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pH Triggered Curcumin Release from PMMA-AA Coated ZnO Nanoparticles for Excellent Anti-Gastric Cancer Therapy

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Abstract

The curcumin loaded PMMA-AA/ZnO nanocomposite potentially inhibited the growth of AGS cancer tumour in male Swiss albino mouse, which showed a promising targeted cancer therapy. Interestingly the given bio-nanocomposite was rapidly cleared from the organs with negligible exhibition of toxicity. From the obtained results it is understood that the apoptosis has been occurred through mitochondrial disruption-mediated pathway. Also these nanomaterials could efficiently hinder the Go/G1 transition along with cycle progression at S-phase transition due to the radiation-induced DNA damage. These findings declared that the auspicious candidate, curcumin could be successfully delivered into the target by the polymer encapsulated ZnO NPs and exhibited a potent activity against gastric cancer cells at molecular and cellular levels as well as cell proliferation in a panel of tumour cells.

Keywords: PMMA-AA Co-polymer; Curcumin; ZnO; Biocompatible; pH-dependent

Introduction

The triumph of cancer treatment lies with primary diagnosis and efficient therapy making them inseparable. Recently, there has been an extraordinary development in the nanomedicinal research with the development of new nanoparticles for the diagnosis and cancer treatment. In general these studies are aimed on creating novel nanocomposites for battling cancer [1]. Gastric (stomach) cancer is a disease in which malignant (cancer) cells form in the lining of the stomach. It is the fifth most common cancer in worldwide. Around 952,000 new cases of stomach cancer were recorded globally in 2012, accounting for seven percent of all new cases of cancer [2]. Surgery is a very important part of gastric cancer treatment, Endoscopic, laparoscopic and robotic treatments for gastric cancer have progressed rapidly with development of surgical instruments and techniques, especially in Eastern countries but it sometimes leads severe side effects [3].

Semiconducting nanoparticle, ZnO has unique physical and chemical properties that show significant advantages in biological and biomedical applications, especially in bioimaging, drug delivery and biosensing fields due to its low cost and low toxic materials and have shown promising performances in biomedical experiments. In this present report such ZnO nanoparticles has been utilized a nano drug carrier to deliver curcumin as drug to the target. For the feasible entrap of the curcumin and its delivery at the specific target, it was encapsulated with the biocompatible polymers such as poly(methyl methacrylate) and abbreviation for AA (Acralyic acid) (PMMA-AA). PMMA is a biocompatible material, also has been extensively studied; it has already been used for about 20 years as an orthopedic implant material in Europe [4].

The drug loaded bio-polymer encapsulated nanocomposites are promising materials to the treatment of severe diseases, such as cancer, infections, and neurodegenerative disorders. Such nano-architectures are attained by the encapsulation of a drug with amphiphilic copolymers in aqueous solution in a self-assembled manner [5], and this approach have led to numerous interesting proven results in the in vitro analysis. Though, the unsafe quick drug release (burst release) [6], in the post-administration is an important limitation in this approach. In order to avoid such obstacles, the drug has been covalently linked to macromolecule in this present work. This approach enhances drug solubility, prolongs in vivo circulation, and reduces hostile effects, the last feature being of vital importance in various chemotherapy treatments. In this analysis of polymeric prodrug, is to link hydrophobic drug (curcumin) to a preformed hydrophilic polymer (PMMA-AA) as previous reports [7-10] to give complete water-soluble bioconjugate, moreover, some of the previously stated limitations of nanomaterials might mitigated by utilizing this approach [5].

Literature Review

These nanocarriers are made of a well-organized amphiphiles of polymer-drug bioconjugate attained between the hydrophobic polymer and a hydrophobic drug (curcumin) tail as discussed in our previous report. It exhibited that the curcumin loaded PMMA-AA/ZnO nanoparticles induce cytotoxicity in low concentration of 0.01 μg mL^{-1}. At this concentration AGS gastric cells are highly sensitive to curcumin loaded PMMA-AA/ZnO nanoparticles (Cur/PMMA-AA/ZnO NPs). Such great sensitivity of our previous nanocomposite material inspired us to evaluate its capability of antitumor analysis with tumour bearing mice models containing AGS gastric cells [11]. The observed apoptotic activity of this bioavailable form of curcumin was also evaluated in terms of loss of mitochondrial membrane potential, which account for apoptotic cell death. Following an injection of Cur/PMMA-AA/ZnO NPs was lower the peritoneal cavity of the mice bearing AGS cells, consequently the growth of the tumours were significantly inhibited and the survival rate of mice was increased. Most importantly they

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could not only control the cancer cell growth but also were rapidly cleared form the organs, resulting in little hepatic toxicities in mice. All these results strongly suggest that these Cur/PMMA-AA/ZnO NPs can be ultimately applied to the treatment of gastric cancer as a nanomedicine (Figure 1).

Previous reports pointed out that certain metal oxide and polymer based nanoparticles as drug carriers, while they could not be decant simply by the animals through faecal matter. This is the major cause for the internal organ damage in animals, e.g., Ag nanoparticles cause chromosomal abrasives [12] and TiO_2 nanoparticles cause brain damage [13]. These results indicated that different chemical characteristic metal oxide nanoparticles displayed their different toxicity level. Interestingly, this present work was observed the easy removal of the Cur/PMMA-AA/ZnO NPs from the tested mice organs. The weights of Cur/PMMA-AA/ZnO Nps and standard drug (5-flurouracil) were found to be same in the tumour bearing mice while, the weights of the saline group was decreased dramatically during the 16 days of systemic therapy in the tumour model.

Experimental Section

PMMA and AA have been chosen as hydrophobic polymers for their interesting properties such as chemical and enzymatic degradability, as well as its biocompatibility as we discussed earlier. Curcumin, the well-known natural medicinal material demonstrated activity against a wide range of solid tumors (e.g., colon, lung, pancreatic, breast, bladder, and ovarian cancers) [14], was selected as a drug model. Earlier we have reported the *in-vitro* evaluations of Cur/PMMA-AA/ZnO NPs [15], while its *in-vivo* evaluations have been carried out in a detailed manner and discussed in this present work. The loss of mitochondrial membrane potential (MMP) is a direct indication of the apoptosis, therefore it is necessary to evaluate the efficiency of Cur/PMMA-AA/ZnO NPs in the loss of MMP in detail. It is well known that curcumin could efficiently induces apoptosis through intrinsic signalling pathways by depolarizing the mitochondrial membrane and triggering the release of cyto-chrome c [16,17]. Very recently, it has been reported that the curcumin has a lot of potential to act as an adjuvant remedy in liver cancer through the loss of mitochondrial membrane potential and also protect against the side effects of the currently available chemotherapeutic agents [18]. The efficient role of the Cur/PMMA-AA/ZnO NPs in reducing the mitochondrial membrane potential (ΔΨm) was measured using a cationic fluorescence dye rhodamine1 [19] which diffuses into the mitochondrial matrix, thus bringing about drastic changes on it. The AGS cancer cell lines were treated with these studied nanomaterials which in turn induced a significant loss of mitochondrial membrane potential (ΔΨm) in a time dependent manner. Based on the IC_{50} values from the cell viability assay, the AGS cancer cell lines were treated with ZnO Nps (0.05 μg mL^{-1}), nanocurcumin (0.05 μg mL^{-1}) and Cur/PMMA-AA/ZnO NPs (0.01 μg mL^{-1}) for 24 and 48 hours. The exposure of Cur/PMMA-AA/ZnO NPs on the AGS cells lead a much lower rhodamine123 staining than the controls as illustrated in Figure 2, signifying that the permeability of the outer mitochondrial membrane increases and consequently, membrane potential (ΔΨm) decreases. After 48 h treatment of cell lines, the percentage of cells is less with a high MMP (ΔΨm) compared to the untreated control cells. These observations conclude that the apoptosis in AGS is triggered by Cur/PMMA-AA/ZnO NPs through mitochondrial disruption-mediated pathway.

The cell cycle analysis was performed to examine the growth inhibiting effect of ZnO nanoparticles, nanocurcumin and Cur/PMMA-AA/ZnO NPs on AGS cancer cells using propidium iodide staining, which is widely used as fluorescent dye for DNA staining. AGS cells were treated with 0.05 μg mL^{-1} of ZnO NPs, nanocurcumin and 0.01 μg mL^{-1} of Cur/PMMA-AA/ZnO NPs based on the obtained IC_{50} values of cell viability assay for 24 and 48 h, respectively. As illustrated in Figure 3, it was observed that the cells which were treated with the respective nanomaterials did not induced any significant changes on G0/G1 fractions but S- cells were decreased in 24 hours, though they did not undergo any further changes after 48 h. These observed time course experimental results illustrated that the cell cycle at S-cells could be arrested at 24 h. This may due to the major proteins, cyclin dependent kinase 1, 2 and 4 (CDK 1, 2 and 4) are considered potential molecular targets of curcumin [20]. Hence, curcumin acts as an adenosine triphosphate (ATP) competitive inhibitor; it downregulated the mRNA and the protein expression of cyclin D1 and suppressed transition of the cells from G1 to S phase, thus prevents invasion of gastric cancer cells. These results indicated that the curcumin could increase G1 cells while S-phase decrease. Therefore, it is obvious that the anti-proliferative effect of curcumin is related to the downregulation of cyclin D1 expression induced DNA damage and finally leads to the specific cell death.

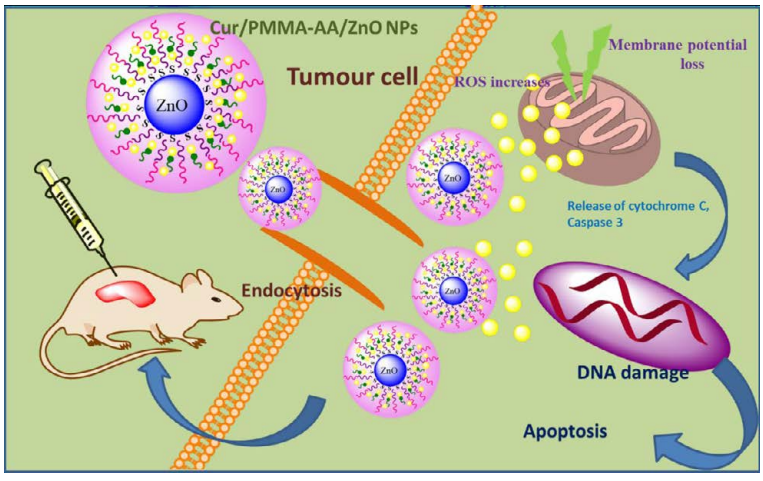

Figure 1: Cur/PMMA-AA/ZnO NPs can significantly reduce the growth of tumour *in vivo* by targeting the mitochondria and initiating the mitochondrion-mediated apoptosis signalling pathway.

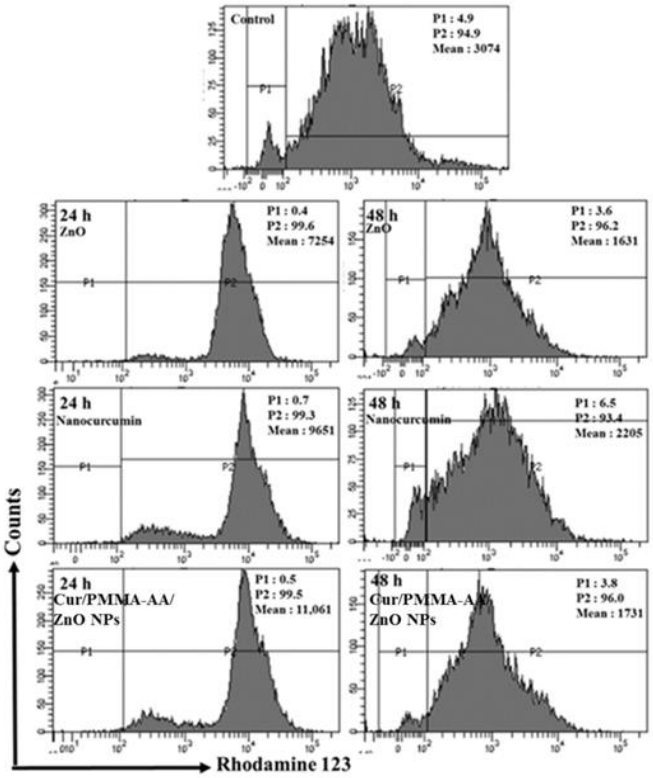

Figure 2: Images of mitochondrial membrane potential loss (ΔΨm) in the AGS cancer cells treated with the respective nanomaterials.

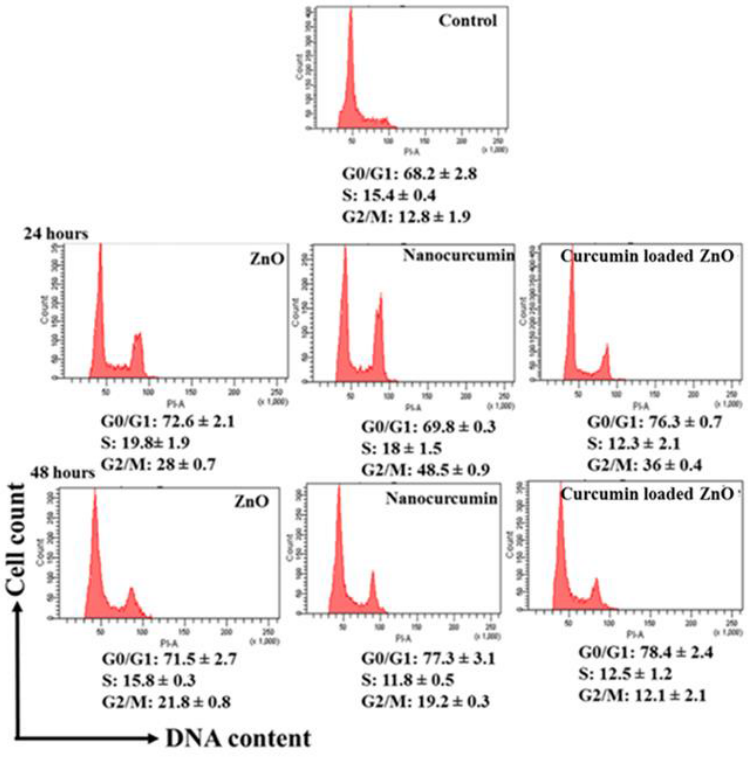

Figure 3: Effect of nanomaterials on AGS cell cycle analysis as measured by propidium iodide flow cytometry for 24 and 48 h.

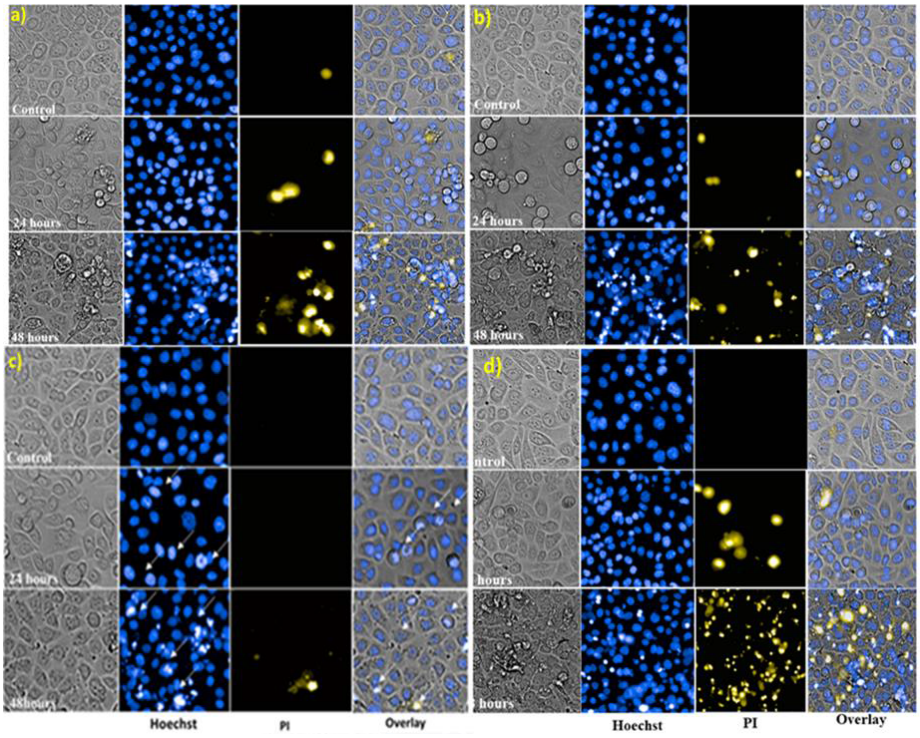

Figure 4: Hoechst staining and Propidium iodide images of AGS cancer cells of a) ZnO NPs b) nanocurcumin c) PMMA-AA co-polymer and d) Cur/PMMA-AA/ZnO NPs.

Apoptosis, also referred as "programmed cell death", is generally associated with cell shrinkage, nuclear and DNA fragmentation, and chromatin condensation induced by the presently studied nanomaterials was specifically evaluated. The observed sample-to-sample changes in Hoechst 33342 and PI staining were measured using HCS and illustrated in Figure 4. The untreated control AGS cells showed normal nuclei, while the cells which were treated with the proposed nanomaterials were exhibited the condensed or fragmented nuclei at longer culture times, since these observations are the characteristic feature of apoptosis (Figure 4), it is confirmed that these studied nanomaterials showed an proficient activity on the cancer cells. The fluorescent signature of PI was used to mark the fragmented DNA which were ejected from the cells during apoptosis, appeared to increase over the course of cell culture for all materials. However, it was particularly pronounced with the Cur/PMMA-AA/ZnO NPs treated cells (Figure 4d). These observations further demonstrated the superior anticancer activity of the nanocomposite relative to its constituent nanomaterials.

Cur/PMMA-AA/ZnO NPs inhibit the growth in tumour bearing mice

Most interestingly, these nanomaterials have also established the tumour growth suppressive effects through *in-vivo* analysis on AGS colon cell carcinoma using male Swiss albino mice models. In the present study, they were divided into five different groups, group 1-normal control, group 2-tumour control, group 3-the positive control were intravenously administered with the commercial drug, 5-fluorouracil (10 mg kg^{-1}), group 4 and group 5 were injected with nanocurcumin (10 mg kg^{-1}) and Cur/PMMA-AA/ZnO NPs (10 mg kg^{-1}) respectively at clinically relevant doses via the lower peritoneal cavity of the mice bearing AGS cells and the anti-tumour activity of each nanoparticles was assessed. The observed results from the tumour control group (G_2) were showed that implantation of these tumour cells in the specified quantity leads to the formation of disseminated tumours as shown in Figure 5a and 5c. On average, there were more than 6 tumours in each mouse, and the tumours from each mouse weighed more than 750 mg. Ascetic fluid is the direct nutritional source for tumour cells and a rapid increase in the tumour growth would be a means to meet the nutritional requirement of tumour cells [21]. The average tumour size of the mice was gradually suppressed with Cur/PMMA-AA/ZnO NPs at the end of 16 days. After the final administration the saline treated mice groups showed no reduction in tumour size, while the inhibition rate of nanomaterials treated was higher than that of non-treated mice. The mice group which was treated with Cur/PMMA-AA/ZnO NPs showed remarkable deduction of tumour size due its higher antitumor activity than those were treated only with the ZnO NPs and nanocurcumin. These observations clearly indicated that the lower peritoneal cavity could supresses the spread of metastasis cancer during the administration of nanoparticles through the fluid to entirety of host body, which illustrated that the ascites fluid plays major role in metastasis cancer. Therefore, from these observations, it could be concluded that even a minimum dose (10 mg/kg) of the Cur/PMMA-AA/ZnO NPs is sufficient to arrest the growth of tumour with decreasing the nutritional fluid volume in the peritoneal cavity and also it increased the life span of AGS bearing mice as shown in the graph (Figure 5d). This could be due to the sustained delivery of curcumin from the carrier, Cur/PMMA-AA/ZnO NPs (Figure 5).

Results and Discussion

The obtained serum test results showed that the AST, ALT, ALP, blood creatinine, total cholesterol (TC), triglycerides (TG) lipid profiles were at normal levels in the group consisting of Cur/

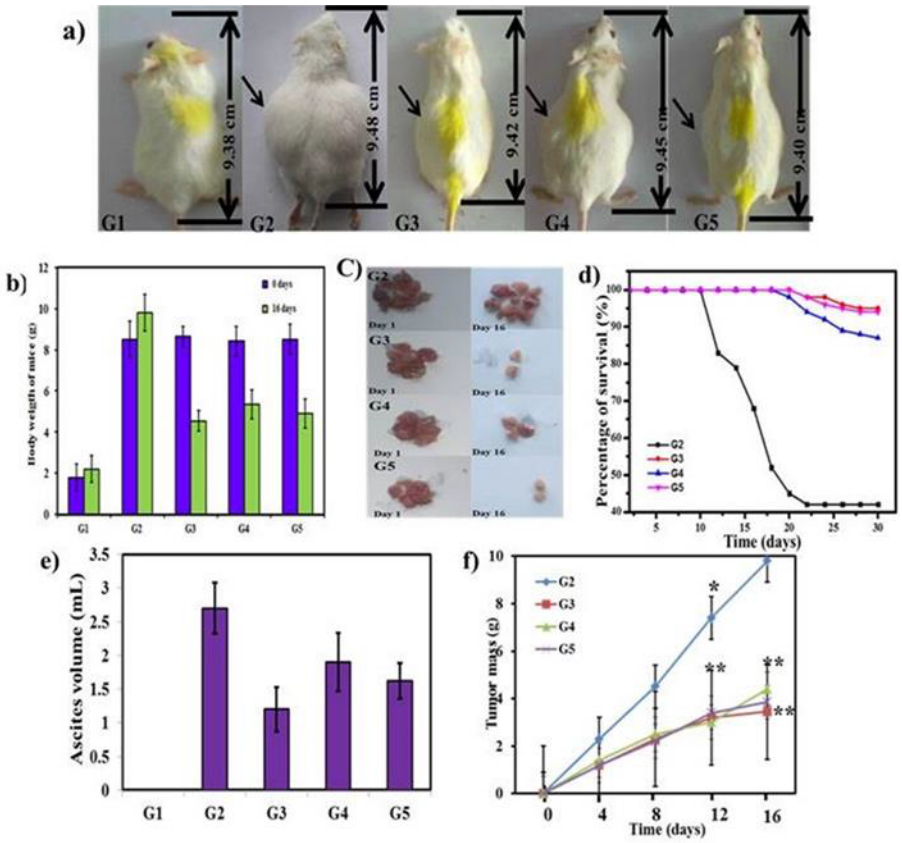

Figure 5: Representative images of mice bearing tumour from the same study after 16 days. (a) The tumours which treated with Cur/PMMA-AA/ZnO NPs group (G5), b) plot of weight changes in the mice bearing tumours. The tumour weights of the mice which treated with saline group were stable, whereas they dramatically reduced with Cur/PMMA-AA/ZnO NPs after 16 days. c) Representative images of stripped tumours, d) Survival plot of mice bearing tumours, the survival of mice treated with Cur/PMMA-AA/ZnO NPs was remarkably longer than the mice in the saline group, e) Ascites fluid were removed on day 16 from Cur/PMMA-AA/ZnO NPs treated and saline mice. f) Plot of tumour mass versus time. Day 0 was the starting of the treatment. The mice bearing tumours were euthanized when exhibiting signs of illness or death. The tumour masses of the deceased mice were not included after the day of death.

Treatment	Total WBC Cells/ml × 10^3	RBC Count Mill/cumm	Hb Gm/dl	PCV%	Platelets Lakhs/cumm
G1	10.30 ± 1.26	4.32 ± 0.90	12.35 ± 1.30	14.20 ± 2.40	3.28 ± 0.75
G2	16.60 ± 1.98	2.65 ± 0.50	6.95 ± 0.90	39.30 ± 3.30	1.92 ± 0.25
G3	13.35 ± 1.65	3.95 ± 1.80	11.87 ± 1.50	15.95 ± 1.65	2.89 ± 0.32
G4	15.30 ± 1.20	3.05 ± 0.62	10.02 ± 1.65	19.32 ± 2.65	2.12 ± 0.56
G5	13.90 ± 2.91	3.65 ± 0.75	11.19 ± 0.95	16.52 ± 2.30	2.98 ± 0.65
G6	12.50 ± 2.31	4.25 ± 0.55	12.09 ± 1.05	15.02 ± 2.20	3.19 ± 0.85

Table 1: Effect of nanomaterials on hematological parameters.

PMMA-AA/ZnO NPs shown in the graph (Figure 6). This may due to the absence of significant statistical differences during the course of analysis between nanomaterials and normal control group respectively with mice, suggesting there is little hepatoxicity was occurred in mice. The liver virtually showed no obvious histological changes in the HE-stained images shown in Figure 7. Group 1 showed liver parenchyma with hepatocyte to appear normal, and central vein, the portal tract are normal, group 2 tumour control showed liver parenchyma with scattered focal area of necrosis of hepatocyte, groups 3, 4, and 5 showed normal hepatic architecture. These attained results exhibited that Cur/PMMA-AA/ZnO NPs were potentially delayed the growth of AGS tumours along with increased the survival rate of tumour-bearing mice without damaging the normal organs.

Generally, myelo suppression and anaemia is the major target of cancer chemotherapy. The anaemia encountered in tumour bearing mice is mainly due to reduction in RBC or hemoglobin percentage and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions. Treatment with Cur/PMMA-AA/ZnO NPs at a dose of 10 mg/kg could significantly brought back the hemoglobin (Hb) content, RBC and WBC count nearly close to the normal levels. This observation clearly indicated that these Cur/PMMA-AA/ZnO NPs at a dose of 10 mg/kg possess protective action on the haemopoietic system (Table 1).

Conclusion

In summary, a novel and biodegradable Cur/PMMA-AA/ZnO NPs has been successfully prepared and assigned for the therapy of AGS colon cancer through both *in-vitro* and *in-vivo* techniques. After curcumin was loaded on the drug carrier PMMA-PA/ZnO, the cellular uptake and *in vitro* cytotoxicity were significantly enhanced,

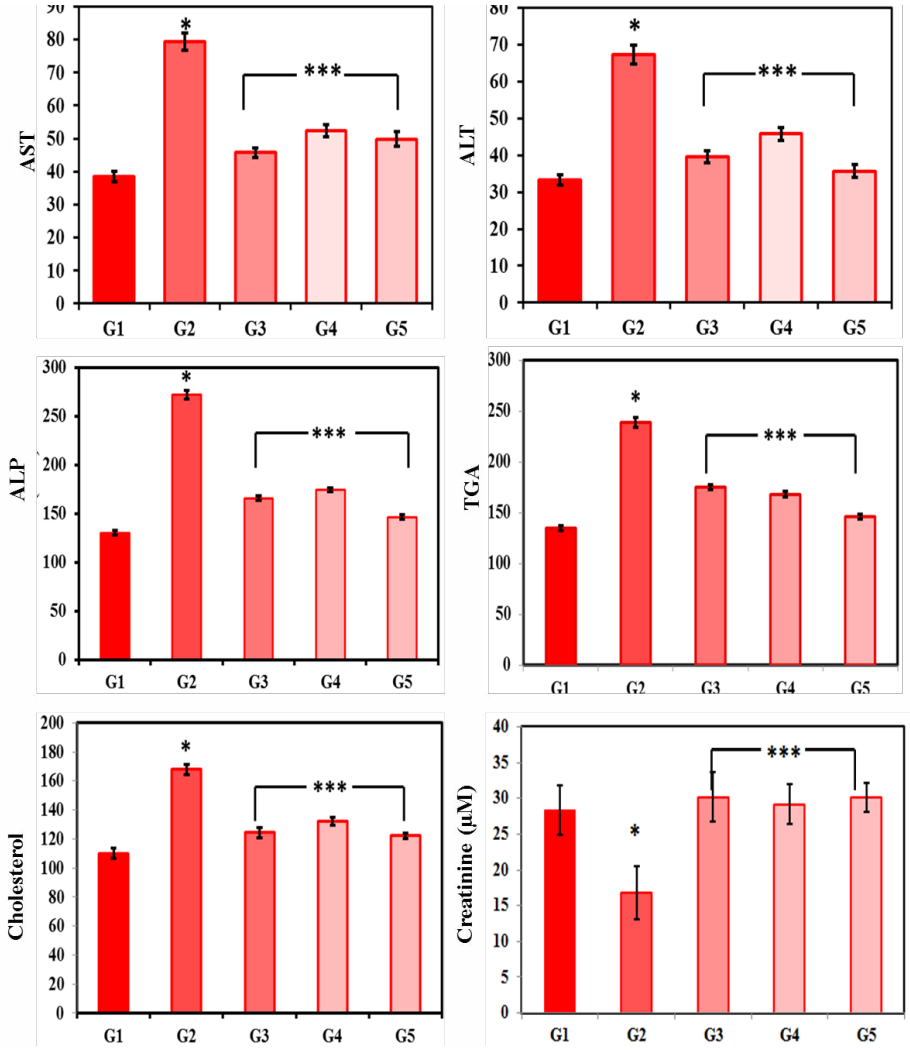

Figure 6: Assessment of toxicity and clearance of Cur/PMMA-AA/ZnO NPs, Aspartate amino Transferase (AST), Alanine amino Transferase (ALT), Alkaline Phosphatase (ALP) and blood creatinine. Total Cholesterol (TC), Triglycerides (TG) lipid profile creatinine levels in the treatment group were the same as in the saline group.

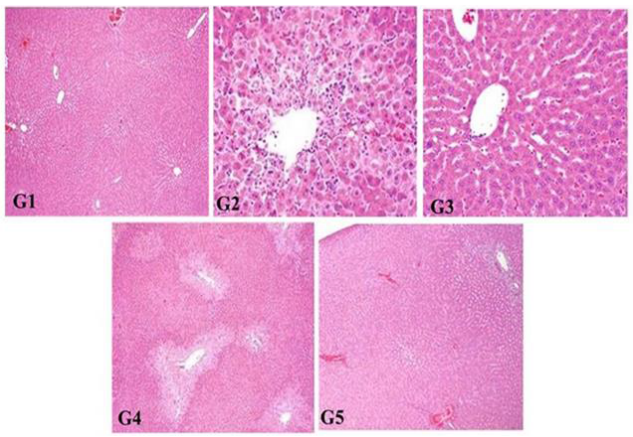

Figure 7: HE-stained images of the showing that section of liver at a total dose of 10 mg kg^{-1} day and 20 mg of 5-fluorouracil.

compared with free curcumin and ZnO NPs respectively. It also can induce cell cycle arrest and promotes apoptosis by a mitochondrial disruption-mediated pathway. All the observed results indicate that the Cur/PMMA-AA/ZnO NPs constitutes a new path towards the clinical implementation with a very negligible risk and consequently highly efficacious nano-vehicle to deliver such hydrophobic anticancer drugs. Furthermore, compared with free curcumin and ZnO NPs, Cur/PMMA-AA/ZnO NPs were more effective in suppressing tumour growth and metastasis. Thus, the studied Cur/PMMA-AA/ZnO NPs in this work showed remarkably enhanced *in vitro* and *in vivo* anti-tumour activities by showing a prolong life span of the test subject without hepatotoxicity. The hemotologiacal parameters of the test subjects were not affected by the studied nanomaterials during the analysis and hence these Cur/PMMA-AA/ZnO NPs could be potent alternate for the commercially available existing anticancer drugs in the near future.

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