Characteristic and cytotoxic activity of different α-Lactalbumin/fatty acids nanocomplex

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Abstract

Theaim of this research w*asto study the possibility of preparing a bovine α-lactalbumin/fatty acids nanocomplex with cytotoxic activity similar to the human alpha-lactalbumin made lethal to tumor cells (HAMLET). So, five different fatty acids with different concentrations were used to prepare nanocomplexes withbovine α -lactalbumin (α-LA) in ratio of 1mg/ml. Different measurements such as the Surface Tension, Circular Dichroism, Turbidity, and Cytotoxic activity for both individual fatty acid and α–LA/fatty acids nanocomplex were recorded. The results showed the surface tension was significantly decreased by adding different fatty acids and the nanocomplexes of α-LA showed a higher affinity to bind fatty acids in cis than in trans conformation. The signal of CD spectra increased with increasing the fatty acid concentrations which refer to the changes of α-LA from fold to unfold. The nanocomplexes of α-LA/fatty acid exhibited very low turbidity values with different fatty acids concentrations. All α-LA/fatty acidsnanocomplexunder test showed cytotoxic activity effect in different extent depending on the type and concentration of fatty acids.*

***Key words**: Nanocomplex;α-lactalbumin; fatty acids; circulardichroism; cytotoxicity activity.*

INTRODUCTION

In the last few years, a remarkable apoptotic-like activity towards cultured cancer cells of a complex between the calcium-free form of human α- lactalbumin and oleic acid has been described [1]. The complex could either be isolated from human milk [2] or formed on a diethylaminoethyl (DEAE) trisacry column equilibrated with oleic acid[3]. The complex was named HAMLET (human alpha-lactalbumin made lethal to tumor cells) and defined as a complex between partiallyunfoldα-lactalbumin and oleic acid. When tested against different cell types, HAMLET showed strongest activity against tumor cells, while mature differentiated cells were not affected [4]. Three molecular are involved in the conversion of α-lactalbumin to HAMLET such as protein unfolding, fatty acid bindingand formation of the biologically active complex. In contrast to human milk, bovine milk did notshow similar natural activity. However, it has been possible to make a complex between bovine α-LA and oleic acid (OA) called BAMLET (bovien alpha-lactalbumin made lethal to tumor cells), capable of inducing cell death in trans-formed cells in vitro. Meanwhile, the OA complex retains its activity upon replacement of human α-LA with closely related amino acid sequences. Human α-LA can be replaced by bovine, equine, porcine, or caprine α-LAs, proteins that share 76 - 79% sequence identity with the human protein [5]. It isof interest to investigate conditions for generating BAMLET-like activity with different fatty acids that could potentially modulate the growth of those cells. So, the objectives of the present work wereto study the bovine α-lactalbumin as a base of nanoparticlesand its ability to formulate nanocomplexes with different unsaturated fatty acids and to study the cytotoxic activity of bovine α-lactalbumin with different fatty acids to form (BAMLET) with same anti-tumor activity similar to oleic acid.

Materials and Methods

Bovine *α*-lactalbumin (*α*-LA) powder was obtained from Protein Biophysics, Molecular Biology Dept., Aarhus University, Denmark. The fatty acids (oleic, eliedic, cis-vaccenic, trans-vaccenic and linolenic) were purchased from Sigma-Aldrich Co., St Louis, USA.

Preparation of α-LA solution: Final concentration of α-LA solutions (1 mg/mL) in a buffer concentration of 1.516 M glycine at pH 9 was prepared and determined using Nano Drop Spectrophotometer (ND-1000) at 280 nm.

Preparation of stock fatty acid solutions: Oleic acid was dissolved in sodium carbonate (0.1 M), while,eliedic, cis-vaccenic, trans-vaccenic and linolenic fatty acids were dissolved in ethanol (5 mg/ml) and stirred to complete their solubility.

Preparation of α-LA/Fatty acid (nanocomplex): mixtures of different fatty acids with α-LA solution were prepared as described by Kamijima*et al*., [6] as follows:individual fatty acid was directly suspended into the *α*-LAsolution with different concentrations as a molar ratio, heated at 60°C for 15 min., to facilitate dispersal of the fatty acid, and then cooled to the room temperature (20±1°C).

Surface tension measurement: The surface tension as a function of fatty acids concentration (Molar Ratio) for all samples were measured at room temperature (20°C), using the Pendent drop (KSV Instrument's, CAM 101). The measurements were repeated six times and the average value was recorded for both individual fatty acid and different *α*-LA/fatty acids nanocomplexes,. The surface tension of distilled water was measured before each experiment for instrument calibration.

Circular Dichroism (CD) spectra: Circular Dichroism (CD) spectra was measured at pH 9 and 37°C to confirm the changes in structure characteristics of the different *α*-LA/fatty acids nanocomplexes. Spectra of CD was measured with a Jasco J-810 spectropolarimeter (Jasco Spectroscopic Co. Ltd., Hachioji City, Japan) equipped with Jasco PTC-348WI temperature control unit. The pass length of the optical cuvette was 10 mm for 250 – 320nm (near UV) and 1 mm for 190 – 250 (far UV) at 37°C. The wavelength setup was; the response time 2 sec., and the scan rate 100 nm / min.,eight scans were recorded and averaged for each spectrum. Baseline spectra were recorded with pure buffer in each cuvette and subtracted

from the protein spectra. The α-lactalbumin was recorded (70.4 µM) for near UV and (14.08 µM)for far UV. The protein concentrations were determined using Nano Drop Spectrophotometer (ND-1000) at 280 nm.

Turbidimetric measurement: The turbidity of individual fatty acid and different α-LA/fatty acids nanocomplexes were changes associated with interactions between fatty acid (oleic acid as a model) and the α-LA. The ITC was performed at 60°C using VP-ITC (MicroCal Inc., Northhampton, MA). The instrument to react with different α-LA solutions (35.2, 70.4, 119.7 and 176.1 µM). Data was conducted with the Origin-ITC software.

Cytotoxic activity using erythrocytes: The interaction effect of individual fatty acid and different α-LA /fatty acidsnanocomplexes on erythrocytes as indication of cytotoxic activity was Examinedas described by Dobrovolskaia*et al*., [7] as follows: fresh bovine blood was collected from the slaughter house and immediately added 10mM EDTA to prevent coagulation. Blood sample was divided into equal volume and centrifuged at 3000 rpm, 4°C for 10 min. The bottom layer (red blood cells) containing erythrocytes were washed using 5mM NaH_2PO_4, 150mMNaCl buffer, pH8. The washing process was repeated 4 times and the washed erythrocytes were stored at 4°C until used.

Stock erythrocytes was diluted by glycine buffer up to 10% solution (1 erythrocytes stock: 9 buffer) and 100 µl of this solution were added to 100 µl of previously prepared α-LA /fatty acids nanocomplexes. All treatments were incubated at 37°C for 3 hrs.thencentrifuged (3000 rpm), at 4°C for 10 min. The supernatant was collected in plate holes (3 for each sample), the color density was measured as OD at 405 nm using plate reader and compared with twocontrol samples(buffer, withoutlysis), and (lysisin water).

Statistical analysis

Data of the three experimental were analyzed using the Statistical Analysis System [8].

Results and Discussion

Surface tension measurements--

measured at 400 nm using Nano Drop Spectrophotometer (ND-1000).

Isothermal Titration Calorimetry (ITC): ITC was used to probe enthalpy

instrument has a 1.42 m cell volume which 2.5 – 5 µL aliquots of titrant are injected. Oleic acid solution (20mM, dissolved in glycine buffer pH9), was dropped wisely from ITC

The surface tensionof different concentrations (molar ratio) of individual fatty acid and different α-LA/fatty acids nanocomplexes is presented and plotted in Fig. (1). Data showed that the surface tension of α-LA solution was (70.6), and the buffer was (71.9). The surface tension value was decreased by increasing the concentration of fatty acids and all α-LA/Fatty acid nanocomplexes had lower surface tension value compared to the individual fatty acid. The surface tension of the α-LA/Fatty acid nanocomplexes was decreased by the type of fatty acid e.g. α-LA nanoparticles had high ability to bind oleic acid more than eledic acid. Fatty acids (cis-form) such as oleic, cis-vaccenic and linolenic decreased the surface tension of α-LA nanocomplex dramatically than the trans-fatty acids (eledic and trans-vaccenic acids). These results are in agreement with those obtained by Svensson*et al*., [9], who reported that fatty acids in the cis conformation are U-shaped around the double bond, with both carbon chains projecting in one direction, while trans fatty acids are rod shaped around the double bond due to the carbon chains on opposite sides of the double bond.This may explain the ability of cis fatty acids that have more ability of binding with α-LA nanoparticles more than trans-fatty acids. Also, cis conformation allows fatty acids a close stereo-specific fit, and the additional critical feature of the fatty acid is the carbon chain length. Based on the analogy with other fatty acid binding proteins, the fatty acid may bind to BAMLET by electrostatic interactions between its negatively charged head

group and basic side chains in the protein, due to the hydrophobic effect, as well as by van der Waal's contacts with the tail, which are optimized for the preferred stereo-specific match (C18:1:9cis). Active complex, *α*-LA has exposed hydrophobic surfaces specially Apo-*α*-LAand adding fatty acids increase this hydrophobic effect may be allow significant effect in decrease of surface tension[9]. Fig.-1), also, indicated that cis-vaccenic and linolenic fatty acids were close to oleic acid in binding with *α*-LA and had similar effect in reducing the surface tension value.

Circular Dichroism (CD) Spectroscopy

The Circular Dichroism (CD) spectra of *α*-LA/fatty acids nanocomplexes with different concentrations of fatty acids in the near and far UV regions are presented in Figs 2 and 3. The spectra of *α*-LA/OA nanocomplex in the near-UV(Fig. 2A) showedthe characteristics of significant minimum signal (negative ellipticities peak) at 266 nm and maximum signal (positive ellipticities peak) at 296 nm. Heated *α*-LA in buffer solution (pH 9) without oleic acid had lower signal intensities compared to *α*-LA/oleic acid nanocomplex (higher concentration, 50 molar ratio), indicating the loss of tertiary structure and the weak negative peakindicated that *α*-LA/oleic acid nanocomplex partially maintain the native-like tertiary structure. These findings are very close to those obtained by Kamijima*et al*., [6] who found that the human *α*-LA had the characteristics of negative peak at 270 nm and a positive peak at 293 nm, indicating the rigid packing interactions of aromatic side chains and the lower signal intensities of heated *α*-LA compared to native *α*-LA is commonly indicating the loss of tertiary structure.

The CD spectra of heated *α*-LA sample without oleic acid or as nanocomplex at far-UV (Fig. 3A) showed significant negative ellipticities and minimum signal (negative peak) at 207nm. Kamijima*et al*., [6] mentioned that CD ellipticity at 222 nm is commonly used as a measure of extent of secondary structures. The spectrum of heat treated complex showed an increase in helicity from that of native *α*-LA. The ellipticity of *α*-LA/oleic acid nanocomplex increased with increasing the oleic acid concentration which means that, *α*-LA changed from fold to unfold and lost the tertiary structure. Therefore, the ability of *α*-LA/oleic acid nanocomplex to kill cancer cell has been shown to increase after losing the tertiary structure. The tertiary structure of *α*-LA/OA complexes were thought to be completely lost from their CD spectra by increasing the concentration of oleic acid (Figs2 and 3-A).

Kuwajima[10]mentionedthat*α*-LA after removing the calcium, in heat-treated samples showed a conformational transition toward the intermediate state that suggested the binding of oleic acid. Thus, heat treatment of *α*-LA would facilitate the exposure of hydrophobic residues to water and binding to oleic acid regardless of the presence of calcium. In terms of practical efficiency, these results may confirm by increasing the loss of *α*-LA conformation by increasing the concentration of oleic acid and by the heat treatment.Curry *et al*., [11] explained the carboxylate head group of the fatty acid interacts with two to four positively charged amino acids, usually arginines, and the carbon chain is coordinated by 6 to 10 hydrophobic amino acids. The crystal structure of human serum albumin has revealed six asymmetrically distributed fatty acid binding sites within the repeating *α*-helical domain structure of the protein. The binding of fatty acids to human serum albumin causes conformational changes with rotations of the three domains of the protein, and adjustments of side chains to make way for the incoming fatty acid. The native *α*-lactalbumin molecule is a hydrophilic, acidic protein, exposing mainly charged and polar amino acids.

The CD for near and far – UV spectraof *α*-LA nanocomplex with different concentration of eledic acid is plotted in Figs. (2 and 3B). It could benoticed that at near UV the CD spectra showed a minimum signal (negative peak) at 265 nm and a maximum signal (positive peak) at 295 nm, which is nearly same as *α*-LA/oleic acid nanocomplex. While, in far-UV (Fig.3B), the CD spectra of *α*-LA/eledic acid nanocomplex showed a minimum signal (negative peak) at

206.7nm, in comparison to 207nm for *α*-LA/oleic acid nanocomplexes. All nanocomplexes of *α*-LA/eledic acid were completely lost their tertiary structure and the *α*-LA can be partially unfolded by the interaction with eledic acid after heating at 60°C/15min.

Circular dichroism (CD) Spectra of *α*-LA with different concentrations of cis-vaccenic acid in the near and far -UV region are presented in Figs (2 and 3C). It could be noticed that the near UV CD spectrum had a maximum signal (positive peak) at 296 nmand a minimum signal (negative peak) at 260 nm. The nanocomplexes of *α*-LA/cis-vaccenic acid had completely lost their tertiary structures and showed same signals as with *α*-LA/oleic acidnanocomplexes. From far-UV CD spectra of *α*-LA/cis-vaccenic acid nanocomplexes (Fig.3C) exhibited a minimum signal (negative peak) at 206.14 nm compared to 207 nm for *α*-LA/oleic acid nanocomplexes.Theseobservations indicated that *α*-LA can also lost its tertiary structure and be unfold after making a nanocomplex with cis-vaccenic acid.

The Circular Dichroism (CD) Spectra of *α*-LA with different concentrations of trans-vaccenic acid in the near and far -UV region are presented in Figs (2 and 3D). The signals pattern at near UV of *α*-LA/trans-vaccenic acid was different than that found with previous examined fatty acids(oleic, eledic and cis-vaccenic). It could be noted that the CD spectra at near-UV had a maximum signal (positive peak) at 296 nmand a minimum signal (negative peak) at 260 nm which is nearly same as *α*-LA/oleic acid complexes. CD spectra of *α*-LA/trans-vaccenic proved that the nanocomplex lost the tertiary structures as compared with *α*-LA/oleic acid. The *α*-LA has to bind higher concentration of trans-vaccenic acid to reach the same effect as *α*-LA/oleic acid nanocomplexes. Far-UV CD spectra of *α*-LA/trans-vaccenic acid nanocomplexes(Fig.3D)showed a minimum signal (negative peak) at 208.4nm, while *α*-LA/oleic acid nanocomplexwasat 207nm. These indicate that *α*-LA can be partially unfolded by interaction with trans-vaccenic acid after heating at 60°C/ 15min.

Data plotted in Figs. (2 and 3E) refer tocircular dichroism (CD) spectra of*α*-LA with different concentrations oflinolenic acid (molar ratio)in near and far-UV region. The near – UV spectra of *α*-LA/linolenic acid nanocomplexes had a maximum signal (positive peak) at 297 nm, and minimum signal (negative peak) at 286 nm with no significant different compared to *α*-LA/oleic acid nanocomplexes. It could be observed that *α*-LA completely lost the tertiary structures as compared with *α*-LA/oleic acid.Linolenic acid can bind and interact with *α*-LA and converted to BAMLET as same as conversion of*α*-LA/oleic acid complexes, *α*-LA can bind higher concentration of linolenic acid. From far-UV CD spectra of *α*-LA/trans-vaccenic acid nanocomplex (Fig.3E)the minimum signal (negative peak) was noted at 205.4nm compared to 207 nm with *α*-LA/oleic acid nanocomplexes. The *α*-LA also lost the tertiary structures and be unfold with linolenic acid.

In general, the signal of CD spectra increased with increasing the fatty acid concentrations which increased the signal refers to the changesof *α*-LA from fold to unfold.

Turbidity measurements

The turbidity values of different concentrations of individual fatty acids and *α*-LA/fatty acids nanocomplexes are illustrated in figure -4 (A and B). Data indicated that the turbidity value was increased by increasing the fatty acid concentrations (Fig 4-A) and fatty acids in cis-form (oleic, cis-vaccinic, linolenic) showed lower turbidity values compared to the trans-form (eledic, trans-vaccinic).The *α*-LA/ OA nanocomplex showed the lowest turbidity(Fig. 4-B), while, *α*-LA/eledic acid nanocomplex indicated the highest turbidity value among all *α*-LA/fatty acid nanocomplexes under test. It can be noticed the *α*-LA/fatty acid nanocomplexes showed lower turbidity values compared to individual fatty acid could be due to the presence of protein (*α*-LA) which decreased the

ability of fatty acid to aggregate in the solution and/or using high pH value (9.0) to prepare *α*-LA solution.

Thermodynamic characterization of *α*-LA/oleic acid nanocomplex

The enthalpy change upon injection of oleic acid into the reaction cell was highly dependent on the initial concentration of *α*-lactalbumin present in the solution as shown in Fig. (5).The enthalpy increased by increasing the titrated amount of oleic acid and be stable after saturated the protein. These results confirmed the surface tension data. Fig. (5), Showsthe titrating oleic acid into protein (***α*-LA)**and gave reproducible transitions seen as peaks or troughs in the enthalpogram. These transitions shift to higher oleic acid concentration as increased the protein concentrations. At low oleic acid concentration, there was an exothermic reaction due to binding of oleic acid molecules to the *α*-lactalbumin. As the oleic acid concentration increased, the number of available binding sites on the *α*-lactalbumin decreased; hence, the exothermic contribution to the enthalpy change associated with binding was decreased. At higher oleic acid concentrations, stable enthalpywas observed because there was no free binding site remaining at *α*-LA in the reaction cell.

Eventually, all of the binding sites on the *α*-lactalbumin became saturated; therefore, any further oleic acid micelles injected into the reaction cell dissociated into monomers, leading to an endothermic reaction. When the concentration of free oleic acid monomers in the aqueous phase increased above the critical micelle concentration (CMC), micelle dissociation no longer occurred, and the enthalpy change was only due to micelle dilution effects. These results are in agreement with those obtainedbyWangsakan*et al*., [12].

Cytotoxic activity of *α*-LA/fatty acid nanocomplexes

The Cytotoxic activity as erythrocytes lysis for individual fatty acid and *α*-LA/fatty acids nanocomplexes are shown in Fig.-6 (A and B). The erythrocytes lysiswas increased by increasing the individual fatty acid concentrations (Fig.-6A) and increasing *α*-LA/oleic acid nanocomplex. It could be seen that at low oleic acid concentration the complex of *α*-LA/oleic acid showed slight lysis but still higher than that of *α*-LA in buffer only.

The erythrocyte lysis increased with increasing the concentration of individual fatty acid or *α*-LA/oleic acid nanocomplex. The complex was more effective in erythrocytes lysis than the individual fatty acid which showed erythrocyte lysis effect too.Complete erythrocytes lysiswas reported at low concentration of OA alone or with *α*-LA nanocomplex compared to other fatty acids. Cis-vaccenic acid showed the nearest observations to OA either alone or as nanocomplex. The fatty acids in cis conformation showed highest activity than in trans conformation and the lowest erythrocyte lysis was observed with trans-vaccinic acid. Brinkmann*et al*., [13] reported that it is not known how OA binds to *α*-LA and whether equilibrium exists between OA bound to *α*-LA and/or OA bound to other OA. There is no consensus to the cytotoxicity can be ascribed to a synergistic effect of *α*-LA and OA or exerted per se by either of the two components. Almost full lysis was observed at a BAMLET concentration of 175 lμg/mL^{-1}, with no lysisat 88 lμg/mL^{-1}. Full lysis at a measured OA concentration of 62 and 51 μm,while, *α*-LA did not lyze the erythrocytes at any concentration under test.The lysis of erythrocytes suggests that the cytotoxicity of BAMLET could consist of two parts (i.e. high concentrations in lysis of the cells, whereas, at lower concentrations, more subtle response was seen, resulting in cell death with features resembling apoptosis and ⁄ or necrosis). Lysis is caused by OA alone, whereas the complex between *α*-LA and OA induces more ordered form of cell death. OA is responsible for more than the simple lysis effect. The hemolysis assay cannot distinguish between lysis and more subtle form of cell death.These results clearly suggest that the cytotoxic function of the α-LA/ fatty acid nanocomplex was similar to that of HAMLET and clearly

formed apoptotic complex where heat treated which changed such mixture to a cytotoxic component. These results are in agreement with those obtained by Kamijima*et al*., [6].

Erythrocytes lysis induced by BAMLET (*α*-LA/fatty acids nanocomplex) and fatty acids depends on the concentration of fatty acids. Cytotoxic complexes can be made with other fatty acids than OA; however, only complexes with *α*-LA and vaccenic acid were reported to be as cytotoxic as HAMLET. Complexes of *α*-LA and five fatty acids with different saturation and double bond conformation were all cytotoxic, but to various extents.

The results also agree with Svenssone*t al*.,[9] who suggested that only complexes between *α*-LA and unsaturated fatty acids in cis conformation can induce cell death. Complexes between *α*-LA and either OA or CV were reported to be the most active causing 100% and 99% cell death, respectively, while complexes with *α*-LA and linolenic acid (Lin) or palmatic acid (PA) showed only minor cytotoxic effect, causing 11% and 27% cell death, respectively. They did not see any cytotoxicity of complexes between *α*-LA and saturated or unsaturated trans-fatty acids. The cytotoxicity of the complexes was correlated to the number of fatty acid per protein means more fatty acids per protein being more cytotoxic.

Conclusions

The *α*-LA nanoparticle has the affinity to bind different fatty acid such as eliedic acid, cis-vaccenic, trans-vaccenic and linolenic acidas well as oleic acid. The *α*-LA nanoparticles have higher affinity to bind the cis-unsaturated fatty acids more than the trans-unsaturated or saturated fatty acids.Increasing binding fatty acids with *α*-LA nanoparticles reflect increasing the nanocomplex availability to kill cancer cell or produce a complex hasmore turmcidicaleffects.The erythrocytes lysis measured indicated that the *α*-lactalbumin/ fatty acid nanocomplexes have more efficiency to erythrocytes lysis than individual fatty acids.In general, all *α*-LA/ fatty acids nanocomplex showed cytotoxic effect in different extent depending on the type and concentration of fatty acid. Nanocomplex contained cis-fatty acids showed almost the same cytotoxicity as BAMLET, while complexes with trans-fatty acids were less cytotoxic. The cytotoxicity of the complexes increased with higher numbers of fatty acids per *α*-LA molecule. The fatty acids alone in buffer solutions showed also a cytotoxic effect to less extent as in complex with protein.

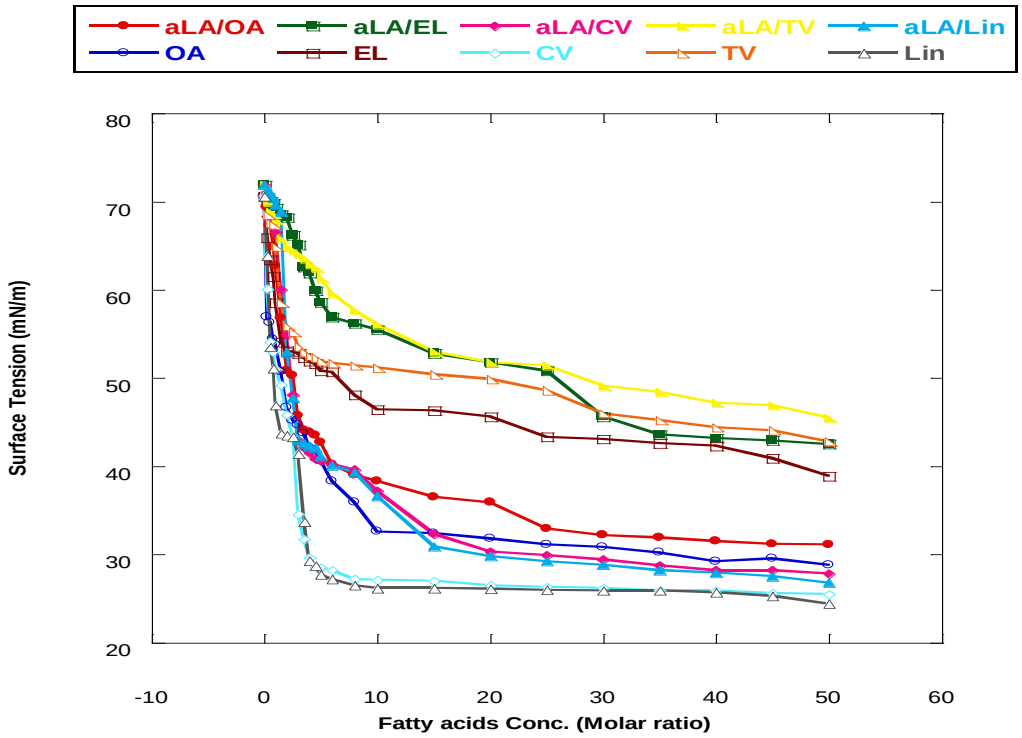

Fig. (1): Surface tension of different concentrations of individual fatty acid and different *α*-LA/fatty acids nanocomplexes.OA= oleic acid, EL= eliedic acid, CV= cis-vaccenic, TV= trans-vaccenic, Lin= linolenic acid

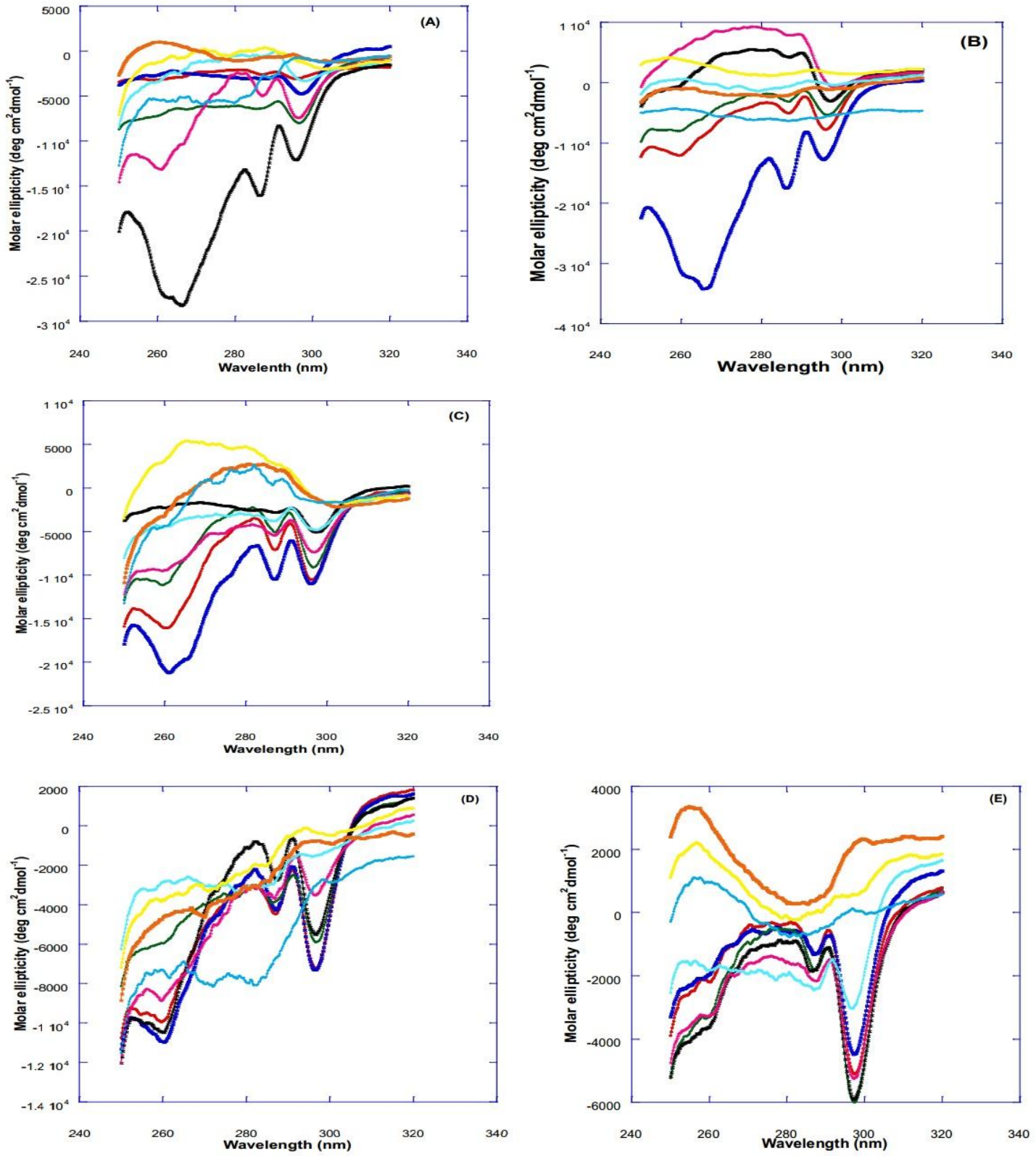

Fig. (2): Circular Dichroism (CD) spectra of *α*-LA/Fatty acids nanocomplex with different concentrations of fatty acids (molarratio) Inthefar-UV region. (A): α-LA with different concentrations of oleic acid; (B): α-LA with different concentrations of eledic acid; (C): α-LA with different concentrations of cis-vaccenic acid; (D) α-LA with different concentrations of trans vaccenic acid;(E):α-LA with different concentrations of linolenic acid.

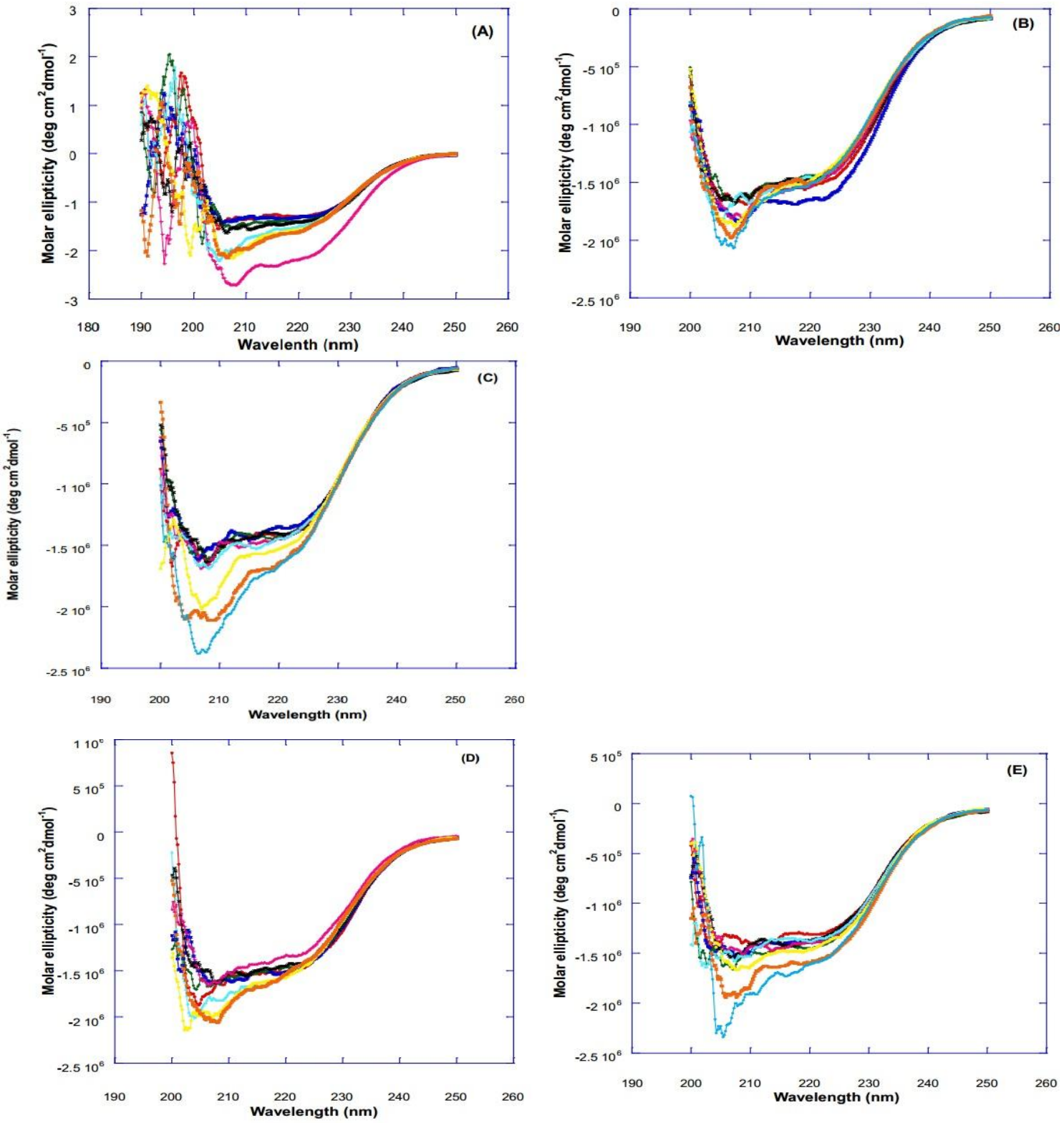

Fig. (3): Circular Dichroism (CD) spectra of α-LA/Fatty acids nanocomplex with different concentrations of fatty acids (molar ratio) in the far -UV region. (A): α-LA with different concentrations of oleic acid; (B): α-LA with different concentrations of eledic acid; (C): α-LA with different concentrations of cis-vaccenic acid; (D) α-LA with different concentrations of trans vaccenic acid; (E): α-LA with different concentrations of linolenic acid.

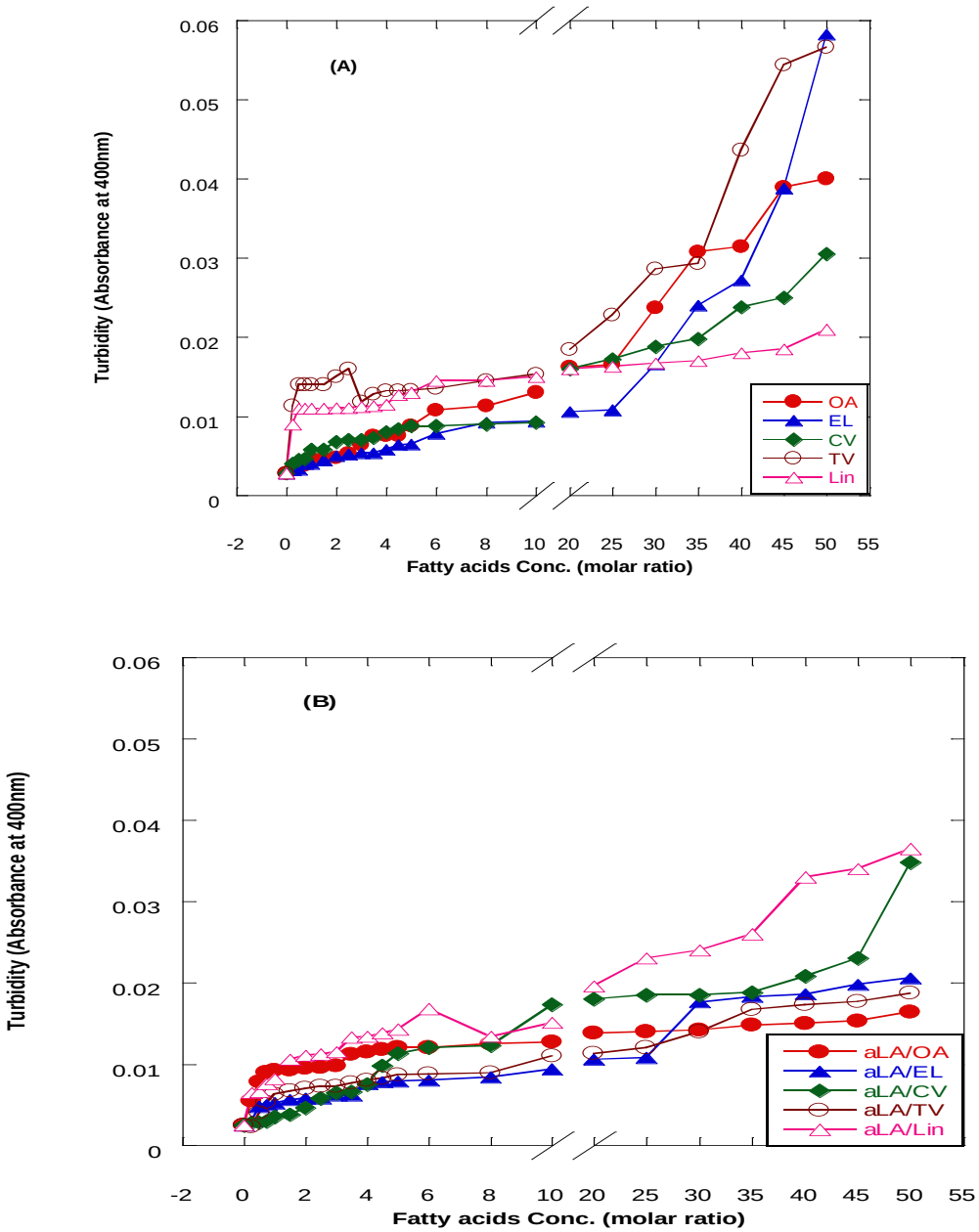

Fig. (4): Turbidity valuesof different concentrations of individual fatty acid and different *α*-LA/fatty acids nanocomplexes.OA= oleic acid, EL= eliedic acid, CV= cis-vaccenic, TV= trans-vaccenic, Lin= linolenic acid

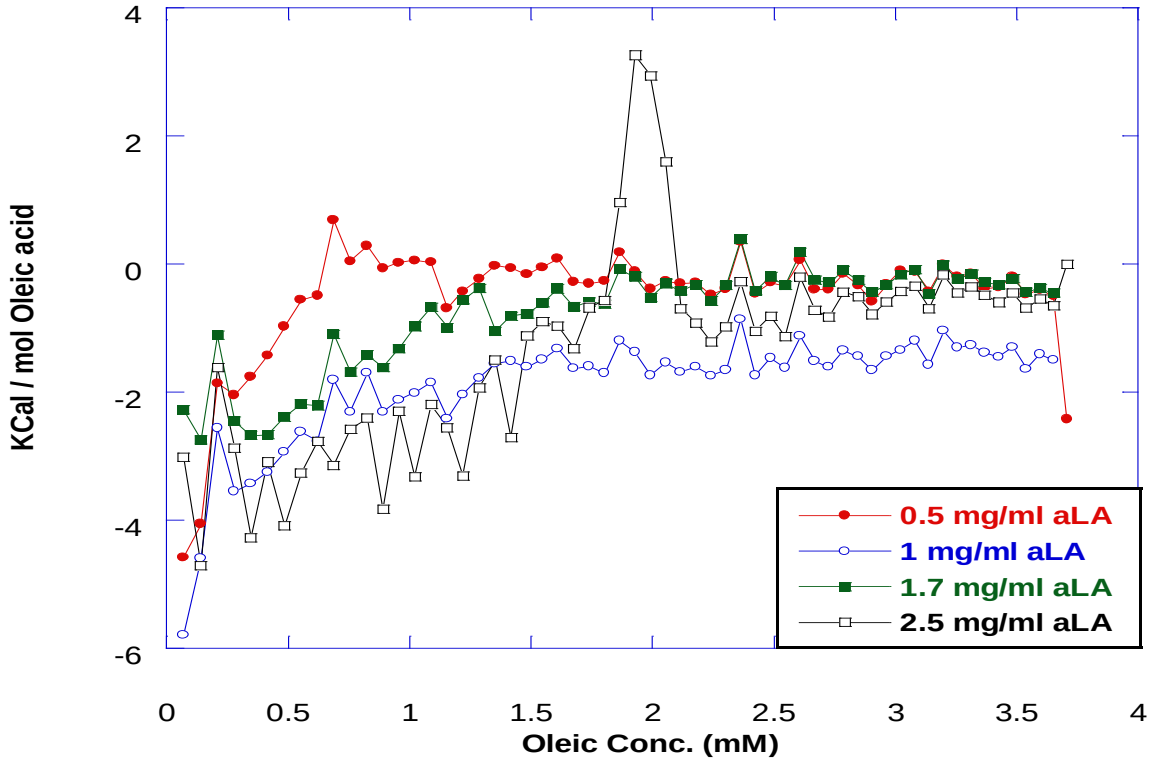

Fig. (5): ITC enthalpogram per mole of oleic acid injected into different concentrations of *α*-LA (µM)

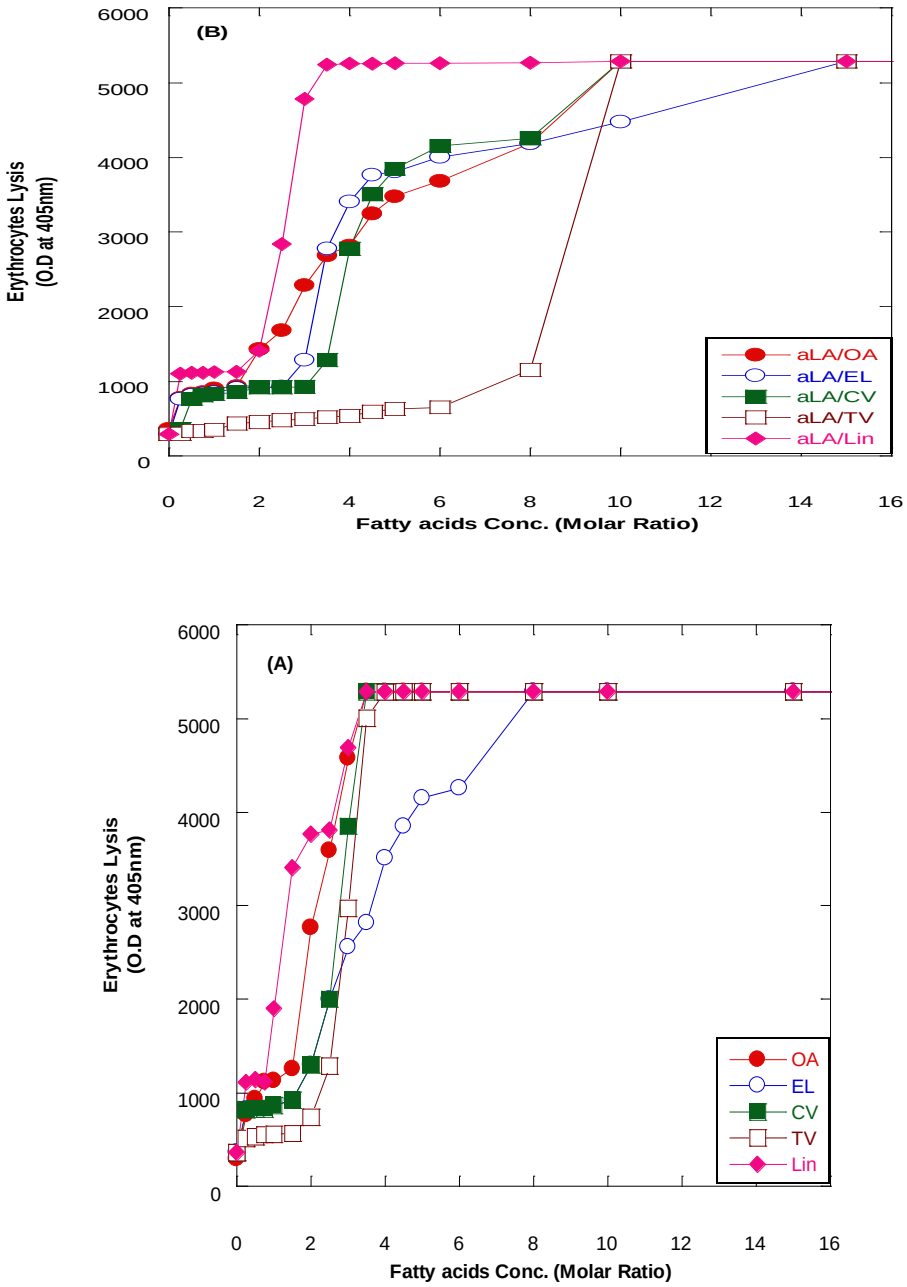

Fig. (6): Cytotoxic activity measurements as erythrocytes lysis for individual fatty acids (A) and (B) *α*-LA/fatty acids nanocomplex.OA= oleic acid, EL= eliedic acid, CV= cis-vaccenic, TV= trans-vaccenic, Lin= linolenic acid

Reference

[1] Mok K.H., Pettersson J., Orrenius S. and Svanborg C. (2007). HAMLET, protein folding, and tumor cell death. BiochemBiophys Res Commun 354: 1-7.

[2] Hakansson, A.; Zhivotovskyt, B.; Orrenius, S.; Sabharwal, H. and Svanborg, C. (1995). Apoptosis induced by a human milk protein. Proc. Natl. Acad. Sci. USA. 92: 8064 - 8068.

[3] SvenssonM., Hakansson A., Mossberg A. K., Linse S. and Svanborg C. (2000).Conversion of alphalactalbumin to a protein inducing apoptosis.ProcNatlAcad Sci., USA; 97: 4221-4226.

[4] Svanborg, C.; Agerstam, H.; Aronson, A.; Bjerkvig, R.; Duringer, C.; Fischer, W.; Gustafsson, L.; Hallgren, O.; Leijonhuvud, I.; Linse, S. (2003). HAMLET Kills tumor cells by an apoptosis-like mechanism – cellular, molecular, and therapeutic aspects. Adv. Cancer Res.; 88: 1 – 29.

[5] Pettersson, J. Mossberg, A., Svanborg, C. (2006). α -Lactalbumin species variation, HAMLET formation, and tumor cell death.Biochemical and Biophysical Research Communications 345, 260–270.

[6] Kamijima, T.; Ohmura, A.; Sato, T.(2008). Heat treatment method for producing fatty acid-bound alpha-lactalbumin that induces tumor cell death.Biochemical and Biophysical Research Communications; 376: 211- 214.

[7] Dobrovolskaia, M. A; Clogston, J. D.; Neun, B. W.; Hall, J. B.; Patri, A. K. and McNeil, S. E. (2008). Method for analysis of nanoparticle hemolytic properties in vitro.Nano.Lett.; 8: 2180 – 2187.

[8] SAS (2004). Statistical Analyses Systems. SAS, User Guide Statistics. SAS Institute Inc. Editors, Cary, NC.

[9] Svensson M., Mossberg A.K., Pettersson J., Linse S. and Svanborg C. (2003). Lipids as cofactors in protein folding: stereo-specific lipid-protein interactions are required to form HAMLET (human alpha-lactalbumin made lethal to tumor cells). Protein Sci., 12: 2805 - 2814.

[10] Kuwajima K. (1996). The molten globule state of alpha-lactalbumin.FASEB J; 10: 102 - 9.

[11]Curry, S.; Mandelkkow, H.; Brick, P. and Franks, N. (1998). Crystal structures of apo- and holo-bovine α-lactalbumin at 2.2 Å resolution reveal an effect of calcium on inter-lobe interactions. J. Biol. Chem. 275: 37021 – 37029.

[12] Wangsakan, A.; Chinachoti, P. and McClements, D. J. (2001). Maltodextrin–Anionic surfactant interaction: isothermal titration

calorimetry and surface tension study. J. Agric. Food Chem., 49: 5039 – 5045.

[13] Brinkmann, C. R.; Heegaard, C. W.; Petersen, T. E.; Jensenius, J. C. and Steffen Thiel, S. (2011). The toxicity of bovine α-lactalbumin made lethal to tumor cells is highly dependent on oleic acid and induces killing in cancer cell lines and noncancer-derived primary cells. FEBS Journal 278: 1955–1967.