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Fabrication of chitosan-alginate microencapsulated curcumin coated scaffold to develop novel cotton crepe bandage

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Medicated cotton crepe bandages have been developed, using natural curcumin conjugated polymer nanoparticle with biocompatible and biodegradable properties. Curcumin (C) has been incorporated into Chitosan (Ch) and sodium alginate (A) microcapsule, which serve as templates for nanofibres. The coating of C-ChA results in chitosan scaffold (CS) containing curcumin-chitosan microcomplex (CS-CChM). Both the substances act as a powerful biomaterial for tissue engineering applications, especially for wound healing. CS-CChM along with C-ChA have been characterized using FTIR and SEM. The free radical scavenging effect is determined by DPPH (2,2-diphenyl-1-picrylhydrazyl) and anti-inflammatory assay. The developed CS-CChM nanofibre shows an impressive mechanical stability which is an essential requirement for wound healing. The bearing of curcumin shows cytotoxic effect. The *in vitro* radical scavenging activity and the anti-inflammatory assay are performed by inhibition of albumin denaturation. These microencapsulated matrix displays potent anticancer activity against mouse fibroblast on both normal (L929) and human lung adenocarcinoma (A549) cells. The cell viability is observed to be 51.4 ± 0.71 and 30.53 ± 0.99 respectively for both. CS-CChM nanofibre is found to be stable against enzymatic degradation, which is the most important parameter for promoting the proliferation of cells, contributing to repair and remodeling of tissues during wound healing applications.

Keywords: Antioxidant, Anti-inflammatory, Biodegradable, Curcumin, Chitosan, Cytotoxicity, 2,2-diphenyl-1-picrylhydrazyl, Radical scavenging activity

1 Introduction

Natural products, embracing the bioactive ingredients, play a vital role in exhibiting their therapeutic properties[1]. Curcumin is a phenolic compound, extracted from the turmeric plant and mainly used as an essential coloring substance, which can absorb the visible light at a wavelength range 420–425 nm. Curcumin has a variety of biological activities and pharmacological actions, such as anti-inflammatory, anti-carcinogenic and anti-virus properties, as well as promising clinical applications due to its low toxicity[2]. In recent years, curcumin has been shown to inhibit cell proliferation in a variety of human cancer cell lines *in vitro* and has been used both to prevent and treat various cancers *in vivo*. However, it has extremely low aqueous solubility and shows rapid intestinal and hepatic metabolism, which results in poor systemic bio-availability and restricts its oral use[3]. The bacterial infection is a major problem in recent days and it interferes with wound healing process that may lead to life threatening complications. To overcome this problem, a new modern therapeutic nano coated drug for wound healing properties has been developed[4].

Chitosan and alginate are well known for accelerating the healing of wounds in humans. Chitosan (Ch), a natural cationic polymer, is biologically renewable, biodegradable, biocompatible, non-antigenic, nontoxic, and biofunctional, and it can accelerate the wound healing process enhancing the functions of inflammatory cells, macrophages, and fibroblasts. The advantages of these biomaterials can be easily processed into different forms, such as membrane, sponges, gels, scaffolds, microparticles, nanoparticles and nanofibre for a variety of biomedical applications such as drug delivery, gene therapy, tissue engineering and wound healing[5]. In medicine, it may be useful in bandages to reduce bleeding and as an antibacterial agent; it can also be used to help deliver drugs through the skin. Alginate, an anionic polymer, is also biocompatible, hydrophilic, and biodegradable under normal physiological conditions. Both the polymers interact to form complexes through chemical binding[6].

This paper reports study on the preparation of curcumin encapsulated chitosan-alginate nanocapsules

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for their antioxidant and anti-inflammatory activities on cotton crepe bandage to use against the pain and inflammation. Normally cotton crepe bandage is used for relieving pain by means of resting the muscles. However, the coating on cotton crepe bandage with curcumin increases its pain relieving property and also reduces the inflammatory response caused by the friction of bones. The cotton crepe bandage fabricated with the curcumin will be a great relief for the chronic arthritic patients. Furthermore, the chitosan as curcumin-chitosan microcomplex coated into the belladonna plaster will enhance reduction in pain and inflammation. Normally the hansaplast is widely used for relieving pain by means of muscle stiffness, nerves swelling, joint swelling, mumps and boils. Coating the hansaplast with the chitosan scaffold containing curcumin-chitosan microcomplex increases its pain relieving property and reduces the inflammatory response.

2 Materials and Methods

2.1 Extraction of Curcuminoids

The extraction of curcuminoids was done using the methodology as reported by Kulkarni[7]. Twenty gram (20 g) turmeric rhizome powder sample was taken into a thimble and placed in a soxhlet apparatus, which was set up with the mid polar methanol solvent (B P 65^{o}C). Methanol (20mL) was added and extracted according to its boiling point for 7 h. After completion of extraction the dark brown extract was cooled, concentrated separately under reduced pressure using rotary evaporator and stored at -20˚C for further testing. The pure curcumin (1mg/mL) was used as standard. Finally, curcuminoids were analyzed by using a spectrophotometric method. The different concentrations (20, 40, 60, 80, 100µg/mL) of the sample were measured at 420 nm using UV – Visible spectrophotometer (Hitachi, U-2900).

2.2 Preparation of Curcumin Loaded Chitosan-alginate (C-ChA)

The chitosan powder (0.2g) in 20mL of acetic acid solution (0.5 % w/v), sodium alginate powder (0.2g) was dissolved in 20mL of deionized water. The solution was mixed in different ratios such as 1:1, 2:1 and 3:1 and blended with a homogenizer until an opaque aqueous solution was obtained. Then the curcumin was accurately weighed and dissolved in absolute methanol. The dissolved curcumin was conjugated with chitosan- alginate. The solution was centrifuged to remove the trapped air bubbles. Finally, the air bubble-free solution was poured into the petri plates, frozen overnight at -40°C, and then lyophilized at 35°C for 24 h in a freeze dryer.

Fabrication of chitosan matrix was done using the method as followed by Amirthalingam *et al*[8]. Chitosan scaffold was fabricated by the freeze gelation method. Briefly, 2 g of chitosan was dissolved in 100 mL of acetic acid solution (2% w/v in water) and this polymer solution was allowed to freeze at -20ºC. The frozen chitosan solution was immersed in a pre-cooled NaOH and methanol solution for gelation of chitosan. Finally, the gelled chitosan was slowly dried at room temperature. The completely dried scaffold was taken in a petridish, and curcumin microcomplex (equivalent to 50mg) suspension in purified water was added onto the surface and slowly dried at room temperature to obtain chitosan scaffold containing curcumin-chitosan microcomplex (CS-CChM) particles.

2.3 Characterization of C-ChA and CS-CChM

FTIR measurements were performed using a Perkin Elmer 2000 spectrometer with Grams as the operating software to determine the functional groups present in the prepared curcumin, C-ChA and CS-CChM samples. The spectra were recorded in the frequency range of 4000−600 cm^{-1} on an infrared spectrophotometer (IR Prestige21, Shimadzu). The structure and composition of freeze-dried purified C-ChA and CS-CChM were analyzed using a 10 kV ultra-high resolution SEM. The 25µL of sample was sputter coated on copper stub, and the images of curcumin coated chitosan were studied using an FEI QUANTA-200 SEM.

2.4 DPPH Radical Scavenging Activity

The free radical scavenging activity of curcumin encapsulated chitosan-alginate extract was measured *in vitro* by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay[9]. About 0.1mM solution of DPPH in 100% methanol was prepared, and 1mL of this solution was added to 500µL of C-ChA dissolved in methanol at different concentrations (20-60µg/mL). This mixture was shaken and allowed to stand at room temperature for 30 min in the dark and the absorbance was measured at 517nm using spectrophotometer. The IC_{50} value of drug was compared with that of ascorbic acid, which was used as standard. The capacity to scavenge the DPPH radicals was calculated using the following formula:

$$\text{DPPH scavenged (\%)} = (\text{Control OD} - \text{Test OD}) \times 100 / \text{Control OD}$$

2.5 Anti-inflammatory Activity

The anti-inflammatory activity of test sample was studied by using inhibition of albumin denaturation technique[9]. The reaction mixture consists of the test extract at different concentration and 1% aqueous solution of bovine albumin fraction. *p*H of the reaction mixture was adjusted using a small amount of 1N HCl. Diclofenac sodium (10mg) was used as a standard drug. The samples were incubated at 37ºC for 20 min and then heated at 57ºC for 20 min. After cooling the samples, the turbidity was measured spectrophotometrically at 660 nm. The experiment was performed in triplicate. Percentage inhibition of protein denaturation was calculated as follows:

$$\% \text{ Inhibition} = (\text{Control OD-Test OD}) \times 100 / \text{Control OD}$$

2.6 Fabrication of CS-CChM into Hansaplast and Crepe Bandage

Fabrication of chitosan scaffold containing curcumin- chitosan microcomplex into hansaplast and crepe bandage respectively was done as per the method of Mohanty *et al.*[10]. CS-CChM hansaplast was prepared using 50µg in 20mL of methanol. The suspension of methanol and CS-CChM was coated into the belladonna plaster. The 50µg of curcumin encapsulated chitosan-alginate was incorporated into a fluid phase of 1.75mL of oleic acid. The mixture of Oleic acid containing curcumin encapsulated chitosan-alginate was coated into the cotton crepe bandage using the padding technique.

2.7 Cell Line and Culturing

The L929 - mouse fibroblast normal cell line and human lung adenocarcinoma (A549) cell line were purchased from National Centre for Cell Science (NCCS), Pune. Cells were maintained in the logarithmic phase of growth [minimum essential medium (MEM) with 10% FBS (GIBCO, USA), 100 U/mL penicillin and 100µg/mL streptomycin] in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C. Once the cells had covered about 80% of the surface, they were trypsinized and then adjusted using haemocytometer for anti-proliferative and cytotoxicity measurements.

2.8 Cytotoxicity Assay

The MTT [3-(4, 5-dimethythiazol-2-yl)- 2,5-diphenyl tetrazolium bromide] assay was used to test the viability of the cells (3×10^5/well). It was seeded in 96 well plates, incubated for overnight in CO_2 incubator. The cells were treated with various concentrations of CS-CChM and the cells were then washed with phosphate buffer saline (PBS). Next, 50µL of MTT (5mg/mL) was added to each well, and the wells were incubated for 3 - 4 h at 37˚C. The purple, blue MTT formazan precipitate was dissolved in 100µL of Dimethyl sulfoxide (DMSO). The absorbance was measured on the ELISA reader at 570 nm. The triplicate values were calculated using the following formula:

$$\% \text{ of cell viability} = (\text{OD of treated cells} / \text{OD of control cells}) \times 100.$$

2.9 Statistical Analysis

Data, expressed as the Mean ± S.D using statistical software SPSS version 17.0, was used for the analysis. *P* value <0.05 was considered significant.

3 Results and Discussion

3.1 Extraction and Estimation of Curcuminoids

Curcuminoids are extracted from the samples through "soxhlet extraction apparatus". After drying the extract, the weight percentage of curcuminoids is calculated (Table 1). Kulkarni *et al.*[7] obtained the maximum concentration of curcuminoids in the methanol extract in the form of yellowish orange color. Concentrations of the curcumin in the extracted dried sample are analyzed on spectrophotometer at 420 nm with respect to standard curcumin. The wavelength at which maximum absorption takes place in UV spectrophotometer at 420nm has been obtained by Hazra *et al*[11]. The results show that the concentration of the curcumin is higher in rhizome turmeric (103mg) than in processed turmeric powder (76mg). Physicochemical characterization of chitosan-alginate interactions due to the positively charged chitosan and negatively charged sodium alginate have been studied for different blend ratio of chitosan-alginate (1:1, 2:1 and 3:1)[6].

Table 1 — Dry weight and percentage of curcumin from different turmeric powder samples

Sample	Solvent	Dry wt. (in 20g turmeric) g	Curcumin present in the extract, %
Fresh rhizome turmeric powder	Methanol	2.06	10.3
Processed turmeric powder	Methanol	1.92	9.6

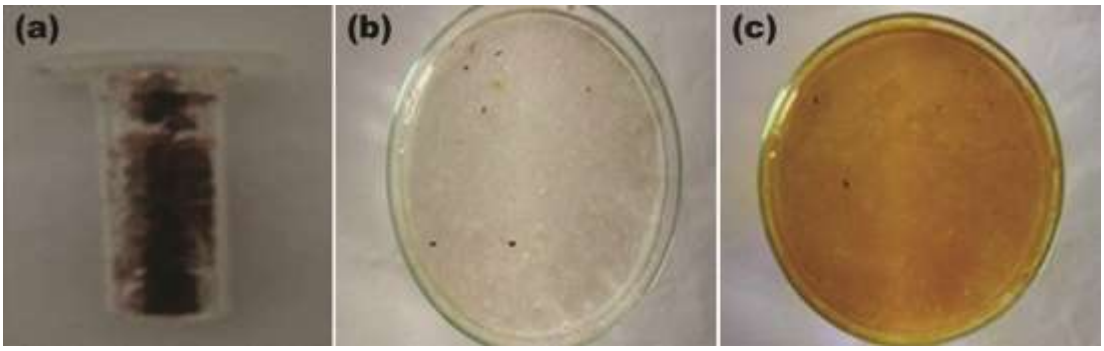

Fig. 1 — Curcumin encapsulated chitosan alginate coated microcomplex (a) curcumin incorporated into the chitosan (C-ChA), (b) chitosan scaffold, and (c) chitosan scaffold containing chitosan – curcumin microcomplex (CS – CChM)

3.2 Curcumin - incorporated Chitosan-alginate

The curcumin-methanol solution is incorporated into the chitosan-alginate that shows highest intensity to prepare the curcumin loaded chitosan-alginate (C-ChA) [Fig. 1 (a)]. Chitosan acts as sustain drug carrier, and the curcumin helps in establishing wound healer, pain relieving and anti-inflammatory activity from the scaffold. The chitosan matrix is prepared by the freeze gelation method. The frozen chitosan polymer solution and gelling liquid are maintained at the same temperature to avoid sudden change in the temperature during the gelling process. The prepared chitosan scaffold is shown in Fig. 1 (b). The excess surface water from the gelled scaffold is carefully removed and the air is dried and stored at room temperature (37˚C). The chitosan support is developed and the results are found in agreement with the studies of Amirthalingam *et al*[8].

The curcumin microcomplex is attached with chitosan through physical interaction. Chitosan in the form of solid support could be converted into a drug delivery system by incorporating curcumin microcomplex in them. The curcumin released from the microcomplex incorporated scaffold would aid wound healing by controlling bacterial infections in the injured skin. The chitosan supported chitosan-curcumin microcomplex (CS-CChM) was shown in Fig. 1 (c). In this study, the chitosan scaffold containing chitosan-curcumin microcomplex (CS-CChM) has been prepared for evaluation of anti-inflammatory, antioxidant and cytotoxic activity.

3.3 FTIR Study

The Fourier transform infrared (FTIR) spectra at different ratio of C-ChA are depicted in Fig. 2. The intensity of the characteristic peak is recorded high as 3:1 (C-ChA) followed by ratios 2:1 and 1:1. This indicates that the degree of ionic interaction between

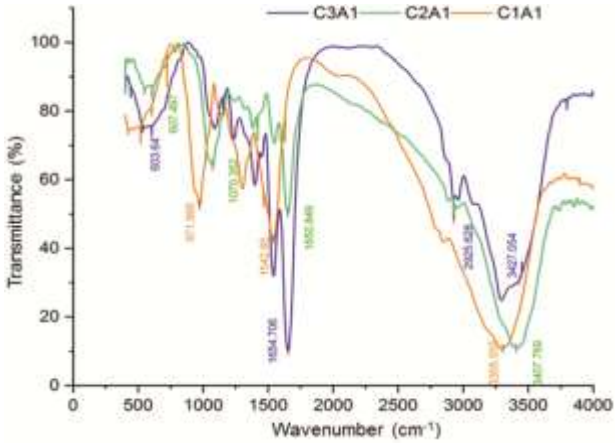

Fig. 2 — Comparative FTIR spectra of curcumin compounds incorporated with chitosan alginate at different ratios [3:1 blue color peak (C3A1), 2:1 green color peak (C2A1), 1:1 orange color peak (C1A1)]

the negatively charged carboxylic ion group of alginate and the positively charged amino group of chitosan in three different ratios of chitosan-alginate inter polyelectrolyte complex is different and found high in 3:1 ratio of chitosan and alginate complex. In the FTIR spectrum, C-ChA (3:1 ratio, blue color peak) exhibits a peak at 3247 cm^{-1} due to amine C-H stretching (phenolic group), besides other peaks at 2925 cm^{-1} (amine N-H stretching), 1654 cm^{-1} (aromatic (C=C) bending stretching and 603 cm^{-1} [primary amines strong halocompound (C-Br) stretching]. Further, the functional group of C-CA (2:1 ratio, green color peak) exhibits amide −NH and −OH stretching frequencies of the broad peak at 3407 cm^{-1} and 1070 cm^{-1} [strong sulfoxide (S=O)] stretching. Finally, C-CA (1:1 ratio, orange color peak) shows the peak at 3305 cm^{-1} for −NH stretching. Peaks for N-O stretching of nitro compound, and alkene (C=C) bending stretching are observed at 1542 cm^{-1}. The peak at 971 cm^{-1} corresponds to the presence of curcumin aromatic

(−C=C−) in C-ChA matrix. The alginate spectrum shows a characteristic band of carbonyl (C=O) at 1640 cm^{-1} and 1424 cm^{-1}, as also mentioned by Dai *et al*[12].

3.4 SEM Study

The cross-sectional and surface morphology of the C-CA and CS-CChM shows the cubic structure with a size range of 35 - 65 nm. The CS-CCM nanofibres have the smooth surface and exhibits improved cell attachment and sufficient oxygen permeability for quick war time action in the skin regeneration process. The particle surface of the microcomplex is found to be smooth. Figure 3 shows SEM micrographs of C-ChA: (a) scale bar 1µm and magnification ×10,000, (b) its surface morphology, scale bar 1µm and magnification ×20,000, (c) scale bar 0.5µm and magnification ×30,000, and (d) scale bar 0.2µm and magnification ×55,000. Similarly, Thi *et al.*[13] reported that PF/Chi NPs loaded with curcumin shows that the particles are spherical and uniform.

3.5 DPPH Radical Scavenging Activity

DPPH in the presence of methanol produces stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. This study illustrates the hydrogen donating ability of C-ChA at various concentrations as compared to standard antioxidant (L- ascorbic acid). The model of scavenging the stable DPPH radical is a widely used method to evaluate antioxidant activities in a relatively short time as compared to other methods. The reduction capability of the DPPH radical is determined by the decrease in its absorbance at 517 nm, induced by curcumin encapsulated chitosan alginate. The IC_{50} value is 41.37 ± 0.71 at 60 µg/mL of methanolic extract of C-ChA. Curcumin is well known to exhibit antioxidant property, as also reported by Das *et al*.[9] Similarly, C-ChA also have the same antioxidant property at different concentrations (Fig. 4).

It is reported that the oxidative stress occurs when the free radical formation exceeds the body's capacity to protect itself and this contributes different biological chronic conditions, such as arteriosclerosis, arthritis, cancer, diabetes and various neuro-degenarations. The primary antioxidants react with free radicals, and this may limit free radical damage occurring in the human body[14]. The observation of this study supports the ability of C-ChA in protection against oxidative damage.

3.6 Anti-inflammatory Activity

Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as a strong acid or base, a concentrated inorganic salt, an

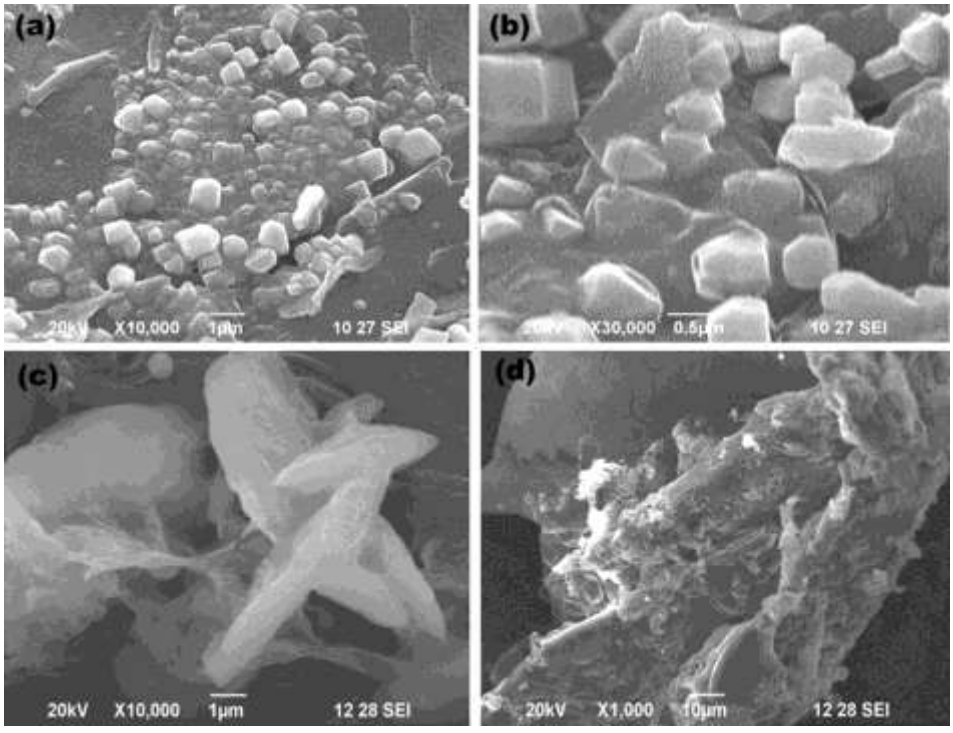

Fig. 3 — SEM micrographs of nanofibres (a) C-ChA (curcumin incorporated chitosan alginate) at × 10,000 & (b) C-ChA at ×30,000; and (c) CS-CChM (chitosan scaffold containing chitosan-Curcumin microcomplex) at ×10,000 & (d) CS-CChM at ×1000 magnification

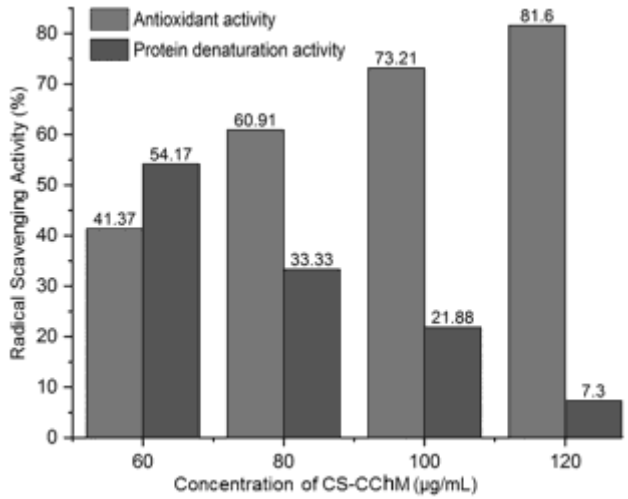

Fig. 4 — Free radical scavenging and anti-inflammatory activity

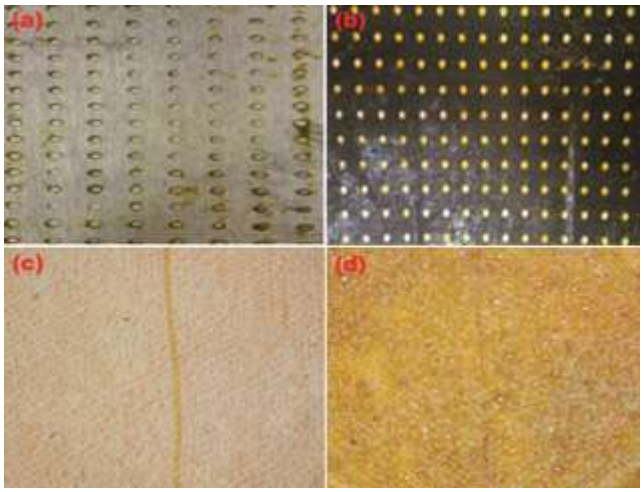

Fig. 5 — Curcumin encapsulated chitosan-alginate fabricated cotton crepe bandage (a) control -front view of hansaplast; (b) fabricated with CS-CChM- back view of fabricated hansaplast; (c) control cotton crepe bandage; and (d) fabricated with C-ChA of fabricated cotton crepe bandage

organic solvent and heat. Most biological proteins lose their biological function when denatured. Denaturation of proteins is one of the well-defined causes of inflammation. Curcumin is well known for its anti-inflammatory property[14], causing the similar anti-inflammatory property in the CS-CChM at different concentrations (Fig. 4). This CS-CChM have shown inhibition of thermally induced protein (albumin) denaturation in a dose dependent manner, where the IC_{50} value is 54.17 ± 0.28 in 60 µg/mL of methanolic extract of CS-CChM and diclofenac sodium is used as a standard. Since inflammation is one of the body's nonspecific internal systems of defense, the response of a tissue to an accidental cut is similar to the response that results from other types of tissue damage, caused by burns due to heat, radiation, bacterial or viral invasion[15].

3.7 CS-CChM Coated into Hansaplast Fabrication

Hansaplast contains 0.2% of *Atropa belladonna* extract. The healing power of belladonna activates body heat against pain reliefs from muscle stiffness, nerves swelling, joint swelling, mumps and boils. The curcumin loaded oleic acid based polymeric (COP) bandages was formulated by Hazra *et al* [11]. Similarly, the CS-CChM coated into the hansaplast increases the relieving property against pain and inflammation. Control and fabricated with chitosan scaffold containing curcumin-chitosan microcomplex coated hansaplast are shown in Figs 5 (a) and (b).

3.8 Fabrication of C-ChA Coated Cotton Crepe Bandage

Cotton crepe bandage, used to reduce the arthritic pain when fabricated with C-ChA using padding technique, causes reduced inflammation and pain due to the enhanced antioxidant and anti-inflammatory activity of the prepared curcumin microcomplex. Crepe bandages both control and fabricated with curcumin encapsulated chitosan alginate are shown in Figs 5 (c) and (d). This study leads to fabricate curcumin microcomplex into crepe bandage and the construction of CS-CChM into the belladonna plaster for enhanced reduction in pain and inflammation[16].

3.9 Cytotoxicity Activity

The cytotoxic effects of cell adhesion/proliferation, against normal mouse fibroblast (L929) cell line and cancer cell line (A549) are studied and the viability of chitosan coated curcumin scaffolds is investigated using phase contrast microscopy and MTT analysis. The results are also compared with normal and cancer cell lines. Some representative microscopic images of cultured L929 cells are shown in Figs 6 (a) and (b). The effect of chitosan coated curcumin scaffolds at different concentrations (5, 25, 50, 75 and 100µg/mL) and the maximum cell viability of L929 cells are studied at 48 h. Cytotoxicity against the mouse fibroblast cell line L929 shows maximum cell lysis at the concentration of 100µg/mL. The IC_{50} value is 75µg/mL (51.49 ± 0.71), which indicates that such concentration could lyse 50% of L929 cell line after 48 h incubation. This significantly decreases the cell viability in treating concentrations as compared to control ($p < 0.05$). Clearly, the MTT assay results reveal that the metabolic activity of L929 cells decreases with the lower concentration of extract in

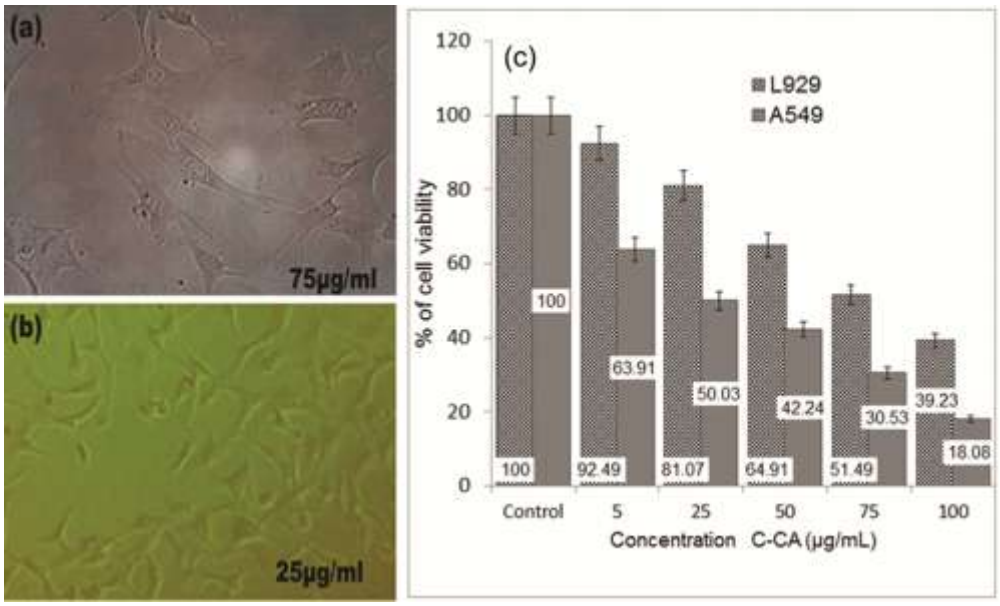

Fig. 6 — MTT assay results of chitosan coated curcumin scaffolds (a) IC_{50} concentration of normal mouse fibroblast L929; (b) IC_{50} concentration of human lung adenocarcinoma A549 cell lines, and (c) graphically showing the percentage of cell viability

chitosan coated curcumin matrix *in vitro.* Verma *et al.*[17] reported that the normal cell lines at higher concentrations show significant cell mortality. Moreover, biocompatibility characteristics of *Nepenthes khasiana* mediated synthesized GNPs are evaluated against normal mouse fibroblasts (L929) cell lines[18].

In the present study the antiproliferation of chitosan coated curcumin scaffolds is made on cancer cell line A549 assessed by MTT assay. Dose–dependent inhibitory concentrations are observed at 5, 25, 50, 75 and 100µg/mL. The IC_{50} (50.03 ± 1.01) value for A549 cells is recorded using 25 µg/mL at 24 h. The results are shown in Fig. 6 (c). There is a significant decrease in the cell viability in lower extract concentrations when compared to control ($p < 0.05$). The chitosan coated HA-DTX conjugate nanoparticles show effective cytotoxicity, causing human breast cancer (MCF-7) and 4 T1 cell lines, with inhibitory concentration (IC_{50}) of 45.34 µM and 354.25 µM respectively. On the other hand, 233.8 µM and 625.9 µM IC_{50} is observed for DTX on 4T1 and MCF-7 cell lines after 48 h incubation. DTX loaded chitosan nanoparticles are found more effective against cancer cells than free DTX drug[19].

4 Conclusion

In this study, a C-ChA nanofibrous scaffold coated with curcumin has been prepared for wound healing application. FTIR and SEM reveal the structural characterization of C-CA and CS-CChM in a stable form. C-ChA coated on the cotton crepe bandage can be effectively used for arthritis against pain and inflammation. Similarly, the matrix can also be used for the above-said diseased condition and they do exhibit cytotoxicity to L929 (normal fibroblast cells) at higher concentration of curcumin, whereas cytotoxicity in cancer cell is achieved at a lower concentration itself, confirming the least toxicity of the prepared scaffold to normal cells at a very high concentration. Hence, the development of novel curcumin coated crepe bandage and chitosan coated scaffold is beneficial for use in diseased condition having inflammation in muscles and joints like arthritis.

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