8.1. Introduction

Biocompatibility was defined at a consensus conference of the European Society for Biomaterials in 1986 as "the ability of a material to perform with an appropriate host response in a specific application" Taking a quicker aspect at this delineation, "appropriate host response" means that the substance doesn't induce any undesirable reactions, like toxic responses, within the tissue where the substance is retained. 'Appropriate' could, however, also refer to a desire to have some positive responses, such as promoting the healing in process and reducing the time until the material or device is functional [1]. The definition above also refers to "a specific application", which means that biocompatibility is contextual. For example, a biomaterial may be biocompatible in one tissue but not in the other and vice versa, or it may be biocompatible for small duration use in a particular tissue, but not in a long-term application in the similar tissue [2].

Biocompatibility highlights the need to consider the suitability of a material both in respect of its potential detrimental effect in the body (toxicity), and in respect of the potential detrimental or beneficial effect of the physiological environment on the performance of the material. It also stresses the need to define a material-biocompatibility only in the precise context of its use. It should be emphasised that it is never possible to define any material chemistry as biocompatible or non-toxic without qualification as to the intended precise use [3]. Amongst various methods to assess biocompatibility and safety of the formulation, Haematocompatibility and Histology studies are widely used by scientists to evaluate whether preliminary in vivo evaluation is ethically justifiable.

Ex-Vivo Histopathology Study

The National Cancer Institute defines histopathology as "the study of diseased cells and tissues using a microscope." Histology is the study of tissues, and pathology is the study of disease. So taken together, histopathology literally means the study of tissues as relates to disease [4]. Histopathology, sometimes also referred to as cellular

pathology, is a complex and important area of study in modern medicine. It is an extremely detailed branch of science that focuses exclusively on the anatomical changes that occur in diseased tissue at a microscopic level. This science is so specific that a histopathologist not only has to perform tests, conduct analysis and gather data, but is also responsible for interpreting the information gathered and making the final diagnosis so a patient's doctor can then move forward to manage the treatment for the specific disease. Scientists specializing in this field look to explore the ways a tissue changes when it comes in contact with a disease and by which means a specific disease changes the tissue in an area of, or throughout, the body. Histopathologists have an array of options when it comes to testing, as they can use a number of stains and various microscopy techniques to examine and analyze the sample. Each stain has specific properties that help scientists identify a particular disease, as not every pathogen is visible with every stain [5]. A common laboratory method that uses two dyes called hematoxylin and eosin that make it easier to see different parts of the cell under a microscope. Hematoxylin shows the ribosomes, chromatin (genetic material) within the nucleus, and other structures as a deep blue-purple color. Eosin shows the cytoplasm, cell wall, collagen, connective tissue, and other structures that surround and support the cell as an orange-pink-red color. H and E staining helps identify different types of cells and tissues and provides important information about the pattern, shape, and structure of cells in a tissue sample. It is used to help diagnose diseases, such as cancer [4].

Haemolytic toxicity study

The National Cancer Institute (NCI) defines haemolysis as "the breakdown of red blood cells due to some diseases, medicines, and toxins more quickly than usual" [4]. Haemolysis (destruction of red blood cells) in vivo can lead to anaemia, jaundice and other pathological conditions, therefore the haemolytic potential must be evaluated for all the pharmaceuticals meant for internalization other than enteral route. Nanotechnology-derived devices and drug carriers are emerging as alternatives to conventional small-molecule drugs, and in vitro evaluation of their biocompatibility

with blood components is a necessary part of early preclinical development. The small size and unique physicochemical properties of nanoparticles may cause their interactions with erythrocytes to differ from those observed for conventional pharmaceuticals, and may also cause interference with standardized in vitro tests. As with any device or pharmaceuticals, nano-carriers intended for biomedical application must be subject to biocompatibility testing before regulatory approval for administration to patients [6].

8.2. Materials and Methods

8.2.1. Ex-Vivo Histopathology Study

Vaginal tissue was obtained from freshly sacrificed goat at a local slaughterhouse. The tissue was then washed thoroughly using Phosphate Buffer Saline and cleaned properly. The vaginal mucosal membrane was separated from the underlying connective tissues with the help of forceps and scissor. The mucosal tissue was cut into small pieces of approximately 1 cm^2 area and submerged into different test formulations and control solutions such that the mucosal membrane to be examined remain completely in contact with the solutions.

Table 8-1: Study sample codes and description for histopathology study

Study Sample	Description
Negative Control (NC)	Phosphate Buffer pH 4.5 solution
Positive Control (PC)	Isopropyl Alcohol (IPA)
P-1	PTX-plain drug suspension in Phosphate Buffer pH 4.5
P-2	PTX-UDNVs formulation
C-1	CBP-plain drug solution in Phosphate Buffer pH 4.5
C-2	CBP-UDNVs formulation

The tissues were kept in different study solutions for the time duration of 1 week and 2 weeks since the formulations are intended to remain in contact with the vaginal mucosa for at least one week when administered intravaginally. At the end of time points i.e. one week and two weeks, the tissue was removed from each tube and

washed properly with distilled water and immediately preserved in 10% formalin solution as fixative. The tissues were then dehydrated in graded concentrations of ethanol, immersed in xylene and embedded in paraffin. The tissues embedded in paraffin blocks were mounted on a Microtome (HM 355S, ThermoFisher Scientific, USA) and thin sections were obtained. The sections were carefully mounted on clean glass slides and stained using haematoxylin-eosin (H/E). The prepared slides were observed under inverted microscope (Nikon H600L Microscope, Nikon Instruments Inc., NY, USA) at 10× magnification and the layers of the vaginal membrane were carefully examined for any pathological changes as a measure of safety. Sections of the vaginal membrane treated with phosphate buffer saline (pH 4.5) and Isopropyl Alcohol were also examined in similar manner to serve as negative and positive control, respectively.

8.2.2. Haemolytic toxicity study

Haemolytic toxicity of the formulation was determined using red blood cell (RBC) lysis assay as per the method described in literature [7, 8]. PTX marketed formulation (GROSS^{TM}-100, Emcure® Pharmaceuticals Ltd.) and PTX-UDNVs formulation were diluted with 0.9 % normal saline solution in the concentration range of 400–4 µg/ml. Similarly, CBP marketed formulation (Carbokem Nova 150mg/15ml Inj, Alkem Laboratories Ltd) and UDNVs formulation were diluted with 0.9% normal saline solution in the concentration range of 300–3 µg/ml. Blood samples were freshly collected from rat retro orbital plexus in heparinized eppendorf tubes and centrifuged at 3500 rpm for 10 min. The plasma supernatant was removed and discarded and the residue was washed and diluted 10 folds using 0.9 % normal saline solution to maintain the environment iso-osmolar to prevent rupturing or shrinkage of the RBCs. A fixed quantity of the RBC solution (200 µL) was dispensed in separate micro-centrifuge tubes and the study samples were prepared by adding a fixed quantity (800 µL) of the test solutions to individual micro-centrifuge tube. Distilled water and normal saline solution were also added to separate micro-centrifuge tubes containing RBC solution. The RBC suspension with distilled water was considered as positive control producing

100% haemolysis whereas RBC suspension with normal saline was considered as negative control producing no haemolysis.

The samples were incubated for 1 h at 37°C. After incubation, few drops of the sample with highest concentrations of test solutions were plated on the glass slide and analysed by the microscope (Nikon H600L Microscope, Nikon Instruments Inc., NY, USA). For the quantitative estimation, the study samples were centrifuged at 3500 rpm for 10 min at 4 ^{0}C to separate non-lysed RBCs. The supernatant was collected and analyzed by UV-Vis spectrophotometer at 540 nm. The absorption value of distilled water containing sample was considered for 100 % haemolysis and the percent haemolysis for other samples was determined using the following equation:

$$\%\, Haemolysis = \frac{(A_{TS} - A_{NC})}{(A_{PC} - A_{NC})} \times 100$$

Where,

A_{TS} = Absorption of test sample

A_{PC} = Positive Control

A_{NC} = Negative Control

8.3. Results and Discussion

8.3.1. Ex-Vivo Histopathology Study

The histology of the vaginal wall reveals that it consists of mainly three layers i.e a) the inner mucosal epithelial stratum and lamina propria containing thin-walled veins, b) the intermediate muscularis stratum, and c) the external adventitial layer. The Mucosa is composed of an epithelium and an underlying layer the lamina propria. A stratified squamous epithelium nonkeratinizing that is normally glycogenated with a basal/parabasal layer, a mid-zone and a superficial layer. A lamina propria that consists of a loose fibro-vascular stroma containing elastic fibres and nerves. The muscularis layer consists of inner circular and outer longitudinal bundles of smooth muscles. The adventitial layer contains numerous blood vessels and nerves within adipose tissue [9].

The histopathological examination of the tissue can provide important information on any kind of irregularity observed in these cellular arrangements caused by the test substance in contact. The substance can be considered as safe and biocompatible if it is non-irritant and does not show toxic effects on the tissues upon specified duration of exposure.

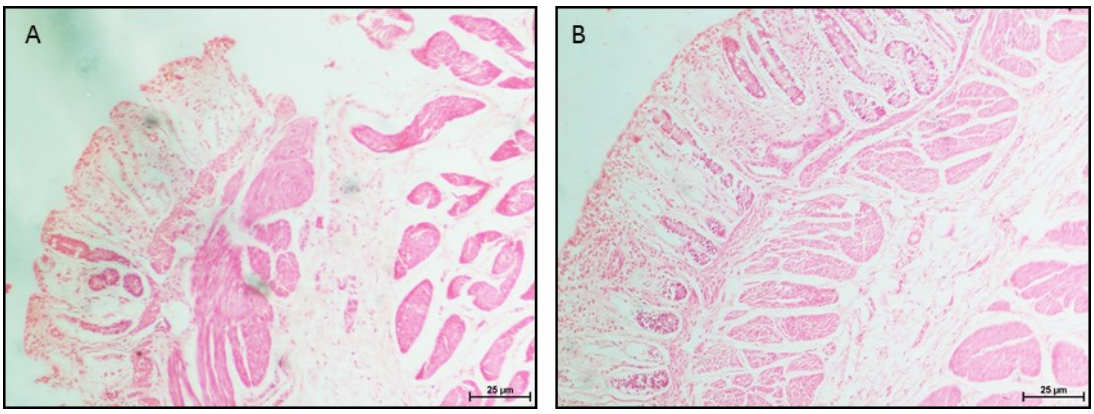

Fig. 8-1: Vaginal tissue treated with Phosphate Buffer pH 4.5 (Negative Control) for A) One week B) Two weeks

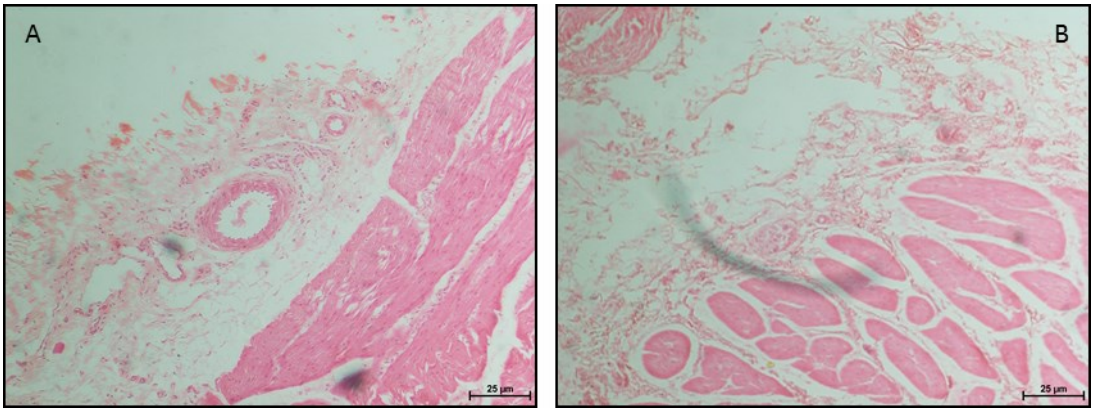

Fig. 8-2: Vaginal tissue treated with Isopropyl Alcohol (Positive Control) for A) One week B) Two weeks

The histology sections of the vaginal tissue treated with Phosphate Buffer pH 4.5 (Negative Control) and Isopropyl Alcohol (Positive Control) represented **Fig. 8-1** in and

Fig. 8-2 respectively after one week and two week exposure. The inner mucosal epithelial stratum corneum with lamina propria layers are clearly visible with no irregularities in epithelium layer for the tissue exposed to phosphate buffer pH 4.5 hence considered as negative control sample. Whereas, the vaginal tissue treated with isopropyl alcohol are showing damaged layers of the tissue. The inner mucosal epithelium and the lamina propria are not distinctly visible which indicates the pattern of damage could be done by an irritant or toxic substance to the vaginal membrane when exposed directly.

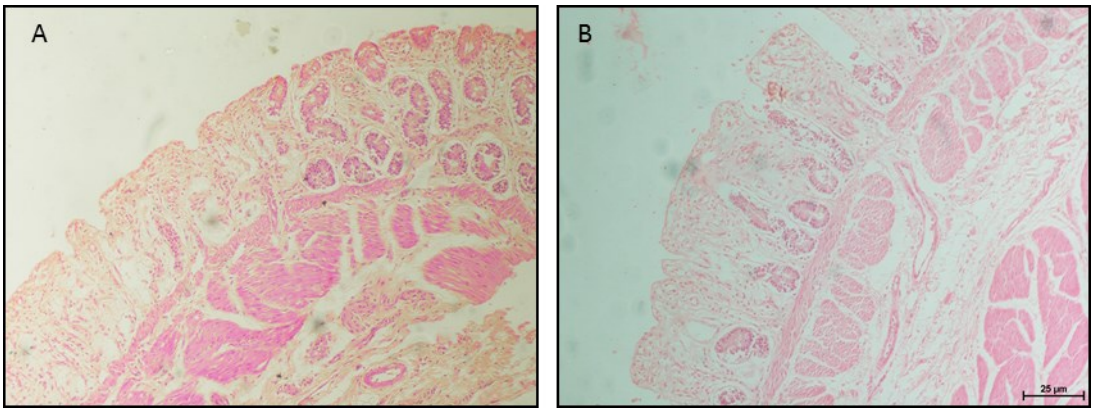

Fig. 8-3: Vaginal tissue treated with PTX-UDNVs formulation for A) One week B) Two weeks

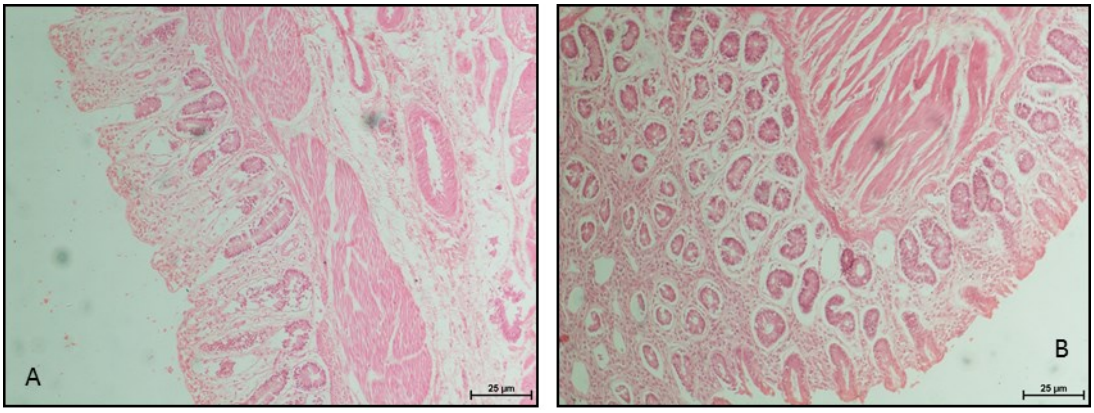

Fig. 8-4: Vaginal tissue treated with CBP-UDNVs formulation for A) One week B) Two weeks

Fig. 8-3 and **Fig. 8-4** represents the histology sections of the tissues exposed to the developed UDNVs formulation of PTX and CBP respectively for the duration of one

week and two weeks. As it is evidenced from the images, the epithelial layer and the lamina propria can be clearly observed with no disruption in the continuity of the layer for both the formulations and time points of one week and two weeks. The appearance of the histology was resembling to that of negative control samples of the tissues which indicates good tolerance of the exposure of formulations to the vaginal tissue.

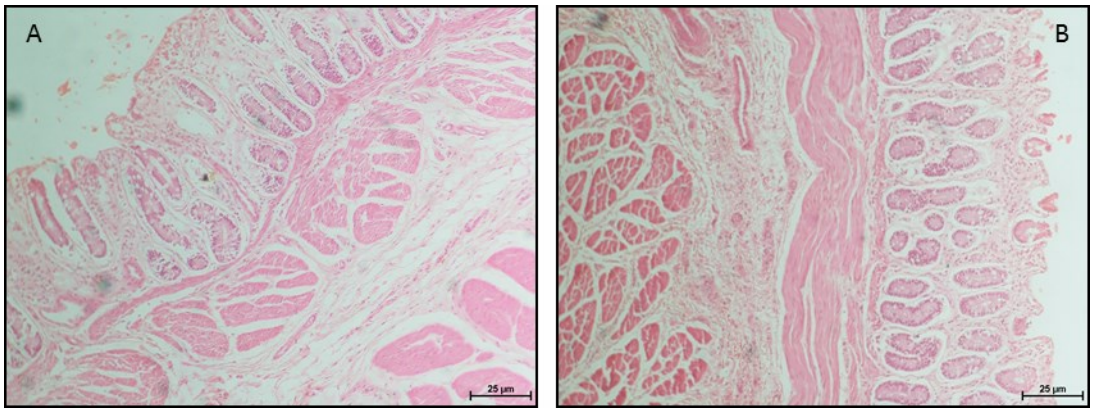

Fig. 8-5: Vaginal tissue treated with PTX-plain drug suspension for A) One week B) Two weeks

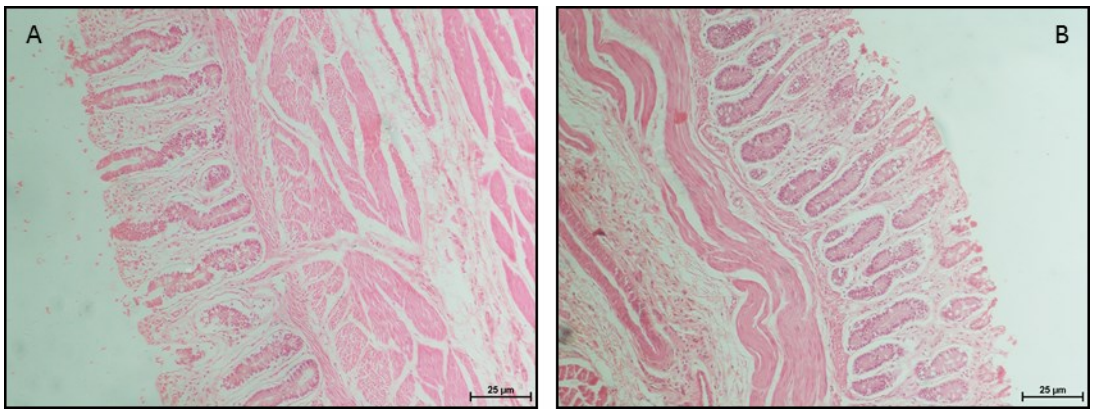

Fig. 8-6: Vaginal tissue treated with CBP-plain drug solution for A) One week B) Two weeks

Fig. 8-5 and **Fig. 8-6** represents the histology sections of the tissues exposed to the plain drug suspension of PTX and plain drug solution of CBP respectively for the duration of one week and two weeks. Both the figures showed that the linings of the epithelium was not observed in uninterrupted fashion indicating damage of the

vaginal epithelium. The little damage was seen for the lamina propria as compared to that of damage caused by the exposure of IPA. The results indicate that the direct administration of the plain drug causes irritation and damage to the vaginal tissues which was avoided via incorporation of these drugs in to UDNVs.

8.3.2. Haemolytic toxicity study

The haemolytic toxicity of the developed formulations of PTX and CBP was assessed qualitatively and quantitatively with comparison to that of marketed formulations of both the drug molecules. The morphological characteristics of the RBCs were observed under microscope (Nikon H600L Microscope, Nikon Instruments Inc., NY, USA) and the results captured. The quantitative haemolysis was assessed using UV Vis spectrophotometer using formula given above and the results were compared to that of marketed formulations.

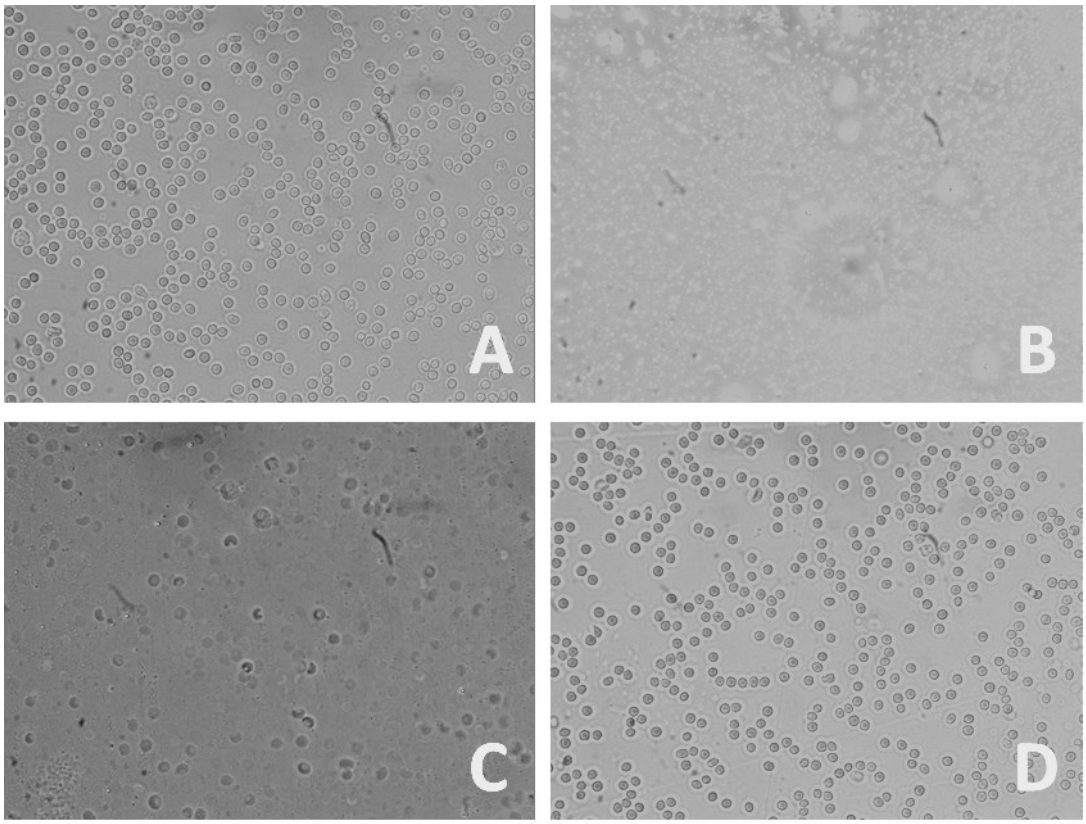

Fig. 8-7: Qualitative examination of haemolysis of RBC when incubated with A) Normal Saline (Negative Control) B) Distilled water (Positive control) C) Marketed formulation of PTX D) PTX-UDNVs formulation

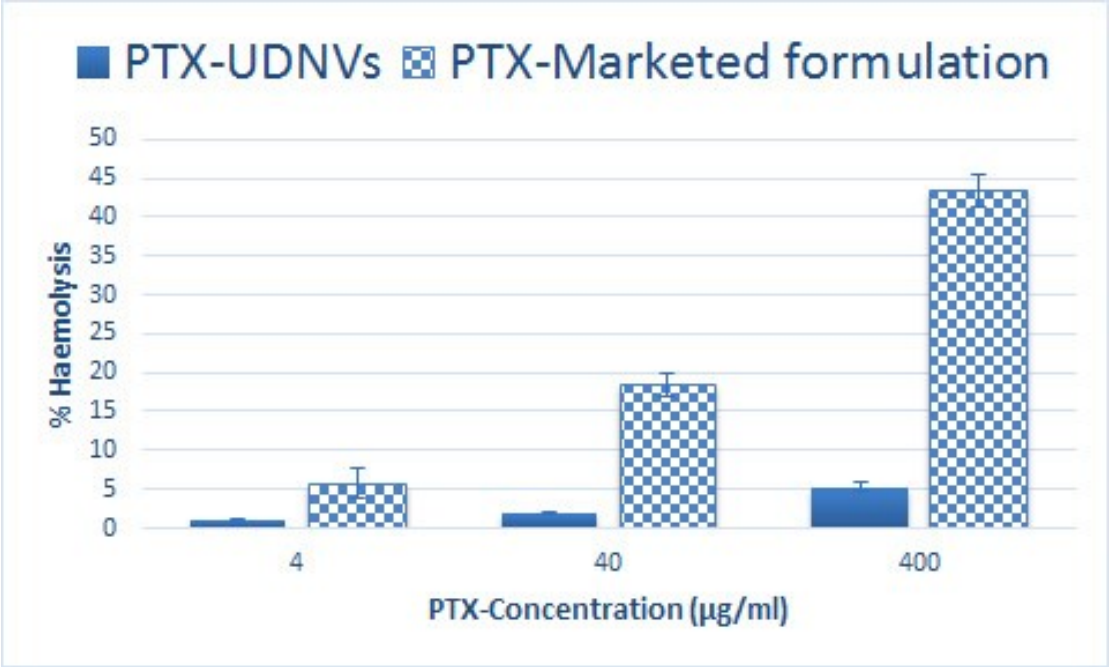

Fig. 8-8: Quantitative measurement of %Haemolysis of RBC when incubated with PTX-UDNVs formulation and PTX-Marketed formulation

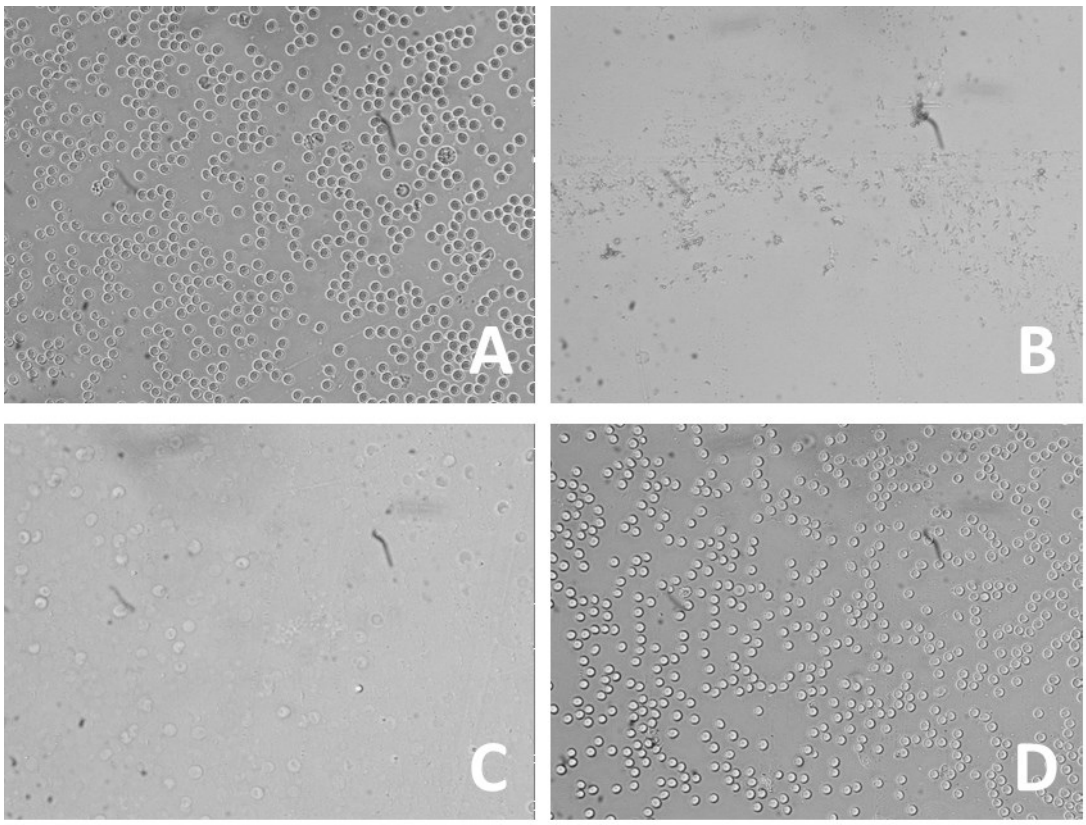

Fig. 8-9: Qualitative examination of haemolysis of RBC when incubated with A) Normal Saline (Negative Control) B) Distilled water (Positive control) C) Marketed formulation of CBP D) CBP-UDNVs formulation

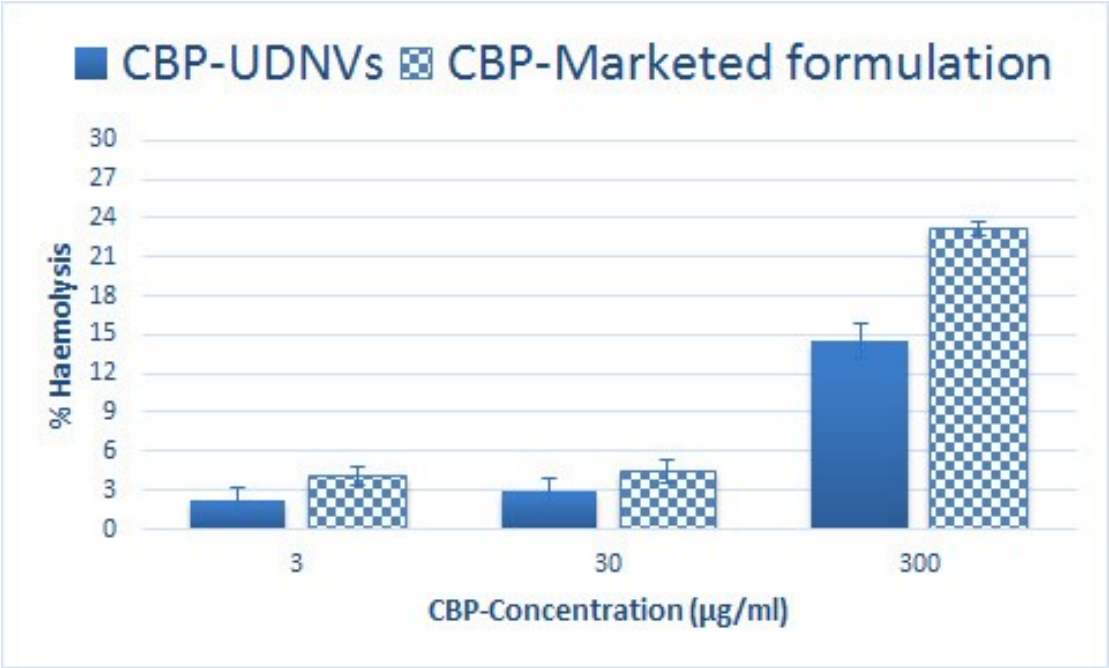

Fig. 8-10: Quantitative measurement of %Haemolysis of RBC when incubated with CBP-UDNVs formulation and CBP-Marketed formulation

Fig. 8-7 and **Fig. 8-8** represent the results of qualitative and quantitative estimation of haemolysis respectively of RBCs when incubated with PTX-UDNVs and PTX-Marketed formulation along with negative and positive control samples. **Fig. 8-9** and **Fig. 8-10** represent the results of qualitative and quantitative estimation of haemolysis respectively of RBCs when incubated with CBP-UDNVs and CBP-Marketed formulation along with negative and positive control samples. The microscopic images of the negative control shows that RBCs were observed in their original biconcave discoid shape indicating no haemolysis. Whereas positive control sample shows no signs of intact RBCs representing complete haemolysis. The reason behind complete lysis of the RBC in positive control sample is hypo-osmotic surrounding of the RBC ultimately lead to the rupturing of the RBC. Whereas, unlike positive control sample, negative control sample provides isotonic environment leading to no lysis of the RBCs. The samples of marketed formulation of PTX and CBP showed little distortion in the shape and membrane of the RBCs indicating intermediate state of lysis of the RBCs due to

direct contact of the drug molecule. Morphologically intact RBCs show a typical biconcave discoid shape that is similar to the shape of a doughnut. The presence of haemolytic agent and the extent of haemolysis represented by the shape change of RBCs from biconcave discoid to slight membrane invagination similar to that of a cup shape [10, 11]. The complete haemolysis may be represented by fusion like cells or absence of structure of RBCs [12, 13]. The microscopic images of the UDNVs formulation show little or no haemolysis whereas in the case of marketed formulations showing slight cup shaped RBCs. The quantitative analysis of the samples represents higher percentage of the haemolysis for marketed formulations of both the drugs as compared to the developed formulation of CBP and PTX. The results indicate that the developed formulation are more safe and biocompatible to that of marketed formulations.

The haemolytic study and histopathology studies revealed that the drug loaded UDNVs formulations of both the drugs were found safe and compatible with biological components. The reason behind the findings might be due to the ability of vesicular system to protect the drug entrapped and reducing the direct exposure of the drugs to surrounding tissues. The observed results are supported by the findings reported by Paromov, V. et al. for Protective Effect of Liposome-Encapsulated Glutathione in a Human Epidermal Model Exposed to a Mustard Gas Analog [14].

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