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In vitro and *in vivo* evaluation of silybin nanoparticles for liver cancer

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ABSTRACT

The most common liver cancer would be Hepatocellular carcinoma. The objective of the study is to prepare silybin nanoparticle and to evaluate for its anticancer potential *in-vitro* and *in-vivo.* Hepatocellular carcinoma was induced in rats by supplementing 100 mg/L of diethylnitrosamine(DENA) in drinking water for 8 weeks. Saline, silybin 30mg/kg body weight and nanoformulation of silybin equivalent to silybin dose were administered orally to the groups divided. Anticancer activity was evaluated by counting the liver nodules and expression levels of antitumor markers. In rats treated with silybin nanoparticles, the number of neoplastic nodules was significantly lower, did not exhibit decrease in mean body weight, the number of liver nodules was found reduced by >93%. Silybin nanoparticles showed improved efficacy and safety compared to silybin for treatment of HCC in rats when administered orally.

Keywords: Hepatocellular carcinoma; Silybin; Nanoparticles; *In-vitro In-vivo*.

1. INTRODUCTION

Hepatocellular carcinoma (HCC), also called malignant hepatoma, is the most common type of liver cancer. Most cases of HCC are secondary to either a viral hepatitis infection (hepatitis B or C) or cirrhosis (alcoholism being the most common cause of liver cirrhosis). HCC is a highly lethal disease, as demonstrated by the equal annual incidence and mortality, and the dismal 8-month median survival without treatment [1] is improved only by a modest 3 months when the best chemotherapy available. [2] On the contrary, when HCC is detected at an early stage, curative treatments such as surgical resection, liver transplantation, and ablative therapies can be used, achieving 5-year survival rates of up to 75%, high lighting the importance of early detection. [3-5] Despite the availability of various diagnostic tools and treatment options, the happening is actually early recurrences and poor outcomes or missed treatment opportunities.

Several nanoparticle (NP) systems are being investigated for use in cancer diagnostics and therapeutics. [6-9] HCC targeting with NPs remains challenging as Kupffercells, specialized macrophages dispersed throughout liver sinusoids that comprise an elaborate reticuloendothelialsystem (RES), take up these particles and interfere with the imaging or delivery of therapeutic payloads. [9-11] Much effort has been devoted to optimizing selective delivery of NPs to tumors while evading the RES, using surface modification of NPs with antifouling polymers such as polyethylene glycol (PEG) and various tissue-specific ligands. [12, 13] Strategies that identify HCC-specific cell surface moieties to conjugate to achieve superior specificity hold the most promise. Vascular endothelial growth factor, epidermal growth factor receptor and small molecules such as galactose have been reported as potential targeting moieties for specific delivery of NPs and drugs to HCC cells. [14-17] However, the demand for novel targeting ligands for HCC-specific delivery remains high as no single receptor is uniformly expressed by the heterogeneous population of HCCs, and the efficiency of existing HCC-targeting ligands is less than ideal. Furthermore, a two-step pre-targeting approach to further enhance the signal for antibody-targeted imaging and treatment has been reported. [18, 19]

Silybin is one of the oldest drug for liver cancer. Although it is considered to be ideal for the treatment of liver cancer, delivery to the liver still needs improvement. Silybin needs to be administered daily to achieve its effects. Nanosized carriers encapsulating silybin can be taken up passively in to Kupffer cells in the liver and can result in increased drug concentration in the liver, thus increasing therapeutic efficacy.

Thus, the objective of this study was to prepare the biodegradable nanoparticles of silybin, and to evaluate it for anti-cancer efficacy both *in-vitro* and *in-vivo.*

2. METHODS

Silybin, Poly-ε-caprolactone (mol wt., 14,000), Polyvinyl alcohol (PVA, cold-water soluble) and Dichloromethane were procured from Sigma-Aldrich, Germany. All other reagents were of analytical grade. Male Wistar rats weighing 150 -180 g were obtained from the animal house of Chinese medicine hospital, Shandong, China.

2.1. Cell culture

Dalton's lymphoma, was used as a tumour model. Dalton's lymphoma (DL) cells harvested from Dalton's lymphoma bearing mice were cultured in RPMI-1640medium supplemented with 10% FBS and antibiotic solution (penicillin 1000 IU and streptomycin 10 mg/mL) in 5% CO_2 incubator at 37°C.

2.2. Animal model

For *in vivo* studies Swiss albino mice were taken, which were housed in well-ventilated cages and fed with standard mouse feed and water ad libitum. The animals were acclimatized to standard environmental conditions of temperature (22°C ± 5°C) for 12 h light-dark cycles throughout the experimental period.

2.3. Tumour model

The anticancer effect of the silybin nanoparticles was determined on Dalton's lymphoma, for which ascites tumour was maintained in mice. 1 × 106 cells/mL was transplanted in the peritoneal cavity of the mice. Dalton's lymphoma ascites (DLA) cells can be propagated as transplantable ascites tumour in Swiss albino mice.

2.4. Isolation of mouse bone marrow cells

Bone marrow cells were isolated from femur bones of approximately 8-10 week old mice by cervical dislocation after mild anaesthesia exposure. The bone marrow was flushed with pre-warmed phosphate buffer saline (PBS) through a 24-gauge needle and single cell suspension was prepared by agitation. The cell suspension was centrifuged at 1500 rpm for 5 min.

The cells were finally resuspended in RPMI-1640 medium supplemented with 10% FBS and antibiotic solution. Cells were seeded in culture plates with supplemented RPMI-1640 medium and maintained in 5% CO_2at 37˚C for 24 h for the experiment.

2.5. Preparation of silybin nanoparticles

Emulsion (o/w) solvent evaporation method was employed in the preparationofsilybinnanoparticles using poly-ε-caprolactone as the polymer. For the preparation, silybin (100 mg) and polycaprolactone (100, 200, 300 or 400 mg) was dissolved in 15 ml of dichloromethane by vortexing. The mixture (organic phase) was added drop-wise to 50 ml of 2 % PVA solution under probe sonication at 40 w for 10 min to obtain a w/o emulsion. This emulsion was placed on a magnetic stirrer to ensure complete evaporation of dichloromethane, leaving nanoparticle suspension. The suspension was centrifuged at 10,000 rpm for 20 min, resulting in the formation of a pellet at the bottom of the tube. This pellet was washed with phosphate buffered saline (PBS), re-suspended and againcentrifuged. The pellet was collected and allowed to dry completely. The powdered particles were collected, weighed and used for further evaluation.

2.6. MTT assay

Evaluation of cytotoxicity was done using MTT assay in Dalton's lymphoma and normal mouse bone marrow cells. DL cells were harvested from DL bearing mice and the mouse bone marrow cells were isolated from the femur bone of a normal adult mouse. 2.5 × 104 cells/mL DL cells and bone marrow cells (BMC) were seeded in RPMI 1640 medium (10% FBS and antibiotic solution) in a culture plate with saline, silybin and silybin nanoparticle. The culture plates were incubated for 24 h at 37°C and 5% CO_2. After incubation, 10 L of MTT (5 mg/mL in PBS) was added to each well and it was incubated for additional two hours at 37°C to allow intracellular reduction of the soluble yellow MTT to insoluble purple formazan crystals. These formazan crystals formed were dissolved in 100 L of DMSO and incubated for 30 minutes at 37°C. **[20]** The absorbance of the solution was read at 570 nm using a microplate reader. Three independent experiments were carried out and five replicates were taken for each experiment. Percentage inhibition was considered by the formula given below:

$$\%\ inhibition = \frac{Control\ abs - Sample\ abs}{Control\ abs} \text{X } 100$$

2.7. Anticancer activity of silybin nanoparticles and silybin in DENA treated rats

HCC was induced in rats by supplementation of 100 mg/L of DENA for 8 weeks. [21] After one week of DENA induction, saline, silybin and silybin nanoparticles were administered orally with oral canula on 1, 7, 14,

21, 28, 35, 42 and 49 days. During the treatment, weight of animals was monitored and the animals were sacrificed at the end of study. Tumour nodules >3 mm in diameter were counted on the surface of each lobe of the liver and the variation in the nodule numbers amongst the groups was statistically evaluated. The anti-tumor effect of the formulations was estimated by comparing the number of animals with more than 40 tumour masses in each of the three experimental groups.

3. RESULTS AND DISCUSSION

3.1. MTT assay

The silybin nanoparticle induced cytotoxic response against DL cells. In the case for normal mouse bone marrow cells the silybinnanoparticledid not induce cytotoxicity.The vehicle group also did not show any effect towards DLcells (Figure 1). The data obtained from the MTT assay clearly showed that silybin nanoparticlecan potentially induce cytotoxicity towards DL cells without affecting the normal cells. Therefore we finally illustrate that the silybin nanoparticle induces cytotoxic effect in DL cells but fails to exert cytotoxicity towards normal cells.

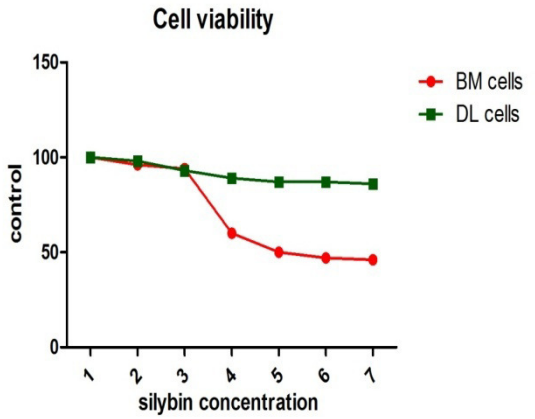

Figure – 1: Cytotoxicity profile of silybin nanoparitcles against DL and BM cells.

3.2. Treatment of hepatocellular carcinoma

HCC was induced by oral administration of DENA and was found evident through the formation of nodules on liver tissue. In the case of rats treated with silybin, the nodule formation was found relatively reduced though some cancerous growth was still observed. In contrast, in the group treated with silybin nanoparticles the nodules in the liver were significantly reduced suggesting highest inhibition of HCC when silybin loaded nanoformulations were employed (Figure 2). The above observations suggest that the silybin nanoparticle formulations are highly effective when compared to silybin as such.

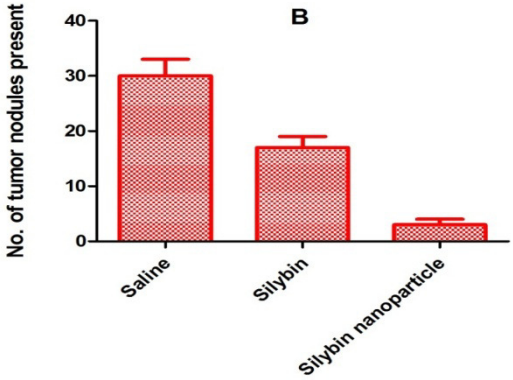

Figure - 2: Tumor nodules on the surface of liver were reduced to a large extent when treated with silybin nanoparticle.

3.3. Efficacy of nanoformulations

HCC bearing rat showed an increase in mean body weight (MBW), which gets drastically reduced when treated with silybin indicating the severe side effects exerted by the drug. Whereas in the case of treatment with silybin nanoparticles, the MBW of animals was significantly restored (Figure 3) with a slight decrease in weight due to cancer inhibition. Thus the nanoparticle encapsulation has reduced the silybin-treatment associated weight loss demonstrating decreased side effects.

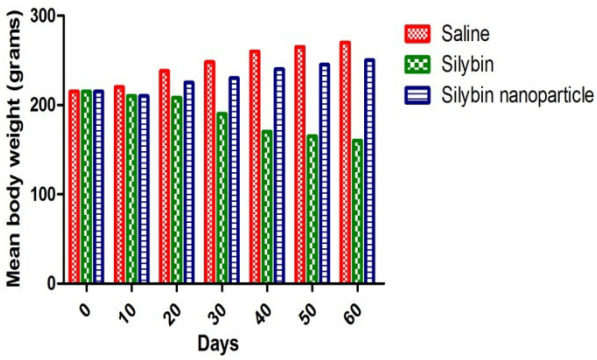

Figure - 3: Body weight of rats was decreased significantly when treated with silybin and gets increased in saline treated. When treated with silybin nanoparticles, a slight increase in body weight was observed.

4. CONCLUSION

The study suggests that the nanoparticle formulation may be useful in the treatment of liver cancer with silybin. In summary, the results obtained involving in vitro and in vivo studies in Dalton's lymphoma cells clearly demonstrated potent anti-cancer activity of silybin nanoparticle. This anticancer activity is due to apoptotic

inducing property and cell cycle delay with the silybin nanoparticle. The results from the study are found to be promising that nano drug delivery is a suitable carrier for silybin for a better therapeutic efficacy.

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