using chitosan-alginate-Enrofloxacin and phytochemical complexes were spherical (Fig. 1) with a diameter ranging from 0.396 mm to 0.764 mm (Table 1). The mean diameter of CS-ALG-EN-SYA (0.764 mm) was significantly ($P < 0.05$) higher than rest of the microspheres. The microspheres of CS-ALG-EN-PIP and CS-ALG-EN-CIA were significantly ($P < 0.05$) higher than CS-ALG, CS-ALG-EN and CS-ALG-EN-CUR. The diameters of CS-ALG, CS-ALG-EN and CS-ALG-EN-CUR microspheres do not differ significantly.

Concentration and loading efficacy
The concentration and loading efficacy of Enrofloxacin and phytochemicals in the microspheres is presented in Table 1. Maximum concentration of EN was achieved in CS-ALG-EN microspheres (10.89 mg/100 mg of microspheres). The addition of phytochemicals decreased the concentration of EN in the microspheres. Among the phytochemical microspheres, highest concentration of EN was observed in CS-ALG-EN-SYA (10.17 mg/100 mg of microspheres) and lowest concentration in CS-ALG-EN-CUR (7.70 mg/100 mg of microspheres).
The loading efficacy of enrofloxacin varied from 9.63% (CS-ALG-EN-CUR) to 13.61% (CS-ALG-EN). Highest Enrofloxacin loading efficacy was achieved in CS-ALG-EN, which decreased with the addition of respective phytochemicals. Phytochemical concentration ranged from 0.087 mg% in CS-ALG-EN-CUR to 3.37 mg% in CS-ALG-EN-CAA. CAA showed higher loading efficacy (84.26%) whereas CUR was lowest (2.19%).

Table 1: Mean diameter, cconcentration and loading efficacy of enrofloxacin and phytochemicals of various microspheres

Microspheres combinations	Mean diameter (mm)	Enrofloxacin		Phytochemical	
		Concentration (mg/100 mg)	Loading Efficacy (%)	Concentration (mg/100mg)	Loading Efficacy (%)
CS-ALG-EN	0.470a ± 0.036	10.89	13.61	---	---
CS-ALG-EN-CUR	0.396a ± 0.027	07.71	09.63	0.087	2.19
CS-ALG-EN-PIP	0.469a ± 0.021	08.59	10.74	1.18	29.61
CS-ALG-EN-CIA	0.606bc ± 0.028	09.98	12.48	3.22	80.51
CS-ALG-EN-CAA	0.565b ± 0.031	09.46	11.82	3.37	84.26
CS-ALG-EN-SYA	0.656c ± 0.031	10.17	12.72	0.54	13.56

Values are mean ± SE. Means with different superscripts are significantly ($P < 0.05$) different. One way ANOVA followed by Duncans *post-hoc* test using IBM SPSS software 19.0 version. CS = Chitosan; ALG = Alginate; EN = Enrofloxacin; CUR = Curcumin; PIP = Piperine; CIA = Cinnamic acid; CAA = Caffeic acid; SYA = Syringic acid
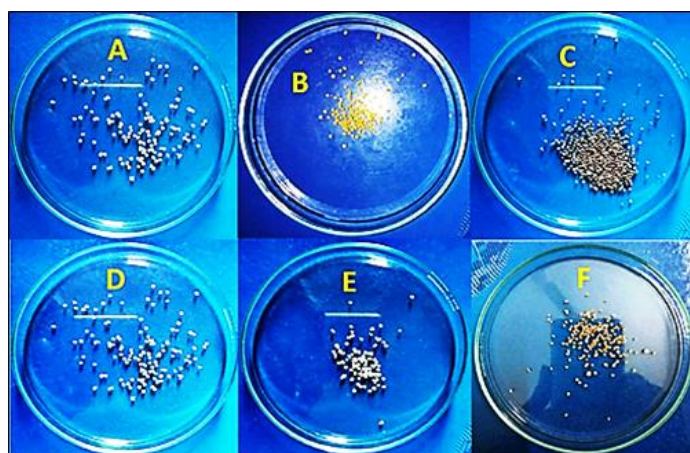

Fig 1: Chitosan-Alginate microspheres containing A: Enrofloxacin, B: Enrofloxacin+Curcumin, C: Enrofloxacin+Piperine, D: Enrofloxacin+Cinnamic acid, E: Enrofloxacin+Caffeic acid, F: Enrofloxacin+Syringic acid.

Enrofloxacin release kinetics

The release of EN from the microspheres was studied in simulated gastric condition involving a pH 1.2 (for 2h) followed by a pH 6.8 (2-26h). The % cumulative release of EN from all microspheres was maximal at pH 1.2 ranging from 27.57 to 49.88% in 2h. At pH 6.8, the release was further increased to 39.26 to 72.95% by 26h. Adding CAA and SYA improved the release of EN from the respective microspheres while CIA, CUR and PIP decreased the same compared to CS-ALG-EN (Fig. 2).
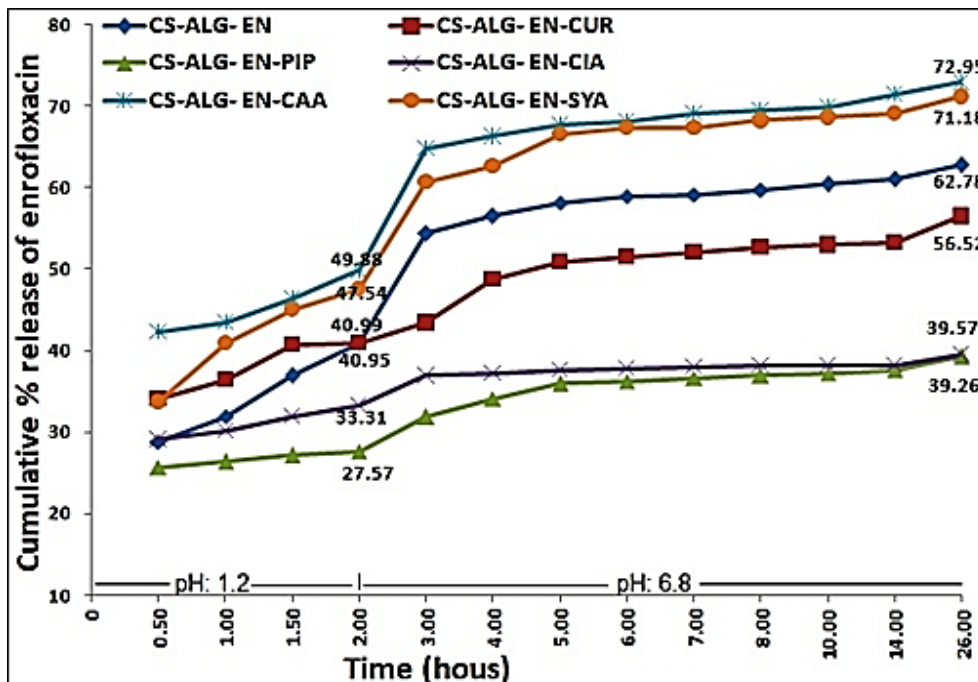
CS = Chitosan; ALG = Alginate; EN = Enrofloxacin; CUR = Curcumin; PIP = Piperine; CIA = Cinnamic acid; CAA = Caffeic acid; SYA = Syringic acid.

Fig 2: Cumulative (%) release of enrofloxacin from various microspheres under simulated gastrointestinal conditions

Release kinetics modeling

The % cumulative release kinetics of EN from the microspheres was fitted to Zero order, First order, korsmeyer-peppas and Higuchi models. R^2 value was used to determine best fit. The release of EN from all microspheres followed korsmeyer-peppas model ($Q = kt^n$) with R^2 values ranging from 0.851 (CAA) to 0.935 (CUR). The values of release component (n), which represents the drug release mechanism ranged from 0.085 (CIA) to 0.228 (EN) (Table. 2).

Dissolution parameters

The dissolution efficacy (DE, %) and mean dissolution time (MDT, min) of microspheres will be automatically generated while investigating mechanism of release kinetic models using kinetic DS software 3.0 version (Table 2). The DE of CS-ALG-EN was 57.77% with a MDT of 124.31 min. The DE of the microspheres was increased by the addition of SYA (65.71%) and CAA (67.81%) while PIP (36.08%), CIA (37.43%), and CUR (51.40%) decreased the same. MDT was similar in PIP (126.28), EN (124.31), SYA (119.34), CAA (109.97) while CIA (84.07) exhibiting lowest and CUR (141.22) exhibiting highest MDT.

Table 2: Model fitting of release kinetics of Enrofloxacin from various microspheres

Microspheres combinations	Release kinetics				Dissolution Efficacy (%)	Mean Dissolution Time (min)
	Zero Order (Q=kt +Q0) (R2)	First Order (1/Q = kt + 1/Q0) (R2)	Korsmeyer and Peppas (Q=ktn) (R2)	Higuchi (Q =k.√t) (R2)		
CS-ALG-EN	0.429	0.388	0.853 (k=14.183; n=0.228)	0.763	57.77	124.31
CS-ALG-EN-CUR	0.572	0.528	0.936 (k=21.29; n=0.142)	0.589	51.40	141.22
CS-ALG-EN-PIP	0.559	0.526	0.904 (k=15.755; n=0.133)	0.548	36.08	126.28
CS-ALG-EN-CIA	0.452	0.429	0.874 (k=22.248; n=0.085)	0.558	37.43	84.07
CS-ALG-EN-CAA	0.453	0.421	0.851 (k=23.59; n=0.17)	0.627	67.81	109.97
CS-ALG-EN-SYA	0.442	0.399	0.872 (k=18.402; n=0.207)	0.753	65.71	119.84

Models are fitted using Kinetic DS software 3.0 version. Root mean square (R2) values for each Model with highest R2 value is chosen.
CS-Chitosan; EN-Enrofloxacin; CUR-Curcumin; PIP-Piperine; CIA-Cinnamic acid; CAA- Caffeic acid; SYA-Syringic acid; k- release rate constant; n- release exponent

In vitro antibacterial activity

The minimum inhibitory concentration (MIC) of various microspheres against MTCC cultures and clinical isolates was presented in Table 3. The MIC (μg mL^{-1}) of pure Enrofloxacin ranged from 0.020 (*E. faecalis* & *S. enterica*) to 2.60 (*P. aeruginosa*). The MIC of EN was significantly (*P*< 0.05) lowered by combining with phytochemicals on *K. pneumonia* (CUR, PIP, CIA, CAA), *P. aeruginosa* (CUR, PIP, CIA, CAA, SYA), *S. aureus* (CUR, PIP, CIA, CAA, SYA) and *S. pyogenes* (CUR, PIP, CAA).

On clinical isolates, pure EN has an MIC (µg mL^{-1}) of 0.254 against *E. coli*, 0.141 against *Klebsiella* spp, 0.325 against *Pseudomonas* spp, *Salmonella* spp and *Staphylococcus spp*. The MIC of EN was significantly (*P*< 0.05) decreased in combination with CUR, PIP, CIA, CAA against *E. coli, Klebsiella* spp, *Pseudomonas spp* and *Salmonella spp*. However, phytochemical combination had no significant (*P*< 0.05) influence on MIC against *Staphylococcus* spp.

Table 3: Antibacterial activity of enrofloxacin and phytochemical loaded microspheres

Culture	Minimum Inhibitory Concentration (µg/mL)						
	EN	CS-ALG-EN	CS-ALG-EN-CUR	CN-ALG- EN-PIP	CN-ALG- EN-CIA	CN-ALG-EN-CAA	CN-ALG-EN-SYA
MTCC Cultures							
Enterococcus faecalis (mtcc 9845)	0.020 (0.009)	0.018 (0.007)	0.015 (0.007)	0.016 (0.006)	0.013 (0.006)	0.019 (0.008)	0.020 (0.009)
Escherichia coli (mtcc 443)	0.033 (0.012)	0.028 (0.010)	0.024 (0.008)	0.028 (0.006)	0.013 (0.007)	0.019 (0.008)	0.023 (0.011)
Klebsiella pneumonia (mtcc 432)	0.650 (0.151)	0.473 (0.133)	0.154* (0.028)	0.170* (0.032)	0.264* (0.034)	0.376* (0.048)	0.404 (0.060)
Pseudomonas aeruginosa (mtcc 3542)	2.600 (0.543)	1.864 (0.334)	1.024* (0.564)	0.968* (0.176)	0.792* (0.432)	0.376* (0.142)	1.344* (0.643)
Salmonella enterica (mtcc 3224)	0.020 (0.010)	0.015 (0.013)	0.014 (0.011)	0.022 (0.013)	0.008 (0.004)	0.014 (0.007)	0.026 (0.013)
Streptococcus pyogenes (mtcc 1927)	0.041 (0.011)	0.033 (0.008)	0.012* (0.006)	0.017* (0.006)	0.032 (0.010)	0.012* (0.005)	0.024 (0.014)
Staphylococcus aureus (mtcc 3160)	0.163 (0.065)	0.122 (0.041)	0.016* (0.009)	0.017* (0.011)	0.020* (0.014)	0.019* (0.011)	0.043* (0.022)
Clinical isolates (number of samples)							
Escherichia coli (15)	0.254 (0.057)	0.168* (0.045)	0.134* (0.054)	0.115* (0.037)	0.109* (0.032)	0.125* (0.036)	0.181 (0.047)
Klebsiella spp (10)	0.141 (0.023)	0.108 (0.021)	0.068* (0.018)	0.075* (0.021)	0.079* (0.020)	0.075* (0.022)	0.101 (0.034)
Pseudomonas spp (5)	0.325 (0.052)	0.273 (0.031)	0.154* (0.028)	0.170* (0.030)	0.192* (0.045)	0.107* (0.038)	0.202 (0.049)
Salmonella spp (10)	0.325 (0.058)	0.273 (0.044)	0.154* (0.043)	0.170* (0.040)	0.158* (0.035)	0.075* (0.021)	0.269 (0.055)
Staphylococcus spp (12)	0.325 (0.102)	0.216 (0.079)	0.308 (0.090)	0.314 (0.086)	0.316 (0.078)	0.251 (0.064)	0.304 (0.077)

Values are minimum inhibitory concentration (µg mL-1) with standard errors in parenthesis

*Indicates significant difference (*P*< 0.05) when compared with pure Enrofloxacin.

One way ANOVA followed by Duncan's post hoc test using IBM SPSS 19.0 version.

CS-Chitosan; EN-Enrofloxacin; CUR-Curcumin; PIP-Piperine; CIA-Cinnamic acid; CAA-Caffeic acid; SYA-Syringic acid.

Discussion

The development and spread of antimicrobial resistance to currently available antibiotics is a worldwide concern and has necessitated the search for new classes of antimicrobials substances and phytochemicals offer effective and economical alternatives not only to treat infection but also to counter bacterial resistance [1].

Enrofloxacin has been developed exclusively for veterinary medicine use [16, 17] and some of its side effects and insolubility in water making its oral administration difficult in animals. An approach to reduce side effects, increase bioavailability and enhance antibacterial activity is administration of enrofloxacin along with various phytochemicals through the chitosan-alginate microsphere matrices.

Chitosan-alginate-Enrofloxacin and phytochemical microspheres were spherical and the diameter of the spheres increased by increasing the number of compounds encapsulated. In an earlier study synthesized chitosan beads containing ciprofloxacin were ranging from 0.60 mm to 0.70 mm in diameter [12]. In contrast synthesized Ciprofloxacin HCl nanoparticles were with a diameter of 457nm [18]. Similarly, synthesized Ciprofloxacin loaded chitosan nanoparticles were ranging from 247±48 to 322±52nm [19]. The higher sizes found in this study are due to the fact that alginate has been incorporated in addition to chitosan to improve the holding capacity of the microspheres. The entrapment efficacy of Enrofloxacin varied from 9.63% to 13.61% while that of phytochemicals 2.19% to 84.25%. In previous studies, entrapment efficacy of ciprofloxacin loaded chitosan nanoparticles ranged from 11.90% to 70.79% [19] and upto 90% [12].

The release of Enrofloxacin was studied under simulated gastric condition (pH 1.2 for 2h followed by pH 6.8 for 24h). Maximum release of Enrofloxacin was observed at pH 1.2 within 2h which ranged from 27.57 to 49.88% for various microspheres and later increased to 39.26 to 72.95% at pH 6.8 by 26h. The dissolution of enrofloxacin was increased by Caffeic acid and Syringic acid while Cinnamic acid, Curcumin and Piperine decreased the release. The release of the drug from microspheres occurred by dissociation. The drug could be associated to the microspheres in three different states: at the nanoparticle surface, in the core as a reversible complex, or in the core as irreversible complex [20]. Generally, drug release follows more than one type of mechanism. In case of release from the surface, drug adsorbed on the surface of microspheres dissolves instantaneously when it comes in contact with the release medium. The early phase of the release corresponds to the release of drugs physically bound to the surface of the nanoparticles and the delayed phase due to the release of entrapped drug due to diffusion of drug from the rigid matrix structure.

The degradation rate of the microspheres depends on the pH of test medium. In acidic elution medium (pH 1.2), the degradation was faster with >50% (for respective microsphere) release in 2h. Conversely, the degradation was found to be minimal at pH 6.8. The faster degradation at pH 1.2 was in contrast with the observations wherein the

complete degradation was reported in 6 hours [21]. The observed faster degradation could be due to high acid solubility of Enrofloxacin, which caused pore formation in the matrix leading to easy ingress of dissolution media and subsequent degradation. An initial burst release of drug was observed for two reasons: the leaching of drug on the microsphere outer surface and faster ingress of dissolution medium and subsequent diffusion of drug. However, on changing the pH from lower to higher level, the drug release slowed. The pH responsive release can best be explained based on charge density on the microspheres, which is an important factor in electrostatic interaction and depends on solution pH. The pH of dissolution medium caused swelling (2h, pH 1.2) and later de-swelling (in pH 6.8) leading to bi-modal drug release. The release of ciprofloxacin depends on its concentration in the microsphere and chitosan [22].

In vitro dissolution studies/drug release is important for the development of new drug. Several theories or models are describing the drug release profile from the Pharmaceutical dosage form [23, 24]. The release of EN from all different microspheres followed korsmeyer-peppas model ($Q= ktn$) with highest R^2 values [25] ranging from 0.851 (CAA) to 0.935 (CUR). The diffusional exponent/release exponent (n) is the indicative of the mechanism of transport of drug through the matrix of spheres, and used to characterize different release mechanisms [26]. In our study the 'n' values of different spheres ranged from 0.085 (CIA) to 0.228 (EN) that suggests drug release followed Fickian diffusion. As per the mentioned methods, if the 'n' value is less than 0.5 in Korsemeyer-Peppas equation it follows the release process of Fickian/Quasi-Fickian diffusion from spheres [25, 27].

Whereas Srinatha *et al.* [12] prepared microspheres that followed non-Fickian/anomalous diffusion. The observed deviation could be possibly due to higher molecular weight of ciprofloxacin (359.4) and the polymer characteristics. A desired release profile could be achieved by modifying a few process parameters, which are discussed above. Further studies are needed to evaluate the performance of these systems *in vivo* and to optimize the formulation.

There have been several documented reports that phytochemicals in combination with antibiotics do produce synergism. Some isolated pure phytocompounds have also been reported to have resistance modifying activities *in vitro*. Diterpene compounds extracted from totara tree have been shown to potentiate methicillin activity against MRSA and reducing the MIC of methicillin against resistant *S. aureus* 256-fold via interference with PBP2a expression [28].

In this study the MIC of Enrofloxacin was significantly lowered in combination with Curcumin, Piperine, Cinnamic acid and Caffeic acid against both MTCC bacterial cultures and clinical isolates. All the phytochemicals used in this study acted mainly on the surface of the micro-organisms while enrofloxacin acted on topoisomerase II leading to inhibition of DNA replication [29, 30, 31, 32, 33, 34, 35]. Synergistic interactions of antibiotic and plant extracts have advantages of increased efficiency, reduced undesirable effects, increased stability or bioavailability of the free agents and obtaining an adequate therapeutic effect with relatively small doses, when compared with a synthetic antimicrobial medication [36]. Plant antimicrobials have been found to be synergistic enhancers even if they may not possess any antimicrobial properties alone, but when concurrently combined with standard drugs [37].

In conclusion, chitosan alginate encapsulation was an effective strategy to encapsulate Enrofloxacin and phytochemical combination. The *in vitro* release of Enrofloxacin from microspheres was influenced by the type of phytochemical combined. The antibacterial activity of Enrofloxacin was improved by combining with phytochemicals with the improvement varying with the type of phytochemical and organism. Further *in vivo* pharmacokinetic-pharmacodynamic studies are required for further development of the formulation for clinical usage.

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