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Chitosan Liposomal microspheres for Ricinoleic acid Encapsulation

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ABSTRACT

Ricinoleic acid (RA) is a C18 fatty acid (FA) with a double bond at the C 9 position and a hydroxyl group at the C(12) position (cis-12-hydroxyoctadeca-9-enoic acid). Recently, RA has been reported as a pro/anti-inflammatory and analgesic agent for topical applications to be considered as an alternative to irritant substances that relive pain. However, RA when it is exposed to air, it reacts with the oxygen and decomposes into short-chain aldehydes and ketones. Moreover, pathologically, small amount of anesthetic agent acts on peripheral nerves, producing reversible block in transmission of peripheral nerves impulses. However, larger amounts effect potentially in the central nervous system and may cause cardiac arrest. Accordingly, extended release formulations for local anesthetic agent such as encapsulation, are highly demanded if the drug will be used for long period. In this work, ricinoleic acid, extracted and characterized, was encapsulated into a new matrix made of phospholipid liposomes and chitosan to protect and control RA release. RA release was controlled by cross-linking the matrices using glutaraldehyde. Spectral and morphology analysis are used to characterize the produced microsphere. The cytotoxicity test is considered to examine the final product biocompatibility. The encapsulation efficiency was investigated by UV- Visible spectroscopy.

INTRODUCTION

Ricinoleic acid (RA), a C_{18}, monounsaturated (at $C_{9\text{-}10}$), monohydroxylated (at C_{12}), and an aliphatic fatty acid, is the main component of the castor oil (Vaisman *et al*., 2008). The use of RA in medical purposes dates back to the 1500 $_{BC}$ when ancient Egyptians chewed the plant seeds with beer to relieve constipation. However, oil has been prescribed to be applied locally for sores and hemorrhoids (Gaginella and Philips, 1975). Recent study results (Gaginella and Philips, 1975; Vieira *et al*., 2000a and 2000b; Vieira *et al.*, 2001) provide evidence that RA shows a pro- or anti-inflammatory and analgesic action following its topical application, and it represents a useful alternative to irritant substances that relieve pain (Vaisman *et al*., 2008). Pathologically, small amount of anesthetic agent acts on peripheral nerves, producing reversible block in transmission of peripheral nerves impulses. However, larger amounts effect potentially in the central nervous system and may cause cardiac arrest (Vaisman *et al.*, 2007). Accordingly, extended release formulations for local anesthetic agent such as encapsulation, are highly demanded if the duration of the drug action is relatively long. Topical application of percutaneous (through unbroken skin) drugs have been developed dramatically via different carries such as; ointments (Raza *et al.*, 2011), creams (Adeyeye *et al*., 2002; Aramwit and Sangcakul, 2007; Jenning *et al*., 2000; Miura *et al*., 2004; Paolino *et al*., 2002; Sadaf *et al*., 2006; Zhou *et al.*, 2010), encapsulated beads (Denkbaş and Odabaşi, 2000; Donthidi *et al*, 2010; Lin and Ayres, 1992; Meyer *et al.*, 1998; Silva *et al*., 2005), gels (Kumar and Katare, 2005; Sahiner and Singh, 2007; Scartazzini and Luisi, 1988; Shchipunov, 2001; Vintiloiu and Leroux, 2008), and films (Vanin *et al*., 2005). Several materials either synthetic or natural have been used for producing such delivery matrices (Kumbar *et al*, 2014). Gelatin (Andreuccetti *et al*., 2009; Bigi *et al*., 2001; Bigi *et al*., 2002; Cao *et al*., 2007; Cheng *et al.*, 2003; Dong *et al*., 2006; Gómez-Estaca *et al*., 2009; Matsuda *et al*., 1999; Sobral *et al*., 2001; Tanaka *et al*., 2005; Vanin *et al.*, 2005; Yakimets *et al.*, 2005), chitosan (Cheng *et al.*, 2003; Gómez-Estaca *et al.*, 2009), cellulose (Adeyeye *et al.*, 2002) and starch derivatives (Donthidi *et al.*, 2010), alginate (Dong *et al.*, 2006; Donthidi *et al.*, 2010; Silva *et al.*, 2005), polyvinyl alcohol

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(Levi *et al.*, 2011), acrylate polymers (Sahiner and Singh, 2007) and many others have been used for performing different matrices for delivery systems. More recently, liposomes, phospholipid vesicles, have been found to be effective as carriers for topical drug administration. Phospholipids molecules spontaneously form bilayer membranes that can be converted to vesicles, namely liposomes. In lipid vesicles, encapsulated drug can be entrapped depending on the drug hydrophobicity (Urano *et al.*, 1988), either on the hydrophilic core or within the hydrophobic bilayer (e.g. fatty acids) (Xi *et al*., 2006). Liposome physical structure would allow it to pass through lipophilic skin layers and to be trapped within the top layer of the stratum corneum cells. Such behavior is useful for both local treatment and for cosmetic formulations. However, liposomal final product is very soft and easy to collapse (Lee *et al*., 2009).

In this paper, free ricinoleic fatty acid was isolated from commercial castor oil and new matrix of encapsulation was made of phospholipid liposomes and chitosan. In particular, commercial lecithin was used as a source of phospholipid and RA impregnated beads was prepared using emulsification technique. RA release was controlled by chemical cross-linking and spectral analysis demonstrated the chemical structure of the new matrices.

EXPERIMENTAL

Materials

A commercial lecithin from soybean was purchased under trade name Lio. A commercial grade castor oil was generously gifted from Fats and Oils department of Food Industry and Nutrition division at NRC. Chitosan (medium molecular weight), acetone, chloroform, ethylene glycol, glutaraldehyde (GTA), ethyl acetate (EtOAc), potassium hydroxide (KOH), hydrochloric acid (HCl), and deuterated acetone and dimethyl sulfoxide (DMSO) were purchased from Sigma and were used throughout this research without further purification.

Methods

Preparation of ricinoleic acid from castor oil

Pure ricinoleic acid (RA) can be obtained by using alkali treatment of commercial castor oil (cold processed) in the presence of alcohol followed by an acidification step to liberate free RA (Salimon *et al*., 2012).

250 g of dried castor oil was transferred to round bottom flask, which has KOH alcoholic solution (60 g dissolved in 500 mL of ethyl alcohol) and equipped with water condenser, for refluxing for 1 hour. The product was concentrated under vacuum distillation. The residual was dissolved in 1200 mL deionized water (the potassium salt of ricinoleic acid is water soluble) and was acidified with conc. HCl up to pH = 1. The free RA was extracted in ethyl acetate (EtOAc) (600 mL) and then was dried over night using magnesium sulfate. The oily organic substance was filtered. The solvent (EtOAc) was evaporated using an evaporator at 50 ^{o}C.

Preparation of RA chitosan/ liposomal microspheres

In the current part, chitosan, non-toxic and biopolymer, was used for hardening the liposomal structure as described below. In typical procedure (Figure 1), 2 gm lecithin (locally available source of phospholipid) was placed to 1.5 gm RA dissolved in 50 mL of chloroform in a 1L round-bottomed flask. The dissolved mixture was stirred for 30 min at room temperature and then concentrated under reduced pressure at 50 ^{o}C for 30 min till a thin lipid layer formed in the inside-wall of the round flask. Afterwards, 50 mL of 2 % chitosan solution was added to the flask and stirring was continued for more 2 hrs at 1500 rpm and sonicated for 1 hr (Takahashi *et al.*, 2006). The final product is very stable milky emulsions.

The chitosan-liposomal microspheres with and without RA were produced by dropping the fresh prepared warm emulsion through a syringe needle to a mixture of 75 mL of commercial oil and 25 mL acetone under magnetic stirrer for 15 mint at 1000 rpm. Small amount of acetone was added to the oil bath and beads were collected and washed several times with acetone (RA dissolves in acetone but lecithin is not) using vortex. Beads were air-dried and stored at 4 ^{o}C for further use. Figure (2) shows the suggested approach of the prepared microspheres.

Preparation of cross-linked microspheres

Fresh prepared beads were cross-linked with 10 mL of 0.5 M of GTA solution in phosphate buffered solution (PBS) at pH 7.4 for different duration (0-2-6 hrs) at ambient temperature with gentle starring (Cheng *et al*., 2014). The cross-linked beads were washed several times with PBS solution followed by distilled cold water and acetone. The final product was directly subjected to a reduction with an excess of sodium borohydride in buffered solution (pH = 4) to give the corresponding secondary amine. Microspheres were air-dried and stored at 4 ^{O}C for further analysis.

Spectral analysis

The chemical structure of ricinoleic acid was characterized with FTIR (Vertex 80v (Bruker)), and H^1 NMR spectrometer, Avance III 500 (Bruker Biospin), using deuterated chloroform solvent ($CDCl_3$).

Morphological characterization

The microspheres size was examined using an optical microscope (Axio Imager.A1m, Carl Zeiss Jena GmbH) equipped with image analyzer (SPOT Insight, Diagnostic instruments inc., Sterling Heights MI, USA; CASCADE II 512, Photometrics, Roper Scientific Inc., Tucson AZ) and a software (Meta Series Software 7.1.1, Molecular Devices). Results were obtained from 5 times measurements.

In vitro drug release experiment

The release rate of RA from gelatin-based liposomal beads were determined by incubating 50mg of RA loaded cross-linked beads in 10 mL PBS (pH 7.4) at 37^{o}C in shaking water

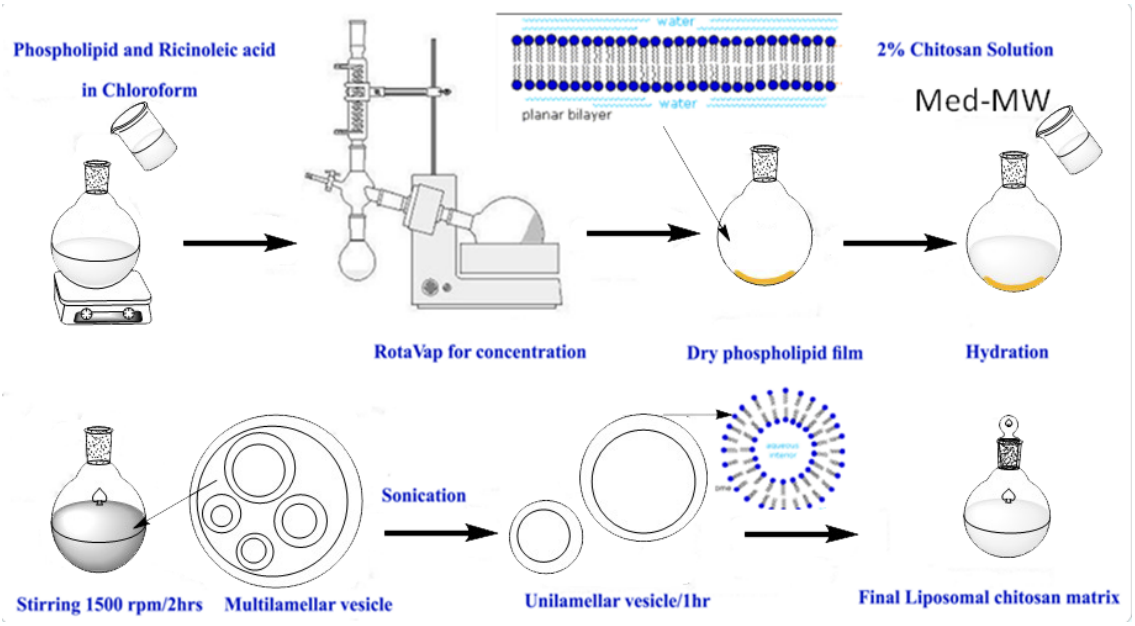


Fig. 1: Schematic diagram of the preparation of RA liposomal/chitosan microspheres..

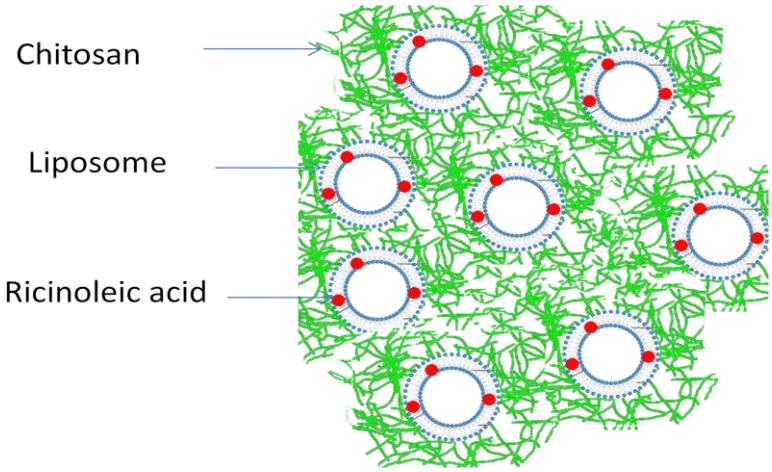


Fig. 2: The suggested approach of the microspheres production in which liposomes surrounded by chitosan chains (green lines represent chitosan chains; red spots represent fatty acid drugs; blue lines represent liposomal phospholipids).

bath. At time intervals, 1 mL of the released medium was taken and 1 mL methanol was added to lyse the liposomes and 2 mL acetone was added to dissolve RA. The 4 mL mixture was filtered through 0.2 µm membrane and the absorbance readings of the supernatant were recorded at 222 nm. The released amount was calculated from standard calibration curve.

Encapsulation efficiency

The encapsulation efficiency was defined as the measurement of the remaining content of the core material which is encapsulated in the wall material compare to the starting core material content:

EE%= [Practical loading drug]/[Theoretical loading drug] X 100

The practical loading drug was measured as mentioned above in the release study.

Cytotoxicity assessment

In general, any substance or formula that comes into contact with an open wound should be clean and liberate no chemical agent that may be toxic or have an adverse effect on healing process. Micro-culture Tetrazolium (MTT) assay is a useful technique for detecting such agents by using the yellow tetrazolium dye to measure the cell viability (Nada *et al*., 2011). The cytotoxicity test of the prepared samples was conducted in adaption from the ISO10993-5 standard test method, using the human skin fibroblasts (HFB4) cell line. The choice of fibroblast cell line was based on their role on producing the protein associates in extracellular matrix (ECM) synthesis and their crucial role in wound healing process. In typical procedure, cells were maintained in Dulbecco's modified eagle medium (DMEM):F12 Medium (nutrient mixture)/10% fetal bovine serum (FBS) and were incubated at 37°C in 5% CO_2 and 95% humidity. HFB4 cells

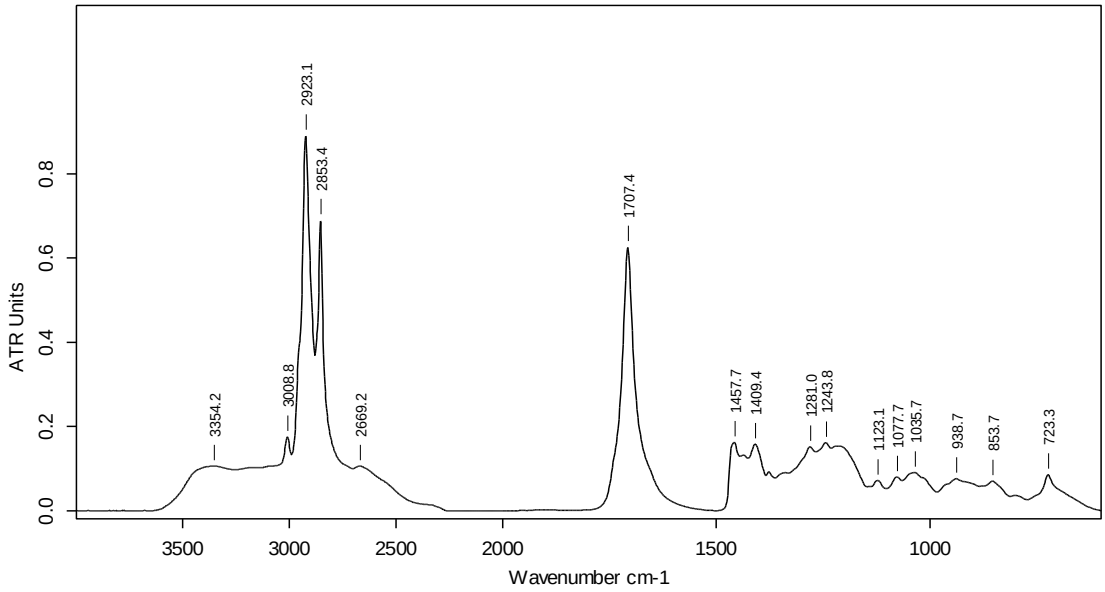


Fig. 3: FT-IR ART spectrum of ricinoleic acid.

were seeded into wells in 96-well plate and at a density of 30000 cells per well. After incubation for 48 hrs the culture medium were replaced by extracted media of different concentrations (from 6.25 to 100 mg/mL) of the tested compounds (each sample was tested in 3 different wells). Samples were sterilized under ultraviolet (UV) light for 20 min in a laminar flow before extraction while the extracted media were filter-sterilized using a 0.22 µm syringe filter. Cell culture medium without additional reagents was used as the control. The plate was incubated again for 24 hrs. The amount of living cells was determined by the MTT assay.

The culture medium was aspirated and replaced by 50 uL of MTT solution (5 mg/mL). Then, the solution was incubated for 4 hr at 37 ^{o}C. The solution was aspirated and 900 µL of DMSO containing 125 µL of glycine buffer (pH= 10) was added to dissolve the formazan crystals. The solution was centrifuged for 10 min to obtain a clear DMSO solution. The absorbance of the DMSO solutions at 570 nm was measured on a plate reader.

Statistical analysis

Results were expressed as a mean value with its standard deviation (mean ± S.D.) of each sample that is repeated three times (n=3). Statistical analysis was performed with student's t-test and differences were considered as significant at p-values under 0.05.

RESULTS AND DISCUSSION

Ricinoleic acid chemical structure characterization

Figure (3) shows the FT-IR spectra of ricinoleic acid. In the IR spectral data of ricinoleic acid a strong O-H absorption band appears at 3354 cm^{-1} indicating that the hydroxyl groups of the acid remains unaffected by the alkali treatment. The strong carboxylic carbonyl absorption peak was observed at 1707 cm^{-1}. Two strong peaks at 2923 and 2853 cm^{-1} indicate the alkyl part of the acid. Figure (4) shows the H^{1} NMR spectrum of the ricinoleic acid. The chemical shifts are: δ 0.91 ppm (3H, H_M), δ 1.2-1.6 ppm

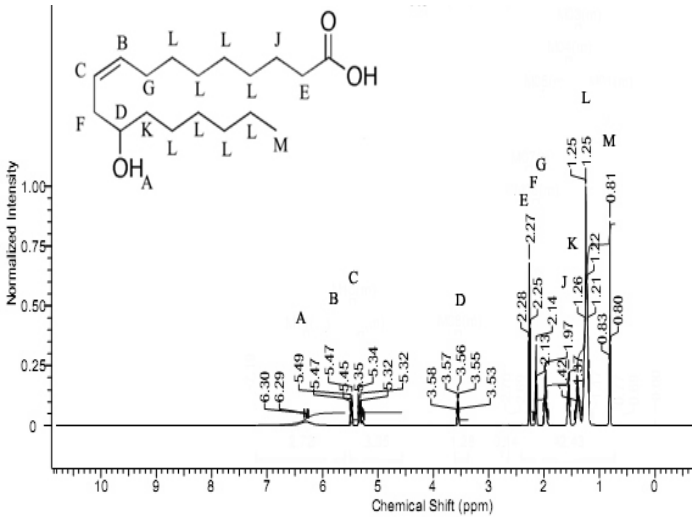


Fig. 4: H^{1}-NMR spectrum of ricinoleic acid in *CDCl$_3$* (300 MHz).

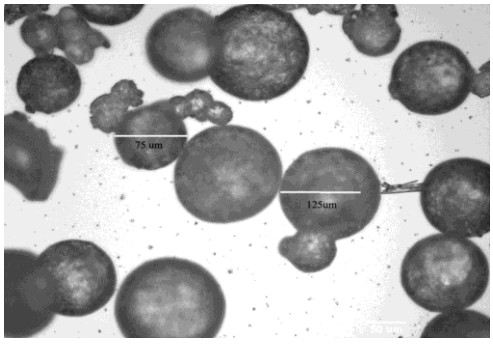


Fig. 5: Micrograph of RA-chitosan/liposomal microspheres

(18H, H_L), δ 1.75 ppm (2H, H_K), δ 2.1 ppm (2H, H_J), δ 2.3 ppm (2H, H_G), δ 2.4 ppm (2H, H_F), 3.4-3.6 ppm (1H, H_C), 3.8 ppm (1H, H_B), 4.1 ppm (1H, H_D) and δ 5.5 ppm (1H, H_A). The characteristic hydroxyl proton at δ 5.5 ppm was observed. The double bond protons are distinguished as two multiplets peaks at 3.8 and 3.4-3.6 ppm.

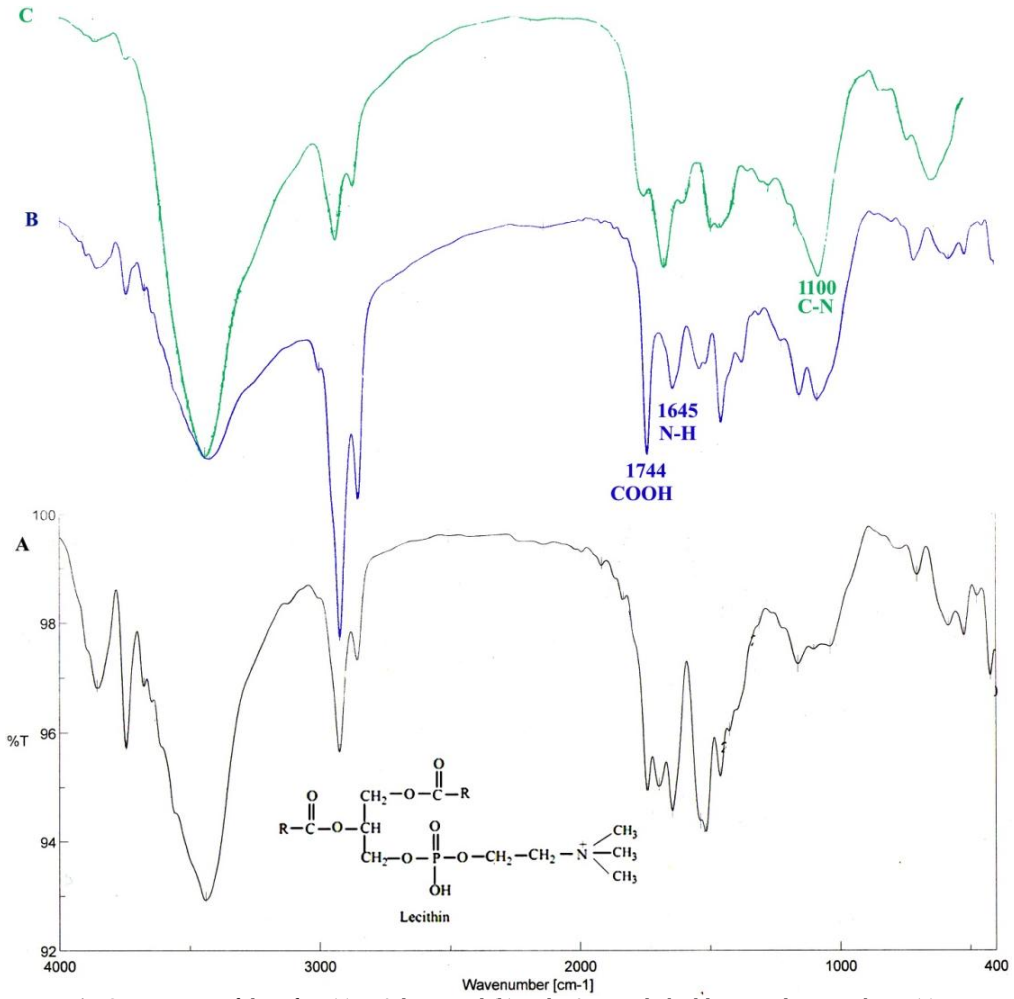
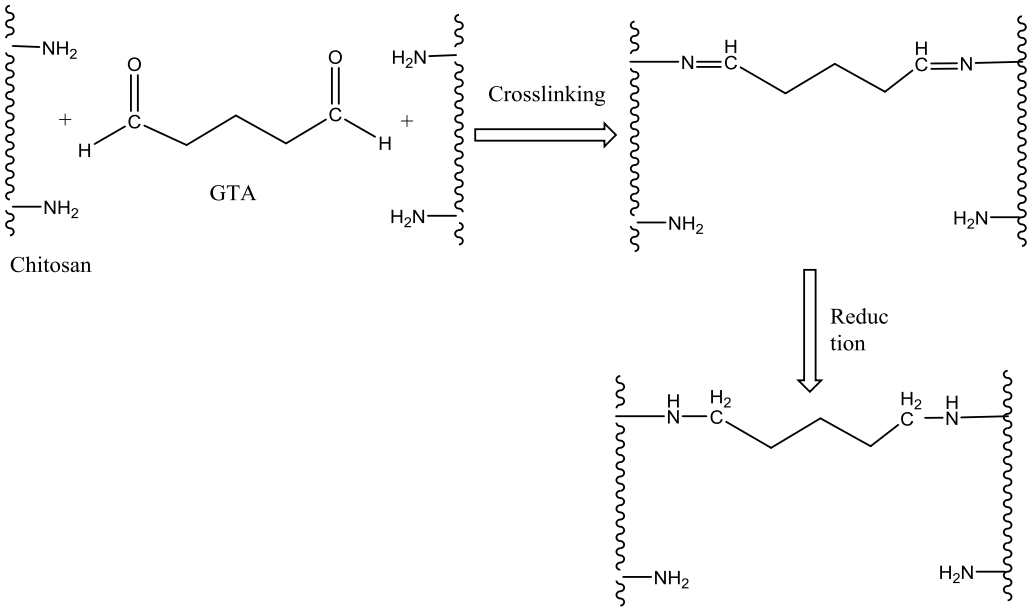


Fig. 6: IR spectra of drug-free (a); RA-liposomal (b) and RA-cross-linked-liposomal microspheres (c).



Scheme 1: Suggested mechanism of the crosslinking of the RA chitosan/liposomal microspheres With GTA.

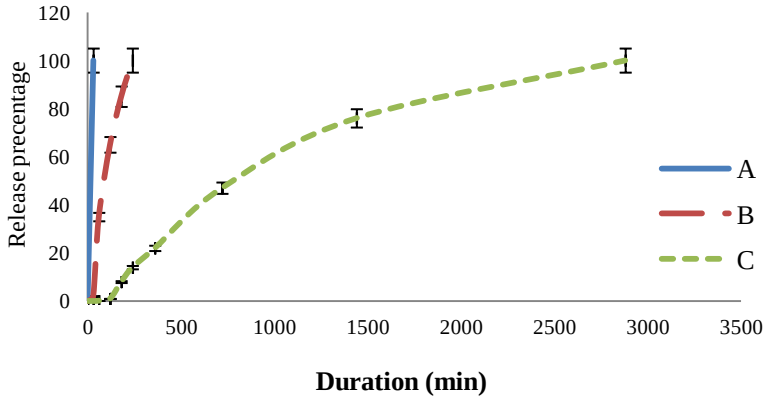


Fig. 7: Release profile of RA from RA-chitosan liposomal beads with curing time with GTA; at 0 time (A); after 2 hrs (B); after 6 hrs (c) in 0.5M GTA solution.

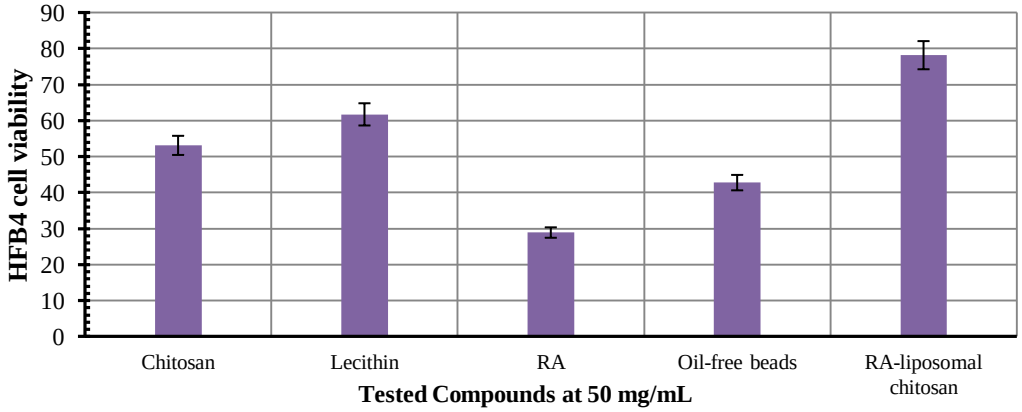


Fig. 8: The cell viability of HFB4 cells of the tested compounds at 50 mg/mL.

Surface morphology of RA and CLA Liposomal/Chitosan microspheres

Figure (5) shows the morphology of the RA chitosan/ liposomal microspheres. The microspheres were quite clear in spherical shape with smooth surface. The diameter range of the microsphere was in range 150±50 µm.

FT-IR spectra of fatty acids liposomal- chitosan microspheres

Figure (6) shows the FT-IR spectra of drug-free (a), RA-liposomes (b) and RA-cross-linked-liposomal microspheres (c).

FT-IR spectrum (a) of the liposomal chitosan (drug-free) microspheres displayed the main characteristic absorption bands of both chitosan and the phospholipid. Literally, C-H stretching at 2857 cm^{-1}, N-H 1645 cm^{-1}, and C-N 1460 cm^{-1} represented the chitosan main characteristic bands. However, the phosphate group of the phospholipid band displayed at 1163 cm^{-1}.Figure 6b shows a characteristic peak of the carbonyl group of RA at 1744 cm^{-1}. The latter is considered as a proof of the fatty acids encapsulation into the chitosan liposomal microspheres.

Figure 6c shows the characteristic peak of the chemical reaction between chitosan and GTA which corresponds the stretching of C-N bond at 1100 cm^{-1}. The amino groups in chitosan in spectrum B diminished which is indicating the formation of a Schiff's base reaction between the amino groups of the chitosan and the aldehyde groups of the GTA as described in Scheme 1.

Encapsulation efficiency

The amount of RA released from the uncross-linked beads after 15 min reached to 90%. This phenomenon is due to the absence of any cross-linking to the microsphere matrix which leads to instance release. Therefore, the encapsulation efficiency is very high to reach 90%. However, when RA-liposomal chitosan beads react with GTA at 0.5 M for different duration, RA release profile has changed accordingly (Figure 7). Within 2 hrs of cross-linking, RA release has delayed to 4 hrs. While after 6 hrs of cross-linking, RA release has prolonged to 2 days. GTA has formed cross-links between the amino groups of the chitosan and resulted in a slower RA release from the chitosan-liposome matrix.

Cytotoxicity assessment

Biocompatibility of the produced liposomal chitosan matrix including the RA was demonstrated by fibroblast cell viability. Material toxicity is defined as greater than 30% cell death (Nada *et al.*, 2014). Figure (8) shows the cell viability of HFB4 cells against pure chitosan (medium molecular weight); pure lecithin; pure RA; drug-free chitosan liposomes; and RA liposomal chitosan bead. Results shown in Figure (8) reveal that all samples have passed the 30 % cell viability except for the RA samples. Such adverse effect for RA on cell viability may be attributed to their surfactant characteristics associated with their

capability to inhibit water transfer to cells and isolate cells. Add to that, RA showed significant cell death more than other for its laxative actions (fluid secretion) (Gaginella *et al*., 1977). Also the increase of cytotoxicity of chitosan is due to the higher amount used (50mg/µL) to prepare the extraction medium. It has been reported that chitosan with different molecular weights showed significant cytotoxicity at concentration higher than 0.741 mg/mL (Huang *et al*., 2004).

CONCLUSION

RA was extracted from commercial castor oil and characterized via spectral analysis. RA was encapsulated into stable chitosan/liposome matrix by cross-linking with GTA. The release profile of RA has changed upon the duration time with the cross-linking with GTA. The RA chitosan-liposomal bead and its constituents were proved to be non-toxic matrix. The new chitosan/ lecithin matrix can be considered as a good approach for protecting RA from environmental degradation and presenting RA for topical applications.

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