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Cytotoxicity Effect Assessment of Theophylline Loaded with Collagen Nanoparticles

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Abstracts

The selective drug delivery of asthma treatment is more challenging. Mullet fish contain several types of medically essential substances, such as collagen, which can be an effective and straightforward method of drug delivery. The present study aimed at extracting collagen from mullet fish scales and forming collagen nanoparticles using ethanol (non-solvent for collagen) to provide a desolvation process and loading of theophylline with the nanoprecipitation method collagen nanoparticles in the presence of glutaraldehyde as a crosslinker. The cytotoxicity effect of theophylline loaded with collagen nanoparticles compared to collagen nanoparticles and free theophylline was evaluated using MTT assay on NHSF cell line at the first dose of 3 mM theophylline with double fold serial dilutions. Our research demonstrated the efficacy of theophylline loading in collagen nanoparticles, and we were assured of the result by energy dispersive X-ray mapping (EDX) and optical microscopy. The order of increase in cytotoxicity was collagen nanoparticles < theophylline loaded with collagen nanoparticles < theophylline for all concentrations. These findings imply that collagen nanoparticles are an effective and safe method to use as a drug delivery system.

Keywords: Collagen extraction; Nanoparticles; Theophylline; Drug delivery; Cytotoxicity

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1. Introduction

Asthma is a chronic inflammatory disorder characterized by chronic narrowing of the bronchial airways due to increased bronchial smooth muscle contraction and hyperresponsiveness. Most clinical manifestations include wheezing and shortness of breath (Gohil et al., 2010). However, there are no selective and effective targeted drugs for the treatment of asthma, and they usually relieve the symptoms with no successful cure for asthma (Dong et al., 2014). Currently, asthmatic medications (immunosuppressants such as corticosteroids or some adrenergic drugs, such as selective β2 agonists), methylxanthine (aminophylline and theophylline), leukotriene antagonists, immunosuppressants such as (cyclosporine, and oral gold) and finally magnesium sulfate injection (in severe cases only), all the medications mentioned above treat symptoms without relieving the contributing factor that caused this asthma. This medication has side effects on the heart (such as tachycardia, blood pressure, increased risk of infections, and many other effects (Ismaiel & Shaffie, 2012).

Theophylline is one of the most commonly used medications in bronchial asthma due to its relatively low price, availability, and effectiveness. However, it is associated with several side effects because it is weak phosphodiesterase inhibitors (PDEs) that result in relaxation of airway smooth-muscle but also can affect many other organs, which is a case side effect (Ford et al., 2010). In addition to the bronchodilator effect, theophylline has other anti-inflammatory and immunomodulatory effects (Persson, 1988).

Several studies have been conducted to find an effective method to achieve the medication to the targeted tissue with fewer side effects. One of these methods is the development of nanoparticle drug delivery systems that decrease metabolism, increase the stability of easily metabolized medications, and reach the targeted tissue (Leu et al., 1984). Furthermore, using polymeric nanoparticles as drug delivery systems has several advantages, including high drug levels in the targeted tissue, decreased degradation, and sustained release of medications (O Elzoghby et al., 2016).

Collagen nanoparticles are an example of polymeric nanoparticles used as a drug delivery system, and there are many studies explaining the use of collagen as a drug delivery system (Calejo et al., 2012; Langasco et al., 2017; Nicklas et al., 2009; Rathore et al., 2020a, 2020b; Vigneswari et al., 2016). In the current study, the desolvation method extracted collagen from mullet scales and molded it as nanoparticles. Then theophylline was loaded into collagen nanoparticles by a simple but efficient method. In our research, we used collagen because of its high advantages as being a natural substance, biocompatible with humans, biodegradable, and does not act as an antigenic agent or cause allergic reactions. (Chan et al., 2020). The effect of theophylline and theophylline loaded with collagen nanoparticles with different concentrations on cell viability has been investigated.

2. Materials and Methods

2.1. Chemicals

Theophylline was obtained from GlaxoSmithKline Co. (Egypt). Mullet fish scales from a free market in the Beheira governorate, Egypt. Acetic acid, sodium hydroxide, ethanol, and glutaraldehyde were obtained from Merk co. Germany. The chemicals used in this research are high purity, and the solvent is HPLC graded. Other reagents and materials used in cell lines and cell cultures used for cell culture were obtained from Merk Co. Germany. Dimethylsulfoxide (DMSO) and thiazolyl blue were purchased from Sigma-Aldrich, UK. These chemicals, materials, solvents, and reagents were obtained from qualified sources and were of high quality.

2.2. Experimental methods

2.2.1. Preparation of fish scales and collagen extraction

Scales of grey mullet fish were isolated by hand and cleared with distilled water. The samples were dried, placed in polybags, and kept at 25 °C until use. Collagen was isolated following the method of Shalaby et al. 2020 with minor adjustments. Noncollagenous proteins and pigments were removed from the fish scales with 0.1N NaOH for two days, then cleared with distilled water. The samples were then extracted for two days with an acetic acid concentration (0.50 M), then homogenized for 3 hours. The supernatants were removed, and the remaining solution was filtrated.

2.2.2. Preparation of collagen nanoparticles and drug loading

The nanoprecipitation technique was used to create collagen nanoparticles using non-solvent (ethanol) (Tarhini et al., 2018). Ethanol was added utilizing a burette with free flow under stirring, which resulted in protein denaturation from stretched to coil conformational change. Theophylline was added slowly to an aqueous solution. To induce particle cross-linking, glutaraldehyde was added with stirring. The solution of drug-encapsulated nanoparticles was centrifuged, lyophilized, and stored for later use.

2.3. Characterization of Collagen Nanoparticles and Drug Load

2.3.1. Optical microscopy

A light microscope was performed to characterize collagen extracted from mullet fish scales, collagen nanoparticles, theophylline, and theophylline loaded with collagen nanoparticles.

2.3.2. EDX and mapping and diffraction analysis

Chemical purity was assessed using energy-dispersive X-ray spectroscopy (EDX), which is used by transmitting the sample with an electron and detected by a special electron microscope. Then the result was analyzed by the EDX analyzer.

2.4. Cell culture and cytotoxicity testing

On the NHSF cell line, the cytotoxic effect of absolute collagen nanoparticles, collagen nanoparticles loaded with theophylline, and theophylline was investigated with serial double-fold dilutions at an initial dose of 3 mM theophylline. Theophylline, theophylline loaded with collagen nanoparticles, and collagen nanoparticles were dissolved in DMSO to make 180 g/mL stock solutions, which were then serially diluted in a complete growth medium to make the different concentrations used in cytotoxicity assays. The cytotoxicity of each drug against the NHSF cell line was determined using an optical microscope and the MTT assay (Mosmann, 1983). This research follows that yellowish (MTT) turned into violet (formazan pigments) in live cells because of the effect on the mitochondria enzyme called succinate dehydrogenase. After a 24-hour incubation period at 37°C, exponentially developing cells were seeded in 96-well flat-bottomed microplates and subjected to drug doses for 48 hours. For each concentration, at least eight wells were used. Following the treatment, each well-received aliquots of 10 ml MTT solution (10 mg/ml in PBS). The MTT-formazan crystals were then dissolved by adding (100 ml/5 percent formic acid to the microplates for another 4 hours at 37 degrees Celsius) (in 2-propanol). At 580 nm, the absorbance was measured using a microprocessor-controlled Labexim LMR-1 microplate reader. The relative growth rate (RGR) was used to calculate the percentage of viability:

$$RGR\ \% = (D_t / D_{nc}) \times 100$$

where D_t denotes the wave absorption of the tested sample and D_{nc} denotes the wave absorption of the negative group (untreated).

3. Results

3.1. Optical microscopy

Analysis of collagen, collagen nanoparticles, and theophylline loaded with collagen nanoparticles using optical microscopy was demonstrated. Optical microscopy reveals the existence of collagen fibrils and the spherical form of collagen nanoparticles—the crystals of theophylline and the attachment of nanoparticles with theophylline.

3.2. Energy-dispersive X-ray (EDX) mapping

EDX mapping and spectra of collagen, collagen nanoparticles, and theophylline loaded with collagen nanoparticles are shown in **Figure 2**. The results showed the fibril shape of collagen, the conformational change of collagen nanoparticles from fibril shape to spherical nanoparticles, loading of theophylline to collagen nanoparticles in addition to the typical carbon (C), nitrogen (N), and oxygen (O) peaks are present in the spectrum **Figure 3,4,5**.

3.3. Cell culture and cytotoxicity testing

MTT assay on NHSF cell line at a concentration of 180 g/mL with double fold serial dilutions was used to investigate the cytotoxicity profile of collagen nanoparticles, theophylline-loaded nanoparticles, and theophylline. The percentage of cell viability in untreated control cells was calculated. The MTT experiment revealed that cells treated with collagen nanoparticles, theophylline, and theophylline loaded with collagen nanoparticles had more than 97% cell viability at lower dosages. At greater theophylline concentrations, cytotoxicity increased significantly, with 55.59 % of cells' viabilities detected at a 180 µg/ml dose. Theophylline loaded with collagen nanoparticles had a lower cytotoxic effect than theophylline alone, with 78.53 % cell viability at about 180 µg/ml of theophylline. The order of increase in cytotoxicity was collagen nanoparticles < theophylline loaded with collagen nanoparticles < theophylline for all concentrations **Table 1**, **Figure 5**.

The collagen nanoparticles-treated cells show a low activity, as determined by an optical microscope 24 hours after incubation (**Figure 7A**). Increasing concentrations of theophylline caused severe changes in cells (**Figure 7B**) compared to untreated control cells; cellular apoptotic components and cellular granules were observed, while the negative group that was not treated exhibited no changes. These changes indicate inhibition of cell growth, survival, and apoptosis. As shown in **Figure 7C**, the effects of theophylline loaded with collagen nanoparticles were less than those of theophylline-treated cells. The collagen nanoparticles-treated cells show a low activity, as determined by an optical microscope 24 hours after incubation (**Figure 7A**), Increasing concentrations of theophylline caused severe changes in cells (**Figure 7B**) compared to an untreated group; cytoplasmic granules and apoptotic bodies were noticed, whereas negative group (not treated cells) exhibited no changes. These changes point to the suppression of cell growth and eventually result in cell death, cell proliferation, viability, and death. The effects of theophylline loaded with collagen nanoparticles treatment were less than those in theophylline treated cells, as shown in (**Figure 7C**).

Table 1. The cytotoxicity profile of collagen nanoparticles, theophylline-loaded nanoparticles, and theophylline using the MTT assay on the NHSF cell line

	Concentrations								
Tested drugs	Conc. 9	Conc. 8	Conc. 7	Conc. 6	Conc. 5	Conc. 4	Conc. 3	Conc. 2	Dose 1
Collagen nanoparticles	99.22%	98.99%	98.48%	98.25%	96.77%	96.54%	95.76%	95.33%	94.40%
Theophylline-collagen nanoparticles	99.30%	98.63%	96.48%	96.69%	93.39%	87.34%	85.90%	83.80%	78.53%
Theophylline	99.59%	98.31%	96.57%	93.48%	89.50%	83.83%	78.48%	65.52%	55.59%
Conc. Means concentrations									

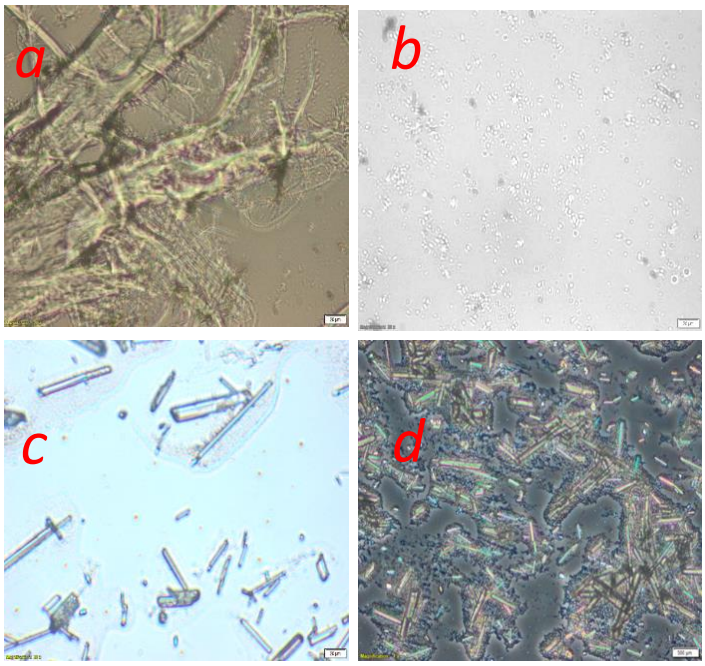

Figure 1. Images of collagen fibers taken from mullet fish scales under a light microscope (20X). (a), the spherical conformation of collagen nanoparticles (b), crystal shape of theophylline (c) and theophylline with collagen nanoparticles (d).

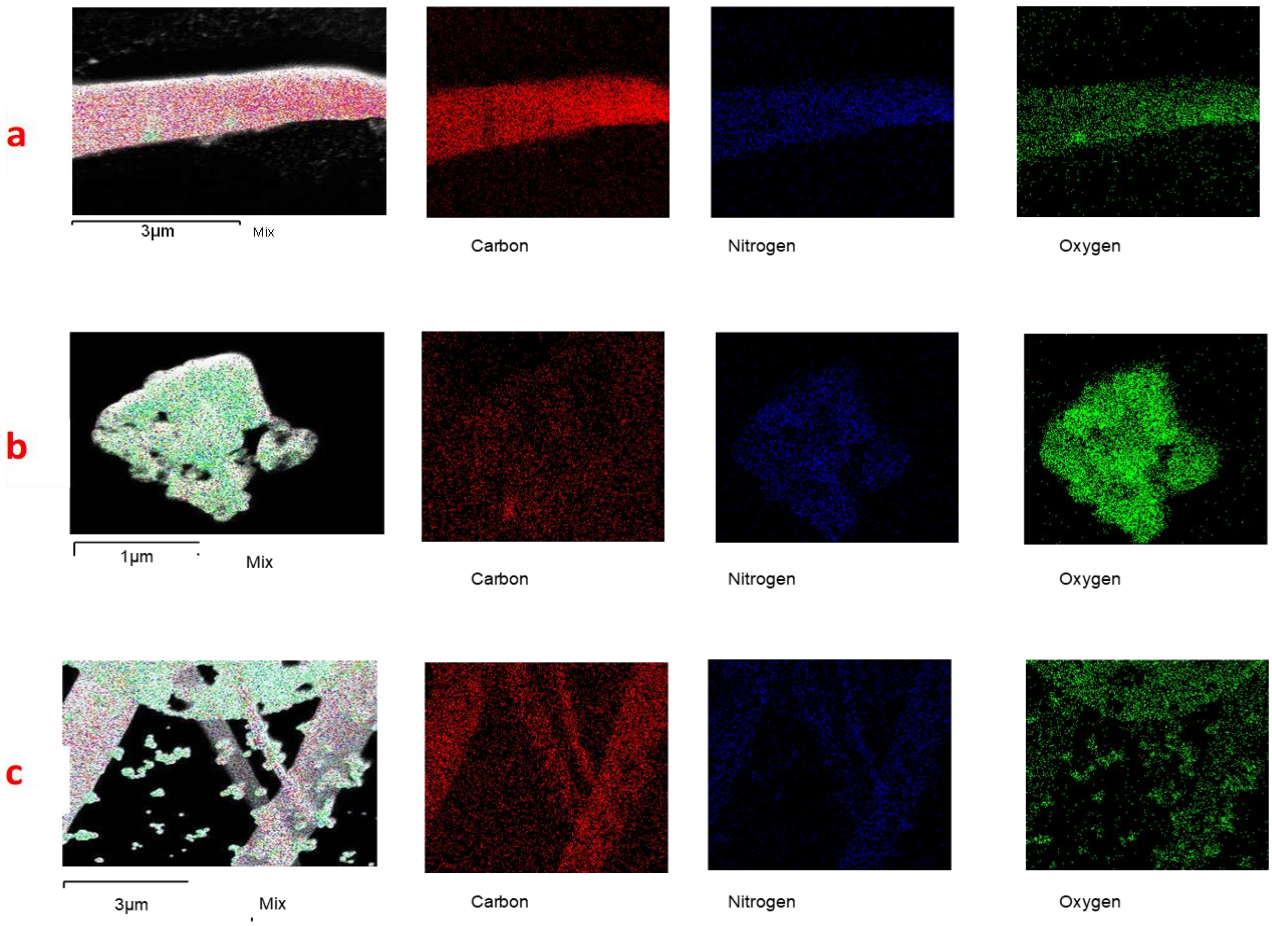

Figure 2. EDX mapping of collagen fibril from mullet scales (a), collagen nanoparticles(b), and theophylline loaded with collagen nanoparticles(c).

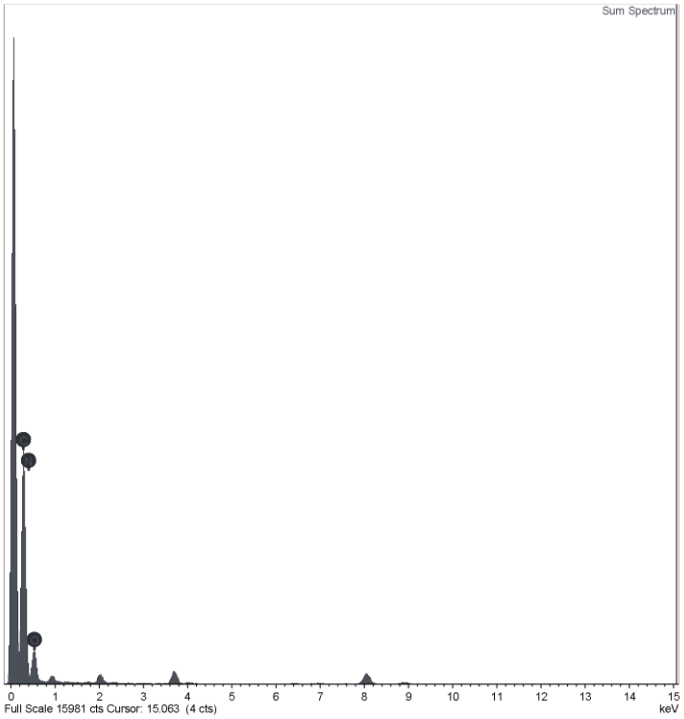

Figure 3. Elemental mapping and EDX analysis of collagen fibers extracted from mullet fish scales.

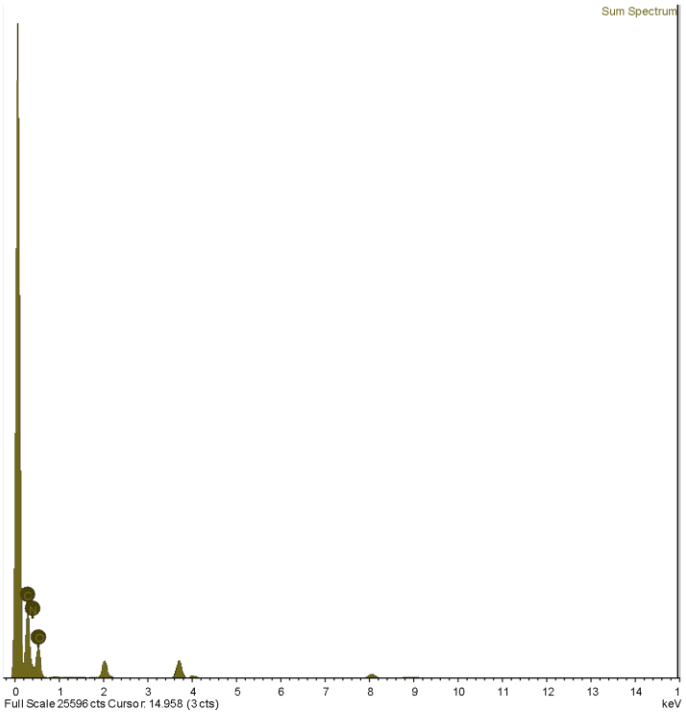

Figure 5 Elemental mapping and EDX analysis of theophylline loaded with collagen nanoparticles

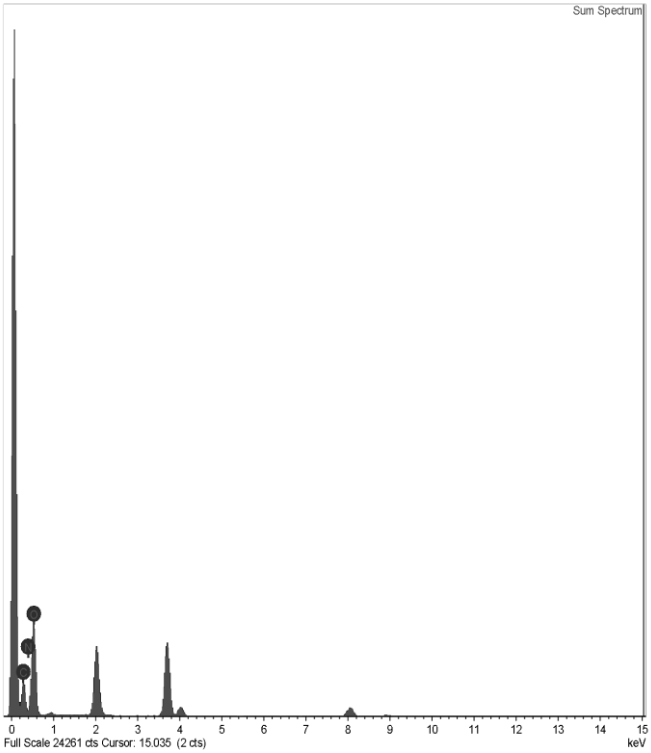

Figure 4. Elemental mapping and EDX analysis of collagen nanoparticles

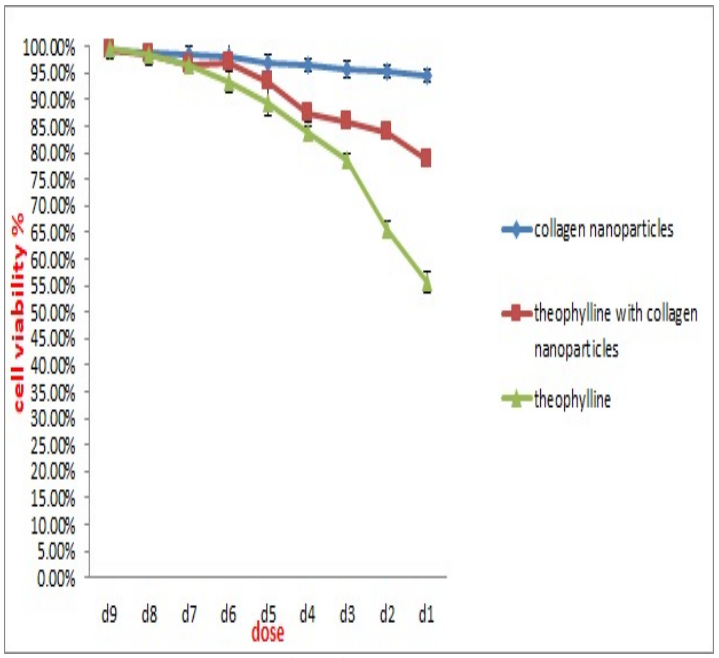

Figure 6 The cytotoxicity profile of collagen nanoparticles, theophylline–loaded Collagen nanoparticles, and theophylline using MTT assay on NHSF cell line at first 180 ug/ml (dose 1) with double fold serial dilutions

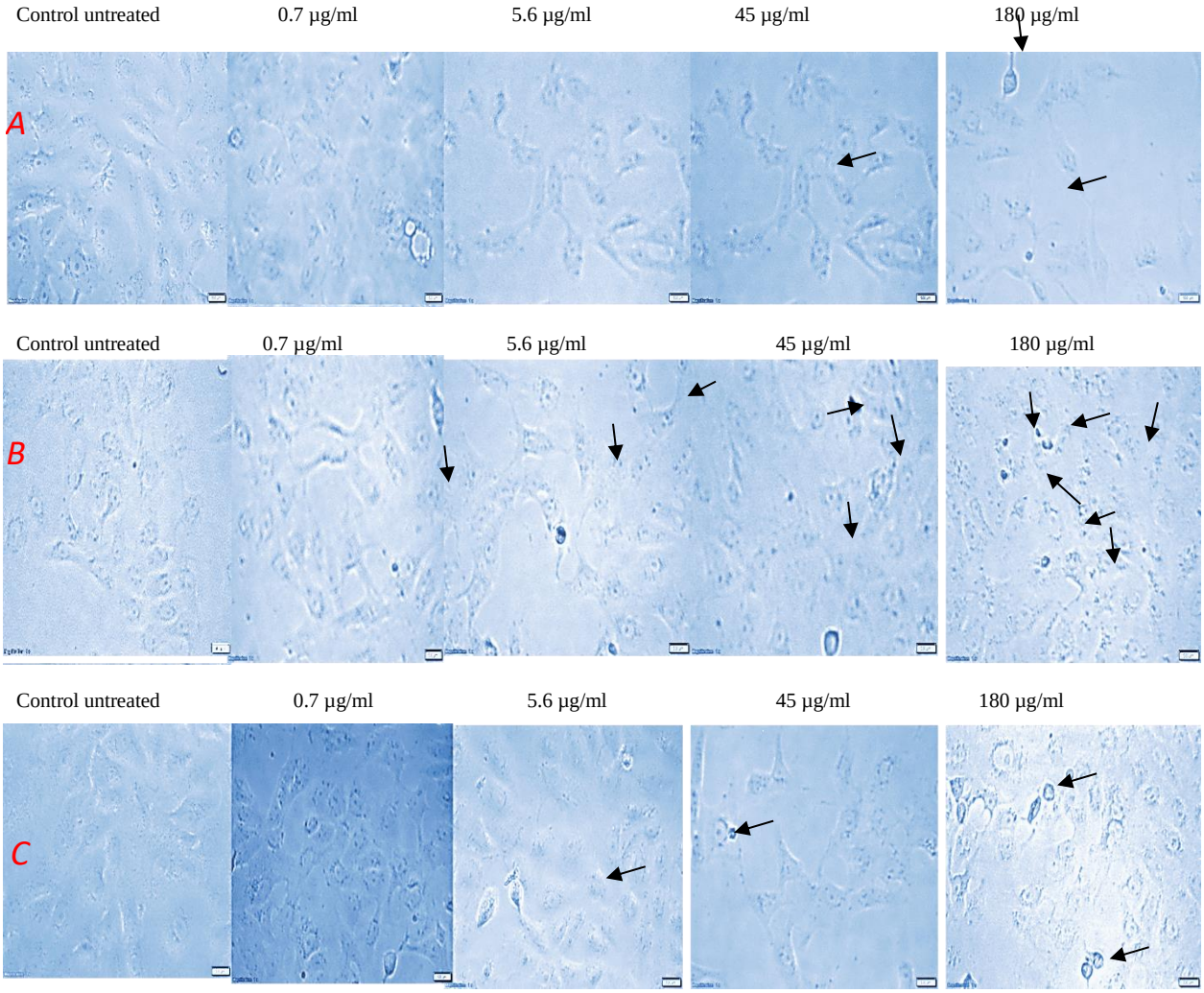

Figure 7 Effect of collagen nanoparticles (A), theophylline (B) and theophylline loaded with collagen nanoparticles(C) on NHSF cell line after 24 hrs incubation. Apoptotic characteristics of the cells included separation from the substrate, shrinking, and the generation of apoptotic bodies (red arrows).

4. Discussion

The collagen was isolated from the fish scale using a basic decalcification process, exposing the collagens, and allowing for dissolution by contact solute-solvent interaction. No pepsin treatment was required since the weak, diluted acetic acid was used to increase the solubility of collagen. Fibrous collagens are found in tissue as covalent cross-linking between individual protein subunits (Sinthusamran et al., 2013). Light microscopy images revealed collagen threads, bundles of collagen fibrils, and fibers that create a fibril system and a pressed compacted sheet-like shape. (Anwar et al., 2022).

The preparation of the nanoparticle approach in this work produced collagen nanoparticles almost rapidly. Several studies, such as Hornig et al., showed that the nanoprecipitation method that we had made is rapid, informal, and have a wide range of medical and none medical application for producing well-defined nanoparticles without the use of any additives (Hornig et al., 2009) (Reis et al., 2006). In our research, the formation of collagen nanoparticles was highlighted using optical microscopic images, revealing distinct collagen strands that turn into spherical accumulations.

The EDX spectrum of collagen showed typical carbon, nitrogen, and oxygen peaks present in the spectrum. The results confirm the high purity of the extracted collagen fibers. The EDX spectrum of collagen nanoparticles indicates the conformational change of collagen fiber to nanoparticles, and the change in percentage of elements in the case of theophylline loaded with collagen nanoparticles shows the loading process (Wang et al., 2014).

In our study, the MTT assay was used to assess the cytotoxicity of theophylline loaded with collagen nanoparticles compared to collagen nanoparticles and free theophylline in NHSF cells. The cytotoxicity of NHSF cells exposed to various doses of theophylline was dose dependent. At lower concentrations, theophylline had the most negligible cytotoxicity. However, cell viability was significantly reduced above a dose of 5.6 ug/ml. The results were similar to the previous report (Uchino et al., 2011). Blank collagen nanoparticles were the least harmful, even at the most remarkable concentration tested. This can be attributed to the natural biodegradable collagen and its additives in formulation creation (Anwar et al., 2022; Chan et al., 2020; Rathore et al., 2020a). Theophylline loaded with collagen nanoparticles had lower cytotoxicity than free theophylline. This is because the drug is successfully captured inside the collagen nanoparticle and released slowly. The cytotoxicity data also demonstrated that the nanoparticles were compatible with normal cells, making them harmless to use as carriers for the medication.

5. Conclusions and future directions

We conclude the successful extraction of collagen fiber from mullet fish scales and the formation of theophylline-loaded collagen nanoparticles. We further guarantee that the nanoprecipitation approach may be modified to

integrate pharmaceuticals with low water solubility and may help provide the best drug delivery system without bioavailability restrictions, such as the solubility and acid liability of medications. Cytotoxicity results revealed that the nanoparticles were compatible with normal cells and thus safe for use in medicines as a carrier. Further research is needed to understand the physicochemical properties of theophylline-loaded collagen nanoparticles and an *in vivo* investigation to adjust for effective drug delivery.

Conflict of interests: There are no conflicts of interest stated by the authors.

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