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Synthesis, Optimization and Characterization of Folate-Chitosan polymer conjugate for Possible Oral delivery of Macromolecular drugs

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Abstract: *Folate-chitosan (FA-CS) conjugate was assorted as a pharmaceutical approach to increase the cellular uptake of macromolecular drugs. It was synthesized by coupling the carboxylic group of the folic acid (FA) with the amino group of chitosan (CS) using carbodiimide. The designed conjugate was suggested to combine the mutual features of both FA and CS. Factors affecting the conjugation process was optimized using Box-Behnken design to maximize the amount of FA that could be coupled with CS. The influence of FA: CS ratio, reaction temperature and time on coupling ratio were studied. The prepared FA-CS conjugate was subjected to in-depth study including FT-IR, ^{1}H NMR, mass spectroscopy, DSC, and XRD studies. The obtained conjugate was amorphous structure where the amount of conjugated folic acid was directly related to FA: CS ratio and the reaction time. On the contrary, increasing the reaction temperature negatively affect the coupling ratio of FA-CS polymer conjugate due to the possible thermal decomposition. The FA-CS conjugate is expected to enhance the intestinal uptake of macromolecular drugs and improve their oral bioavailability.*

Keywords: *Box-Behnken, Chitosan, Conjugate, Folic acid, Optimization.*

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I. INTRODUCTION

Oral administration is enviable route for drug delivery; as it is noninvasive and offering better patient compliance [1]. Efficient oral delivery of therapeutic proteins and peptides remains an elusive goal to be accomplished. Despite the worthwhile outputs of oral macromolecules administration, enzymatic degradation, digestion by proteolytic enzymes (pepsin, trypsin and chymotrypsin) and poor permeability across intestinal epithelium hamper its stability and bioavailability [2, 3]. Therefore, several modern approaches have been pursued to tackle these inherent obstacles with maintaining the structural protein integrity until reaching the systemic circulation [4]. These approaches comprise the use of small molecule permeation enhancers, enzyme inhibitors, chemical modification, and the incorporation into nanocarriers systems such as: liposomes, microspheres, nanoparticles and mixed micelles [5, 6& 7]. The polymeric nanoparticles were widely investigated for oral delivery due to their ability to deliver drugs with small molecular weight as well as macromolecules such as proteins, peptides and genes [8]. Polymeric nanoparticles can be considered as a prospective candidate in drug delivery due to their stability, high drug loading capacity and improving cellular uptake compared to the other delivery systems [9, 10]. Furthermore, it could conquer the enzymatic degradation and protect the encapsulated drug [11].

Among different polymers, chitosan (CS) has been widely used for delivery of therapeutic agents. The CS is a naturally occurring linear cationic polysaccharide obtained from chitin deacetylation. It is broadly employed as a drug carrier as it is a non-toxic, biocompatible, biodegradable and mucoadhesive polymer [12]. The mucoadhesion action of CS was owing to its cationic charge that allows electrostatic attraction with the negatively charged mucus [13]. Owing to its mucoadhesive action, CS can perform as permeation enhancer which facilitates the diffusion process across epithelial cells [14]. Chemical modification of CS chains or its combination with other polymers is a developed approach in pharmacy to achieve high protein bioavailability, trigger endocytosis and improve cellular internalization at the intestinal level [15, 16]. The CS can be

functionalized with specific ligands to increase its targeting efficacy and cellular uptake. Mostly, targeting ligands could be oligosaccharides, small peptides, proteins, antibodies fragment and even small molecules. The targeting ligand can attach to nanoparticles surface either covalently or noncovalently [17]. Folic acid (FA) is an example for small molecules that, is widely employed as a targeting moiety because of its several benefits comparing to peptides and antibodies [9]. The FA has MW 441 Da, stable over a broad temperature and pH values, inexpensive, nontoxic and nonimmuogenic [18]. Moreover, it has a high specificity in recognizing folate receptors abundantly present in many human epithelial cell surface [19]. Towards this goal the present study was aimed to synthesis, optimize and characterize the FA-CS conjugate as a promising targeted polymer for improving the oral delivery of macromolecular drugs such as polypeptides and proteins. The formation of FA-CS conjugate was confirmed via: Fourier transform infra-red spectroscopy (FT-IR), proton nuclear magnetic resonance spectroscopy (^{1}H NMR), differential scanning calorimetric measurements (DSC), mass spectroscopy and x-ray diffraction (XRD). The coupling ratio was assessed for selecting the conjugate containing the highest amount of FA coupled to CS polymer.

II. MATERIALS AND METHODS

2.1. Materials

Low molecular weight (LMW) Chitosan (CS) (MW 50000 - 190000 D), 1-ethyl-3-(3-diethylaminoproply) carbodiimide hydrochloride (EDC), glacial acetic acid, and trifluoroacetic acid (TFA) were purchased from Sigma, USA. Folic acid (FA) was procured from BDH Chemicals, England. Anhydrous dimethyl sulfoxide (DMSO) was purchased from Fluka Chemicals, Switzerland. Acetone, acetonitrile and methanol (HPLC grade) were purchased from Riedel-de Haen Gmbh, Germany.

2.2. Experimental design

A Box-Behnken design with three factors each at three levels was constructed to statistically estimate the influence of different variables on the synthesis of FA-CS conjugated complex using Design Expert® software (Version 9.0.6.2, Stat-Ease Inc. Minneapolis, MN, USA). The exploited independent factors' effects included FA: CS ratio (X1), reaction temperature (X2) and time (X3) on the coupling ratio (response) was implemented. The different factors were optimized to maximize coupling ratio of FA to CS. Selecting the optimized statistical model was based on the highest adjusted R^2, predicted R^2 and the lowest predicted residual sum of squares (PRESS). The levels of the independent parameters used in the experiment design and the dependent response were elucidated in Table 1.

Table 1. Box-Behnken design for optimization of folate - chitosan conjugated complex

Independent variables	Levels			Dependent variables	
	Low	Medium	High	Response	Constraints
FA: CS ratio	0.2	0.6	1	Coupling ratio (%)	Maximize
Coupling reaction Temperature (°C)	30	35	40		
Coupling reaction Time (h)	1	3	6		

2.3. Synthesis of folate – chitosan conjugated complex

Hence, FA is water insoluble and photosensitive molecule, the experiment work was carried out in dark place to avoid its decomposition. Folate conjugated chitosan complex was prepared at two consecutive steps [20]. Briefly, FA and EDC at stoichiometric ratio of 5:1 were dissolved in 5mL anhydrous DMSO [21]. The obtained solution was allowed to react for 1.5h at room temperature to activate FA carboxylic group moieties (COOH). The activated FA solution was dripped slowly onto 20mL of 1%v/v acetic acid solution of CS (1:5, 1:1.6 and 1:1) under magnetic stirring (500rpm) at different reaction temperature (30, 35 and 40 ^{o}C) and time (1,3 and 6hr). The modified polymer was allowed to coagulate by adding 300mL acetone and then collected by centrifugation at 12000rpm for 30min. The excess FA was removed from the precipitated FA-CS conjugate by dialysis against DMSO for two days then against deionized water for another two days. Finally, the obtained yellow colored FA-CS conjugate was dried by lyophilization at -80°C for 48h.

2.4. Characterization of folate – chitosan conjugated complex

2.4.1. Coupling ratio studies

A total number of 17 formulae for the FA-CS conjugated complex were developed according to Box-Behnken design Table 2). Subsequentaly, they were subjected to coupling ratio study for determining the amount of FA conjugated to CS polymer in relation to the different experimental variables. An aliquot of 10mg of FA-CS conjugate was dissolved in 100mL of 2%v/v acetic acid solution. The folic acid content was quantified using a validated HPLC method. A reverse phase C_{18} column (Kromasil 100-5 phenyl®, 300X4.6

mm, 5µm) was used at 25°C. The mobile phase consisted of 0.1%v/v TFA and acetonitrile (80:20 v/v) was eluted at a flow rate of 1.5mL/min and the FA was monitored at a wavelength 290nm. The coefficient (R^2) of the FA calibration curve in acetic acid in the concentration range of 0.1–5 µg/mL was 0.9994 and the respective limits of detection (LOD) and quantification (LOQ) were 0.05 and 0.1µg/mL, respectively. The CV% ranged from 0.87 to 3.36% and the accuracy for FA determination was within acceptable range (not more than 3%) with mean% drug recovery of 99.04%. The coupling ratio, expressed as degree of FA substitution on CS amino groups, was calculated using the following equation:

$$\text{Coupling ratio} = \frac{W_{FA}}{W_{FA-CS} - W_{FA}} \quad (1)$$

Where W_{FA} is the determined amount of folic acid in the prepared conjugate and $W_{FA\text{-}CS}$ is the amount of FA-CS conjugate.

2.4.2. Fourier transform infra-red spectroscopy (FT-IR) study

FT-IR spectra of FA, CS and FA-CS conjugate were recorded on an FT-IR spectrometer in the range 4000-400 cm^{-1}.

2.4.3. Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR) Study

FA was dissolved in 1ml of deuterated DMSO and FA-CS conjugate solution (10mg/mL) was dissolved in deuterated water containing 1% vv deuterated TFA). These solutions were analyzed for complex formation by NMR spectrometer at 70°C where no solvent peaks could interfere with sample peaks.

2.4.4. Mass Spectroscopy Study

The mass spectra of FA, CS and FA-CS conjugate (2mg) were recorded in the positive ion electron impact mode at an ionizing energy of 70 eV

Table 2. Box-Behnken design for folate chitosan conjugates

Formula No.	FA: CS ratio	Temperature (°C)	Time (h)	FA Coupling ratio
F1	1:1.6	40	3.5	0.15
F2	1:1.6	40	3.5	0.151
F3	1:1.6	50	1	0.13
F4	1:1	50	3.5	0.22
F5	1:1.6	40	3.5	0.149
F6	1:5	50	3.5	0.056
F7	1:1.6	30	6	0.25
F8	1:1	30	3.5	0.25
F9	1:5	30	3.5	0.078
F10	1:1.6	40	3.5	0.151
F11	1:5	40	6	0.071
F12	1:5	40	1	0.056
F13	1:1.6	40	3.5	0.148
F14	1:1	40	1	0.15
F15	1:1	40	6	0.28
F16	1:1.6	30	1	0.11
F17	1:1.6	50	6	0.12

2.4.5. Differential Scanning Calorimetric Measurements (DSC)

Accurately weighed 4mg of FA, CS and FA-CS conjugate was sealed in an aluminum pan. The samples were heated from ambient temperature to 300°C at a constant rate of 10°C/min. An empty pan was used as a reference.

2.4.6. X-Ray Diffraction (XRD) Study

The XRD patterns of FA, CS and FA-CS conjugate were conducted using X-ray diffractometer. Samples were irradiated by a secondary monochromatic Cu Kα radiation (K=0.154 nm) at a voltage and a current of 40 kV and 30 mA, respectively. The scanning rate was 1.2°/ min in 2θ angle range of 3-70°.

III. RESULTS AND DISCUSSION

3.1. Coupling ratio of FA-CS conjugates

The coupling ratio of the synthesized FA-CS conjugates was presented in Table 2 and ranged from 0.056± 0.003 to 0.28± 0.01. Equation (2) elucidates the effect of different variables on FA-CS coupling ratio (Y1):

$$Y1 = +0.15+0.08*X1-0.02*X2+0.034*X3 + 0.029*X1X3 – 0.038*X2X3 \quad (2)$$

The positive signs FA: CS and reaction time indicate the direct relation with FA: CS coupling ratio. Increasing FA: CS ratio and/ or reaction time provide higher conjugation opportunities between FA carboxylic groups to CS chains amino groups. These results obtained by F15 which showed the highest coupling ratio (0.28) owing to maximum FA: CS ratio (1:1) and long reaction time (6h). On the contrary, FA-CS coupling ratio is inversely proportional with conjugation reaction temperature, as observed in F6 which have the lowest coupling ratio (0.056) due to high temperature (50 °C) of the reaction.

This might be attributed to the possible decomposition of the complex. Fig .1 demonstrates the response surface for the effect of FA: CS ratio (X1), reaction temperature (X2) and time (X3) on FA-CS coupling ratio.

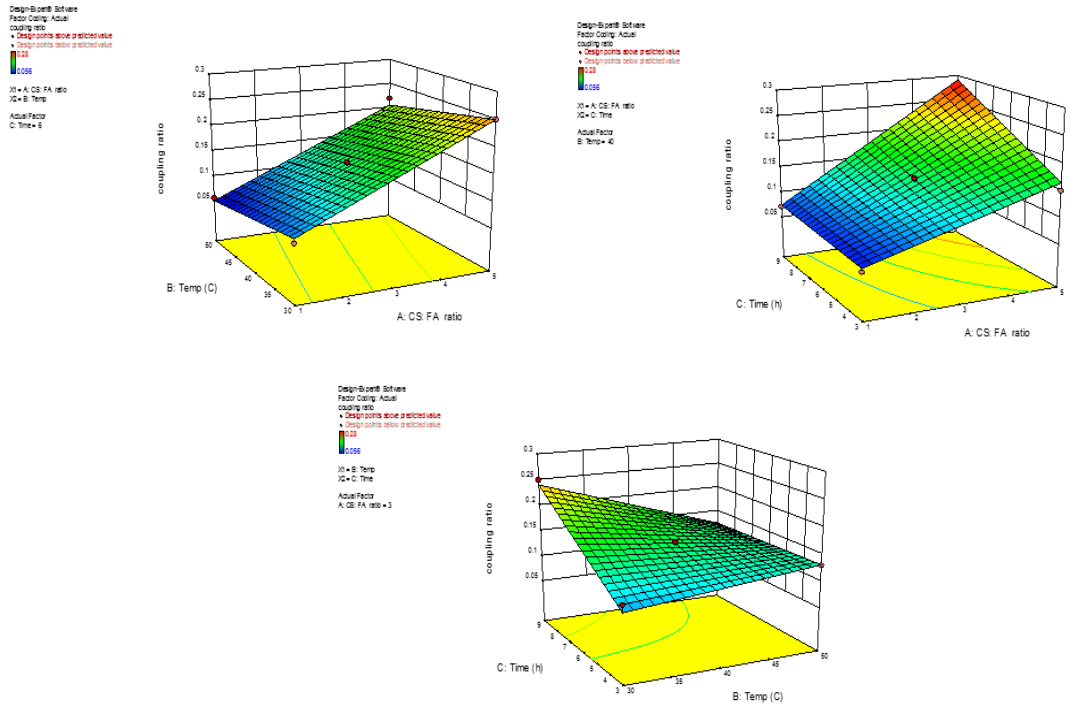

Figure 1. Response 3D plots for the effect of FA: CS ratio (X1), reaction temperature (X2) and time (X3) on FA-CS coupling ratio**.**

The figure illustrated that increasing FA-CS ratio was the most predominant factor affecting the coupling ratio. This result was related to the amount of activated FA carboxylic groups that increase chance for conjugate with amino groups of CS via covalent amide bond

3.2. Optimization of Folate Conjugated Chitosan Complex

The 2FI model was nominated as the statistical model defined the influence of different reaction variables on FA-CS conjugation as it had the least PRESS value with adjusted and predicted R2 values of 0.9777 and 0.9293, respectively. To validate this model, one conjugated formula was selected based on the highest coupling ratio (F15) to be used as a checkpoint. Table (3) demonstrates the reaction condition, the predicted and experimental response. The linear correlation plots between experimental and predicted values for the desired response showed high R^2 (0.997). Therefore, this model was appropriate for predicting the most proper reaction conditions to conjugate FA with CS. The obtained FA-CS conjugate was subsequently subjected to further structural characterization.

Table 3. The experimental and predicted coupling ratio of the optimized FA-CS conjugate

FA: CS ratio	Reaction		Coupling ratio		
	Temperature (°C)	Time (h)	Exp.	Pred.	% pred. error
1:1	40	6	0.28	0.291	2.02

3.3. Characterization of Folate Conjugated Chitosan Complex

3.3.1. FT-IR studies

Spectra of CS, FA and FA-CS conjugated complex were presented in Fig 2**.** FA is an intricate structure consisted of p-amino benzoic acid, glutamic acid and a hetero-bicyclic pteridine [22].

By inspecting FA FT-IR spectrum, the following characteristic peaks could be observed; the broad peak between 3400 and 3600 cm^{-1} described the stretching of -OH and –NH of the glutamic acid and pterinic portion, respectively. Moreover, the strong band at 1696 cm^{-1} was assigned to the stretching of different –C=O groups. The band at 1607 cm^{-1} resulted from the bending of –NH groups. Finally, the band appeared at 1480 cm^{-1} could be attributed to C–C vibration of pterinic ring [23]. The CS FT-IR spectrum showed a broad absorption band in the range of 3000- 3500 cm^{-1}, attributed to an overlap between O–H and N-H bands due to intramolecular hydrogen bonds. The peaks around 2885, 1650, 1424, 1380 cm^{-1} resulted from the stretching vibrations of aliphatic C–H, amide I (-NH) deformation of –NHCOCH3, amide II and amide III, respectively. Finally, the band around 1080 cm^{-1} was attributed to the vibration of C–O–C bonds [24].

The FA-CS FT-IR spectrum showed an obvious increase in the intensity of the absorption peak at 3430 cm^{-1} due to the overlapping of the vibration of O-H and N-H functional groups. The new two peaks appeared at 1633 and 1026 cm^{-1}could be attributed to the vibration of C-N confirming the conjugation of FA and CS. The intensity of the stretching FA carbonyl group decreased and slightly shifted from 1696 to 1634 cm^{-1}. Furthermore, the CS amide absorption peak at 1650 shifted to 1633 cm^{-1}, which was related to the overlapped between the absorption peaks of the newly formed C–N bond [20].

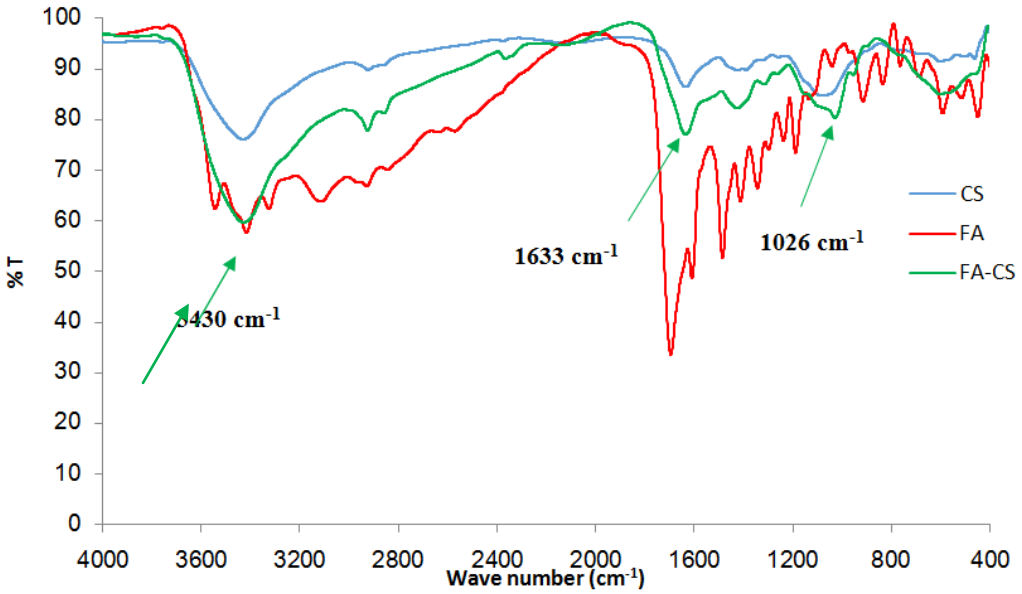

Figure 2. FT-IR Spectra of Folic Acid, Chitosan and Folate Conjugated Chitosan Complex.

3.3.2.^{1}H NMR Spectra of Folic Acid and Folate Conjugated Chitosan Complex

The ^{1}H NMR spectra of FA and FA-CS conjugate were illustrated in Fig (3 A&B). Values of FA ^{1}H NMR was: δ 11.52 [20/23-COOH], 8.59, 7.5 and 6.5 [7, 13/15 and 12/16, aromatic-H], 8.25 [18-CONH], 7.00 [2-NH], 4.78 [19-CH], 4.32, 2.25 and 2 [9, 21 and 22-CH_2] (Fig. A). Inspection of Fig. B reveals the disappearance [23-COOH] peak confirming the successful coupling of COOH group of FA with NH_2 group of CS via EDC-mediated reaction. By comparing the ^{1}H NMR spectrum of FA and FA-CS, a noticeable overlapping of vibration peaks in FA-CS spectrum could be noticed. Values of FA-CS ^{1}H NMR were: δ 8.10, 6.8 [13/15 and 12/16, aromatic-H], 7.10 [2-NH], 4.1 [5'-CH_2OH, alcohol], 3.70 [2'-CH], 3.10, 2.90 and 2.25 [22, 21 and 4'-CH_2] and 1.90 [3'-OH] [25, 26 & 27] (Fig. 3 B).

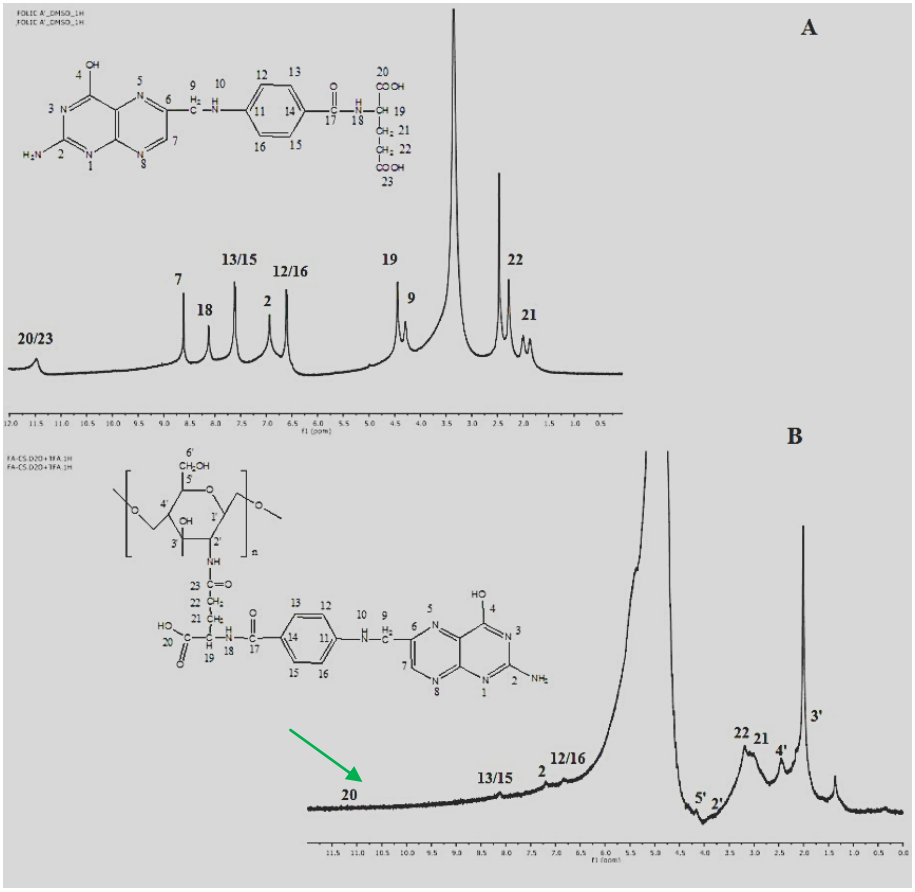

Figure 3. [1]H NMR Spectra of (A) Folic Acid, and (B) Folate Conjugated Chitosan Complex.

3.3.3. Mass Spectra of Chitosan, Folic Acid and Folate Conjugated Chitosan Complex

The molecular weight of the FA, CS and FA-CS conjugate was extracted from the mass spectra as the heaviest ion fragmented (Fig. A-C). From interpretation of mass spectra of FA and CS (Fig 4 A&B), it could be noted that the molecular weights of FA and CS were found to be 441 and 213 g/mole, respectively[28, 29] Moreover, the FA-CS conjugate mass spectrum showed a molecular ion peak [M] at m/z= 636 g/mole. These results revealed the molecular weight of the conjugate equals to the parent molecules, FA and CS molecular weights minus 18 g/mole. Consequently, the coupling reaction between FA and CS were confirmed to occur between FA carboxyl group and CS amino group with a loss of water molecule.

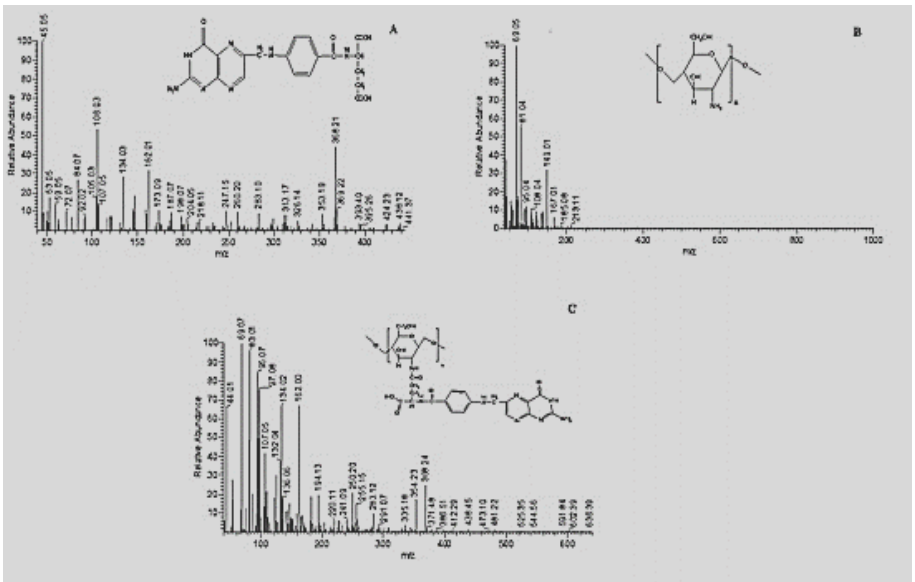

Figure 4. Mass Spectra of (A) Folic Acid, (B), Chitosan and (C) Folate Conjugated Chitosan Complex**.**

3.3.4. DSC of Chitosan, Folic Acid and Folate Conjugated Chitosan Complex

The DSC is one of the main analytical techniques used for the thermal characterization of different pharmaceutical powders. DSC thermogram could be used as an indicator to correlate the structural alternations during conjugation reactions. The DSC thermograms of FA, CS and FA-CS are displayed in Fig. 5. The FA showed a characteristic peak at 177.7 °C, which is corresponding to the loss of glutamic acid moiety [30]. In addition, CS exhibited two broad endothermic peaks at 100 and 257°C corresponding to the evaporation of water content and the decomposition of polymer chains, respectively. It could be seen that conjugation of FA with CS was followed by a complete disappearance of FA characteristic peak and an obvious shifting of CS peak from 257°C to 236.6°C with detrimental reduction in its intensity. Moreover, no characteristic endothermic peaks around 100°C due to the lyophilization of FA-CS conjugate. These results could confirm the successful conjugation of FA with CS.

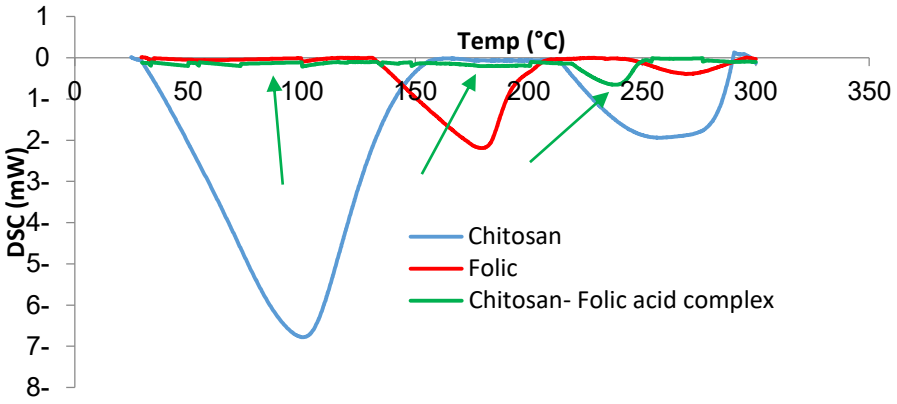

Figure 5. DSC Thermograms of Folic Acid, Chitosan and Folate Conjugated Chitosan Complex.

3.3.5. XRD of Chitosan, Folic Acid and Folate Conjugated Chitosan Complex

The XRD study, which is a versatile nondestructive technique, was conducted to detect the crystalline behavior of different samples [31]. Powder XRD patterns of FA, CS and FA-CS conjugate were displayed in Fig (6 A-C). By observing the XRD of FA, sharp peaks at 2θ value of 5.60, 10.85, 13.06, 16.60, 17.30, 19.35, 21.75, 26.68, 27.79 and 29.49° due to its crystalline properties (Fig. 6A). The diffraction pattern of CS reveals obvious intense peaks 2θ value of 19.85 and 28.10° indicating the high degree of crystallinity of CS (Fig. 6B). Contrastingly, as seen in (Fig. 6C), the X-ray diffractogram of FA-CS conjugate showed an amorphous structure devoid of any crystallinity. The conjugation of FA with CS could hinder the association of CS molecular chain in solid state. This could be attributed to the complex structure of FA if compared to CS amino group [20].

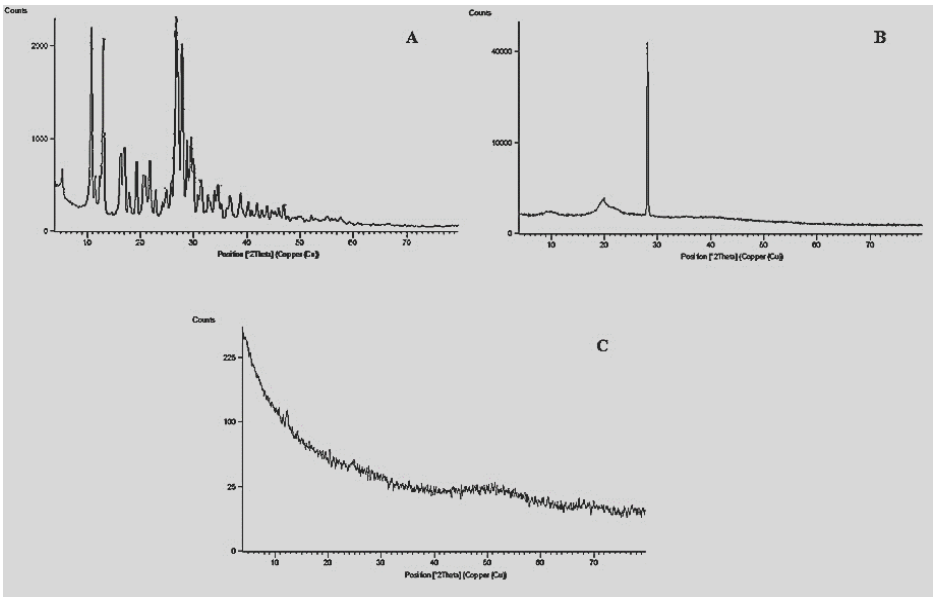

Figure 6. XRD of (A) Folic Acid, (B), Chitosan and (C) Folate Conjugated Chitosan Complex

IV. CONCLUSION

To sum up, the developed FA-CS conjugate was prepared simply by coupling the activated FA carboxyl group with CS amino group. Different analytical tools as FT-IR, H^1-NMR and mass spectroscopy were used to verify and characterize the prepared conjugate. Moreover, FA-CS complex were assessed by DSC and XRD. The results revealed the successful coupling of FA with CS through the Carboiimide chemistry. The FA-CS conjugation was confirmed by the appearance of C-N vibration band in the FT-IR and the disappearance of FA carboxyl group peak in ^{1}H NMR spectrum. FA-CS conjugate is an amorphous compound with a molecular weight equals the sum of FA and CS molecular weights with a loss of water molecule. The obtained conjugate is elected for further studies to prove its efficacy in oral macromolecule delivery.

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