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Photodynamic Potential Of Curcumin Nanoparticle With Chitosan On Adherent Canine Tumour Cell Line A72

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Abstract

Cancer is a highly incomprehensible and complex disease. Current treatment strategies are associated with adverse toxicities like multidrug resistance by cancer cells, destruction of normal cells, hair loss etc. The present work was aimed to use the natural photo sensitizer curcumin from *C. longa and C.aromatica* in nanoscale along with chitosan for the Photo Dynamic Therapy of cancer using adherent canine tumour cell line A72.Curcumin was extracted from *C. longa and C.aromatica* with water, acetone and ethanol. Light Source used for PDT was 12V Philips Endura LED 7W MR16 dimmable lamp. Photo sensitizers and nano materials were characterized by AFM and UV-Vis spectrum. Photosensitizers from 50µg/50µl to 0.393µg/50µl was used for toxicity assay . Toxicity of all photosensitizers were evaluated in darkness and light by MTT assay. Acetone extraction of *C.longa* yielded comparatively more curcumin than ethanol and water. Yield of curcumin was 0.25%, 0.16and 0.05% in acetone, ethanol and water respectively . UV-Vis absorption spectrum of curcumin showed peak at 425 nm. In darkness the toxicity of curcumin to A72 was 9% and curcumin nano particle was 11% at a concentration of 50 µg/50µl .Cellular toxicity by light alone was found to be 2%. Photodynamic toxicity in the presence of photo sensitizer was increased to 18 % and 25 % for curcumin and curcumin nano particle respectively at 12.5 µg/50µl. Curcumin nano particles along with chitosan destroyed 43 % of cells attesting the key role of chitosan in improving the toxicity and the availability of curcumin. Thus formulation of a natural photo sensitizer with chitosan could be a viable alternative to synthetic photo sensitizers.

Keywords: Curcumin nanoparticle,Cannine cancer cells, Photodynamic Therapy, Photosensitizer

Introduction

Cancer is a major cause of morbidity and mortality. More than ten million people are diagnosed with the disease annually. Cancer is a highly incomprehensible and complex disease that develops by a multistep carcinogenesis process entailing numerous cellular physiological systems such as cell signaling and apoptosis (Reichert and Wenger, 2008). Initially, cancers start as localized diseases, but many cancers metastasize which makes them incurable. Conventional cancer treatment strategies are chemotherapy, radiation and surgery (Singhal, *et al.* 2010). Challenges in current cancer therapy includes nonspecific and systemic distribution of antitumor agents, suboptimum drug concentrations reaching the target site, cytotoxicity to

normal cells and development of multiple drug resistance (Das *et. al*. 2009).Chemotherapy and ionizing radiation delivered at doses sufficient to destroy tumours are known to be toxic to the bone marrow. Neutropaenia and other forms of myelo suppression are often the toxicity of these therapies. Most of the commonly used cancer therapies are immunosuppressive. The ideal cancer therapy should be able to kill the primary tumor, activate the immune system to recognize and destroy metastatic tumor cells.

Photodynamic therapy (PDT) is an increasingly recognized alternative to treat various cancers in clinical practice (Huang *et.al*. 2008). Compared to conventional therapies, PDT has many advantages including minimal invasiveness and selectivity (Celli *et.al*.,2010; Lovell *et.al*.,2010). It is an effective and selective method for damaging diseased tissues. The efficiency of PDT depends on the nature of the Photosensitizer (PS), the pharmaceutical formulations, cellular uptake, physical localization and the concentration of PS the in target tissue, light source, intensity of light and the absorption properties of the PS.

Basic principle of PDT (Dai *et.al*. 2012) involves the activation of PS by absorption of light at specific wavelength based on the chemical nature of PS. This leads to excitation of the PS from its ground state (singlet state) into a relatively long-lived electronically excited state (triplet state), as illustrated in Fig.1.The excited PS can react directly via a Type I photo-oxygenation process with substrate (e.g., protein, lipid), leading to free radical intermediates that react with oxygen to generate various reactive oxygen species. Alternatively, the triplet can transfer its energy directly to oxygen to form singlet oxygen (Type II reaction), which is assumed to be the key agent of cellular damage.

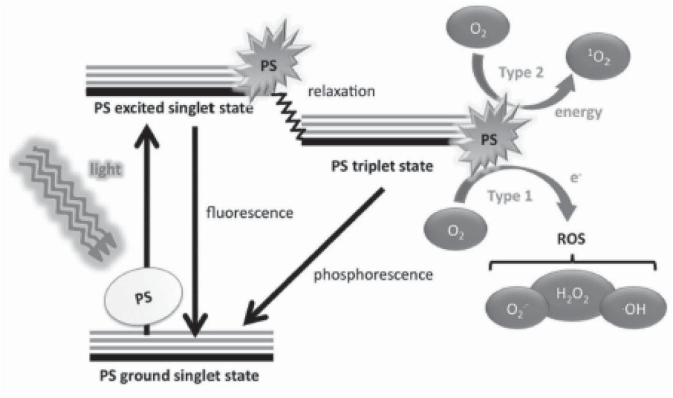

Figure 1: Principle Of Pdt(Dai *et.al*.,2012)

This moiety is highly cytotoxic, with a short half–life (<0.04 ìs) and a short radius of action (<0.02 ìm) (11). As a result, only cells that are immediate viscinity to the areas of reactive oxygen species (ROS) production are directly destroyed by PDT *(*Dolmans *et.al*.,2003). ROS act as signalling molecules of central processes such as proliferation, apoptosis and necrosis.

An ideal PS should be chemically pure and of known specific composition (this is especially significant for toxicity testing), have a high quantum yield for singlet oxygen production, have a strong absorption at a long wavelength (for maximum tissue penetration), have excellent photochemical reactivity, have minimal dark toxicity, and only be toxic in the presence of the correct wavelength of light , have strong preferential accumulation and retention in tumor tissue, rapid clearance from the body after treatment, and be easy to synthesize and administer to a patient intravenously (Wiedmann*et.al*.,2004).

Photofrin®, the first generation PS was approved in Canada in 1993, for treatment of bladder cancer. Japanese government approved Photofrin® to treat early stage lung cancer in 1994. Photofrin® was approved as the first PDT drug by the United States FDA in 1995 to treat patients with high grade dysplasia in Barrett's esophagus, and then in 1998 for patients with non-small cell lung cancer.(Usada *et.al*.,2006). However, Photofrin® is not metabolized and cleared quickly from the body. It has persistent photosensitivity for 4 to 12 weeks after treatment . Other PS are benzoporphryn derivative (Levy *et.al.*, 1990).aminolevulinic acid, Meta-tetra (hydroxyphenyl) chlorine-m THPC, (Triesscheijn, *et.al*.,2006).

However, there are limitations in the clinical application of existing PS. Most PS molecules are hydrophobic and can aggregate easily in aqueous media. Aggregated PS cannot be simply injected intravenously (Trindade, 2001). Third generation PS have improved tissue penetration, drug clearance, selective affinity of the PS to tumour tissue. Targeting moieties, such as single-chain monoclonal antibodies, enable the PS to kill tumour cells while minimizing healthy cell damage (Josefsen *et.al.* 2008).

Although third generation PS have been prepared for selective targeting, their selectivity is not high enough for clinical application.Therefore, numerous approaches have been proposed to offer high selectivity, high efficacy by incorporating PS into various delivery carriers such as liposome, polymer nanoparticles, gold nanoparticles, quantum dots, magnetic nanoparticles, silica nano-particles

etc.Nanomaterials are promising owing to their sub-cellular and sub-micron size.They can penetrate deep into tissues through fine capillaries, cross the fenestration present in the epithelial lining.

The present work was aimed to use the natural PS curcumin from *C. longa and C.aromatica* in nanoscale along with chitosan for the PDT of cancer caused by adherent canine tumour cell line A72.

Materials and Methods

Nano particle of curcumin was obtained by ball milling of turmeric. Curcumin was extracted from *C. longa and C.aromatica* with water, acetone and ethanol. Scanning Electron Microscopy (SEM) images were obtained with FESEM: ZEISS operated at 5.0 kV. UV-Vis spectra were measured in Shimadzu UV-2450 UV-Visible spectrophotometer. The Fourier transform infrared (FTIR) spectra were recorded on a Shimadzu FTIR Spectrometer. A72 adherent canine tumour cell line was obtained from TANUVAS, Chennai and maintained in RPMI-1640 medium supplemented with 10% foetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin and grown at 37^0C in a 5% CO_2 humidified environment. Light Source used for PDT was 12V Philips Endura LED 7W MR16 dimmable lamp. PS and nano materials were characterized by AFM and UV-Vis spectrum. PS from 50µg/50µl to 0.393µg/50µl was used for toxicity assay . Canine carcinoma A72 cells (3×10^5 cells / well) were seeded in 96-well plates and the toxicity of all photosensitizers were evaluated in both darkness and light by MTT assay (Kwitniewsk,*et.al*.,2009).

Results and Discussion

Turmeric (*C.aromatica* and *C.longa* L.) belongs to the Zingiberaceae family and genus Curcuma. The word turmeric is thought to be derived from the French word, terre-merite, meaning merit of the earth. It is the plant's rhizome, also known as root stalk which can be defined as a horizontal stem of a plant from which a variety of plants can grow. It is a herbaceous perennial plant, Turmeric occupies an important position in the life of Indian people as it forms an integral part of the rituals, ceremonies and cuisine. Due to the strong antiseptic properties, turmeric has been used as a remedyfor all kinds of poisonous affections, ulcers and wounds (Ammon and Wahl, 1999).

Acetone extraction of C.longa yielded comparatively more curcumin than ethanol and water. Yield of curcumin was 0.25%, 0.16 and 0.05% in acetone, ethanol and water respectively (Figure 2).

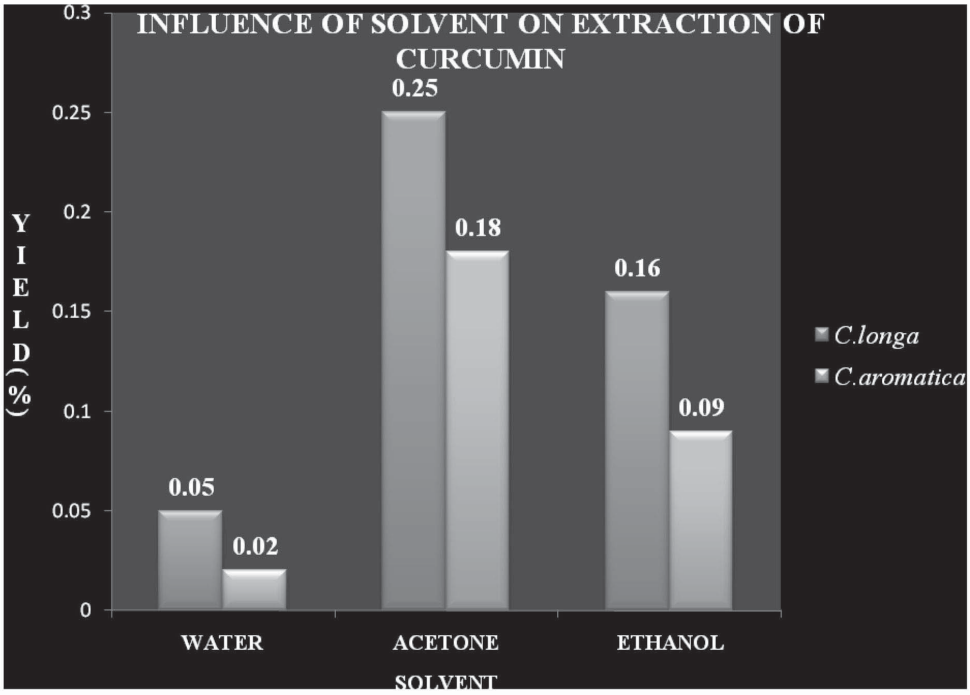

Figure 2: Influence Of Solvent On Extraction Of Curcumin

The colouring principle of turmeric was isolated in the19th century and was named curcumin. Curcumin is a polyphenol with diaryldiheptanoid with á and â unsaturated ketones. Considering the various biological activities of curcuminoids, attempts were made by several researchers to isolate curcuminoids from turmeric rhizomes by solvent extraction using organic solvents (Xu *et.al*.,2004).Yield of extraction depends on the choice of solvent and the extraction method. Antioxidant activity assessed with curcumin from *C.longa and C.aromatica* showed that the activity was superior in *C.longa* (data not shown here) and hence cytotoxicity was evaluated with *C.longa* only.

The AFM and SEM images of curcumin and chitosan nano particle are shown in Fig 3-5.The images showed a narrow size distribution of nano particles prepared similar to the report of Tsaia *et.al*. (2011).

Photobiology of the pigments is essential for its application as PS. PS absorb light at a specific wavelength dependent on its chemical structure. For PDT application, the absorption and emission spectrum of PS should be the strongest for successful therapy.UV-Vis absorption spectrum of curcumin showed a characteristic broad band from 400-500 nm with a maximum absorbance in 425 nm as represented in Figure 6. The maximum absorbance is due to the excitation of its extended ð conjugation system. On absorption of light energy, it is excited from ground state to the first excited state and oscillates between the ends of the chromophore .The photophysics of curcumin is very much dependent on solvent polarity ,as the solvent influences the tautomerisation (Bong ,2000; Khopde *et.al*.,2000).The colour of the PSs was light sensitive and fade away when exposed to light. The results are in accordance with the observation of Tang, 2010.

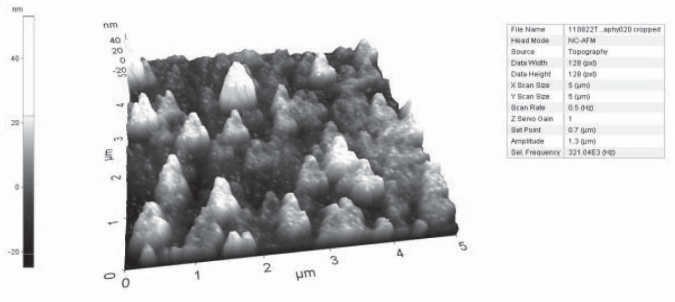

Figure 3: Afm Image Of Curcumin Nano Particle

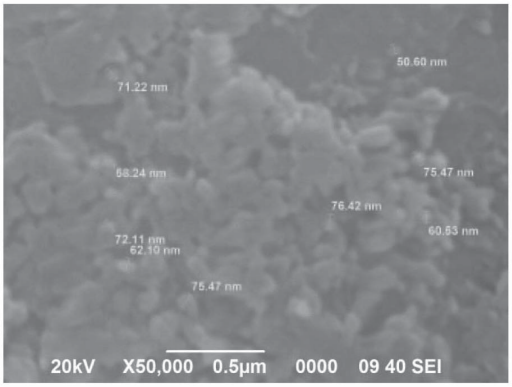

Figure 4: Sem Image Of Curcumin Nano Particle

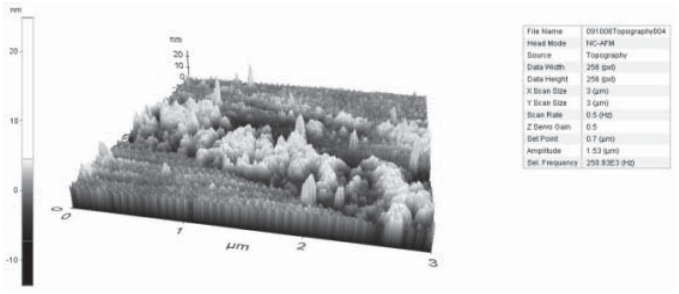

Figure 5: Afm Image Of Chitosan Nano Particle

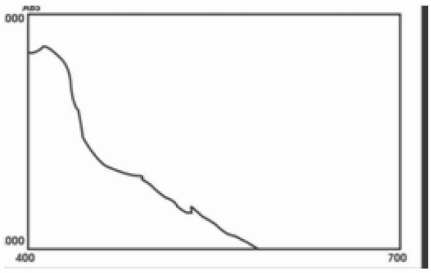

Figure 6: Uv-Vis Absorption Spectrum Of Curcumin Nano Particle

The influence of curcumin and curcumin nano particle on the cyto totxicity of A72 in darkness is shown Figure 7. PS without light illumination causes damage to the cells in concentration dependent manner. At 50µg/50µl of curcumin 9% of cells were destroyed. The destructive effect decreased to 6% and 2% at 6.25785µg/µl and 0.785µg/µl. Thapliyal and Maru, (2001) supported the dose dependent anti cancer activity of turmeric powder. Ozaki *et al*. (2000) reported that curcumin induces apoptosis in cells. Curcumin modulates cell cycle progression as well as the cross talk of various pro- and antiapoptoticfactors.(Kapadia *et al.*, 2002).At all concentrations of analysis curcumin

nanomaterial exhibited comparatively higher toxicity.At 1.57 µg/50 µl the toxicity was 9% for nanoparticles where as curcumin showed the same toxicity only from 12.5 µg/50 µl. In most cells curcumin sequentially induces activationof caspase-8, cleavage of BID, loss of mitochondrial membrane potential, opening of transition pores, release of cytochrome C, caspase-9 activation, caspase-3 activation and cleavage of PARP and inhibitor of caspase-activated deoxyribonucleaseICAD), thus leading to DNA fragmentation and apoptosis.

The cells were exposed to light for 20 minutes at a distance of 60cm in the absence of PS and the viability of A 72 cells were calculated from MTT assay. Cellular toxicity was found to be 2%.Temperature increase during the illumination duration was only $5^{0.}$ C. So the result confirms that the light intensity for 20 minutes is not cytotoxic. Li *et.al.* 2010 supported the present observation that 77.38% of inhibition was found only after 3 days of continuous illumination. The visible light exposure may directly induce photoproducts through the direct excitation of DNA (Hoffmann-Dörr *et.al*., 2005). Visible light might act on mitochondrial PS, such as cyclooxygenase(COX), cytochrome P 45 isoenzymes, and flavin protein to influence the electron transport system and generate substantial reactive oxygen species (ROS). The ROS, including peroxynitrite anion (ONOO)-, are able to penetratethe nuclear membrane and cause various oxidative DNA modifications, cause nuclear DNA damage, which then further triggers downstream sig *invitro* nals andinduces cell death. (Osborne *et.al*. al.2010).

Developments of PDT for cancer mainly focus on the selectivity of PS and the light source for illumination. The photodynamic toxicity of PS to A72 is given in Figure 8. It is clearly evident that at 50µg/50µl of PS the toxicity was 18 %, 25% for curcumin and curcumin nano particle respectively.

The effect of blending chitosan nano particle with curcumin nano particle on the photodynamic toxicity of A72 is represented in Figure 9. When curcumin nano particle was blended with chitosan (40µg of CNP+40µgChitosan /50µl) the toxicity towards cells was significantly increased.At 12.5µg/50µl the toxicity of curcumin, CNP and mixture of CNP and chitosan was 25%,30% and 43% respectively. At lowermost concentration 0.393µg/50µl, the toxicity showed by CNP + chitosan was 35%. Chitosan is a biocompatible carrier for photsensitizers. It has been found that the choice of delivery vehicle can influence the tumor selectivity of the PS.

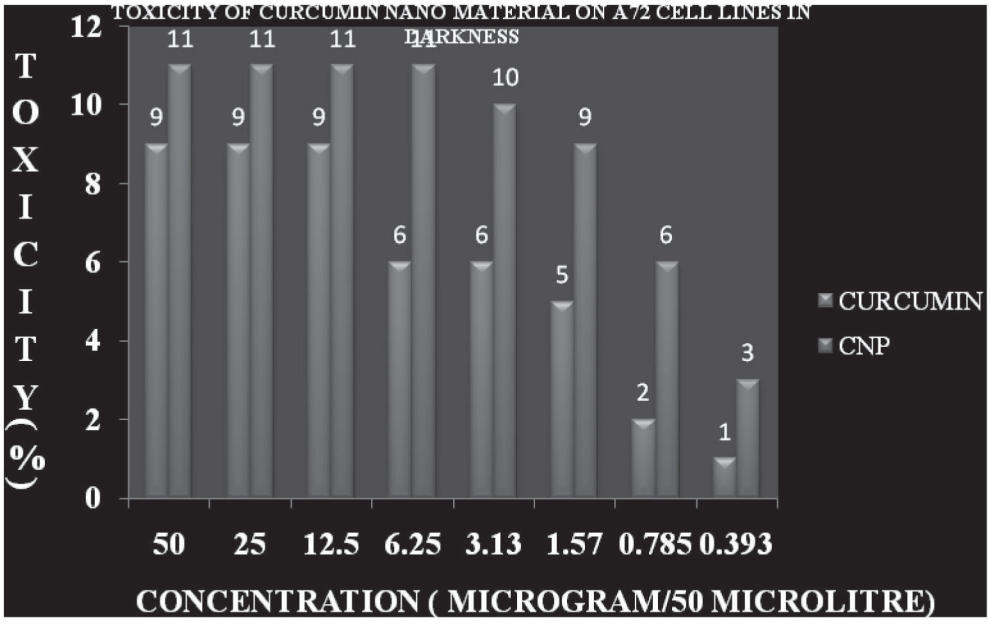

Figure 7: Toxicity Of Curcumin Nano Particle To A72 Cell Lines In Darkness

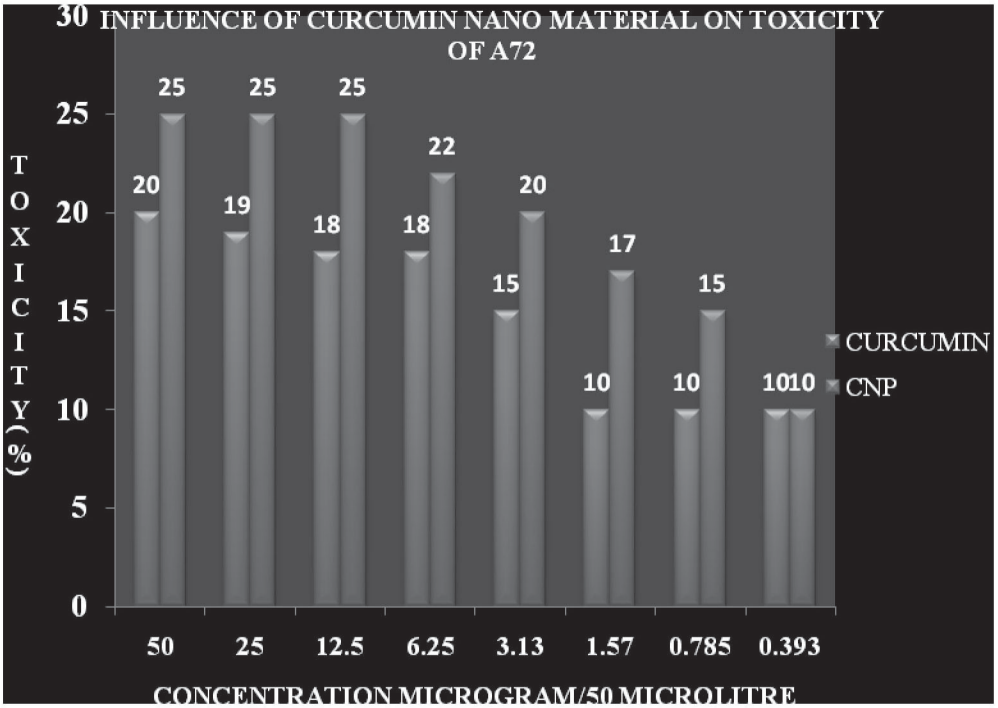

Figure 8: Photodynamic Toxicity Of Curcumin Nano Particle To A72 Cell Lines

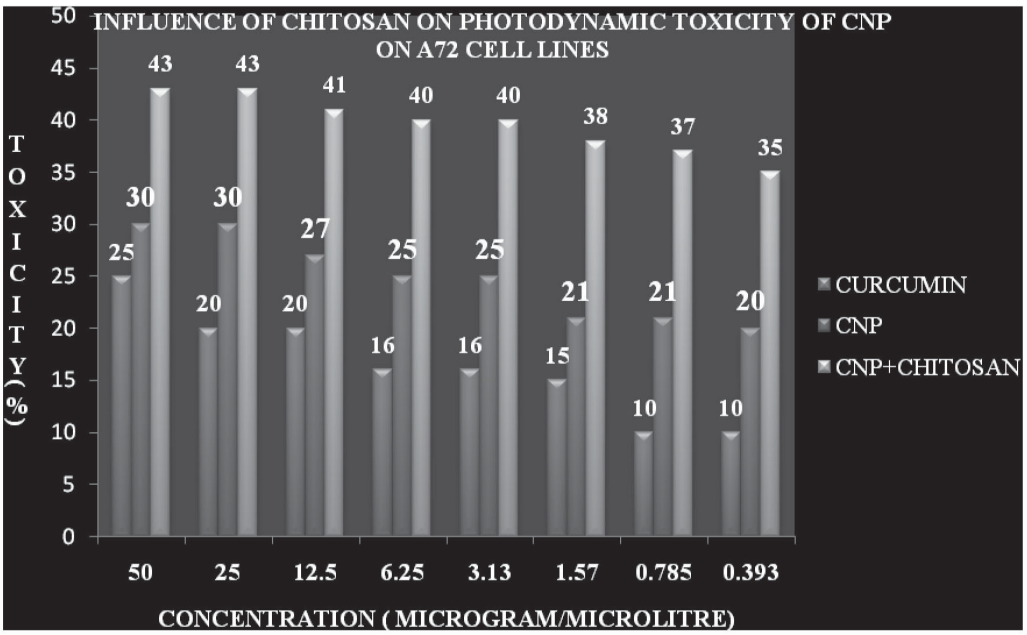

Figure 9: Influence Of Chitosan On Photodynamic Toxicity Of Curcumin Nano Particle To A72 Cell Lines

Conclusion

The study clearly demonstrates the destructive effect of curcumin and the role of chitosan in increasing the bioavailability and the toxicity of curcumin nano materials to A72 canine cancer cell lines. Thus formulation of a natural photo sensitizer with chitosan could be a viable alternative to synthetic photo sensitizers.

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