Suez Canal University Medical Journal Vol. 20 (1), 2017
Pages 20-28

Evaluation the Safety of Thymoquinone Loaded on Gold Nanoparticles in the Treatment of Hamster Buccal Carcinogenesis

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Abstract

Background: **Oral cancer is one of the most prevalent cancers. Thymoquinone (TQ) has a potential therapeutic activity against cancer cells.** ***Aim:*** **This study aimed to compare the effect of TQ and TQ loaded on gold nanoparticles (GNPs) as systemic therapeutic agents through intra-peritoneal injection for 3&6 weeks after painting with DMBA (3 times/week for 14w).** ***Material and Methods:*** **This study was carried out on ninety male Syrian golden hamsters (n=90), its age ranged from 6 to 7 weeks, weighting 90-110 gm, purchased from Theodore Blhars Research Institute, Cairo, Egypt. Characterization of GNPs was achieved through using Ultraviolet-Visible spectroscope and transmission electron microscope (TEM). Analysis for total white blood cells (WBCs) & lymphocytes count and lymphocytes % was done. To ensure the drugs safety, liver and kidney toxicity enzymes were measured.** ***Results:*** **Formation of spherical and well dispersed GNPs (27nm). There was marked increase in WBCs (13.8), lymphocyte count (8.2) and liver enzymes (99.6-104.4) after DMBA painting. These markers were reduced obviously after using of GNPs-TQ for 6w to 6.1, 5.4, 56.7, and 57.5 respectively. Histopathological evaluation showed regression of carcinogenesis and regeneration of striated muscle layers when treated with GNPs-TQ.** ***Conclusions:*** **GNPs-TQ is considered a promising chemo-therapeutic agent in treating carcinoma, without toxic effect on liver and kidney enzymes.**

Key words: Oral cancer, GNPs-TQ, WBCs and liver enzymes

Introduction

Oral squamous cell carcinoma (SCC) is the sixth most common cancer. It is a multifactorial disease which was brought by a combination of causes and predisposing factors[(1)]. The overall 5-year survival rates represent 50% of population[(2)]. DMBA is a polycyclic aromatic hydrocarbon (PAH). On metabolic activation, it produces the ultimate carcinogen, dihydrodiol epoxide, which causes DNA damage[(3)]. One of the best characterized animal models for OSCC is the buccal pouch of Syrian golden hamster to act as a model for oral carcinogenesis[(4)]. One area of interest was the addition of natural products to chemotherapy regimens. Thymoquinone is considered the main active constituent of black seed[(5)]. Despite these promising

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outcomes in preclinical settings, the applicability of chemo-prevention to human has met with only partial success due to poor bioavailability of agents[6]. Nanocarriers were found to modify drugs by increasing their bioavailability, efficacy, stability, solubility, decreasing their toxicity associated with high doses, and sustaining their release[7]. Gold is known to be a noble metal. Several reasons made the researchers to use GNPs in cancer nanotechnology: ease to synthesize GNPs by several simple, economically cheap, safe and reliable methods. GNPs could be easily synthesized with different shapes as spheres, rods, tubes, or wires by changing the reaction conditions. It can be easily characterized due to presence of the characteristic surface Plasmon resonance (SPR) bands[8].

Material and Methods

Chemicals: Tetra-chloroauric acid, Trisodium citrate to get GNPs solution, Thymoquinone and propylenegylcol to get thymoquinone solution, Chemical carcinogen DMBA and heavy mineral oil to get DMBA solution. All the previous chemicals were purchased from Sigma Chemical Company, USA. Serum aspartate amino transferase (AST), serum alanine amino transferase (ALT), and serum creatinine (CR) were obtained from Chema Diagnosta, Monsano, Italy. Serum urea (UR) was obtained from Spinderact, Santa Coloma, Espania. These were used for serum analysis of liver and kidney enzymes.

Gold nano-particles preparation (GNPs) & loading by TQ: All the steps were performed at room temperature. All the glassware's were rinsed with 10% nitric acid, and then washed with deionized water. Turkevich method[9] was used to prepare GNPs. A concentration of TQ solution of 0.001mg/100gm was prepared in propylene glycol through a magnetic stirrer for 2 hours. The GNPs-TQ solution was prepared by mixing equal amounts of both (1:1). The mixture was stirred for 2 hours and the formed solution was kept in 5°C. Transmission electron microscope was used to determine the morphology of GNPs, GNPs-TQ by high resolution transmission electron microscope (TEM) using JEOL JEM 2100 (Japan). Pictures of nanoparticles were taken in the Egyptian Petroleum Research Institute. Ultraviolet-Visible Spectroscope was used to determine the maximum absorption, GNPs, TQ, and GNP-TQ were studied by ultraviolet-Visible (UV-VIS) spectroscope. It was recorded at room temperature with samples in a quartz cuvette using T90+UV-VIS Spectrometer, PG Instruments Ltd (wave length 250-850 nm).

Experimental Design: The chemical carcinogen DMBA was dissolved in heavy mineral oil to get 0.5% solution. The carcinogen was topically applied to hamster left buccal pouches (HBP) by using number (4) camel hair brush. The experiment was held at the animal house in Pharmacology and Toxicology Department, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt. This study was carried out on ninety male Syrian golden hamsters (n=90), its age ranged from 6 to 7 weeks, weighting 90-110 gm, purchased from Tiedor Blhars Research Institute, Cairo, Egypt. Animals were housed, five per cage, with controlled temperature and humidity. They were fed a given tap water *ad libitum*. The hamsters were divided into: group A1: Twenty animals were served as "*negative control*" which didn't receive any treatment. Group A2: Ten animals were considered as "*positive control*". DMBA was painted to the left cheek pouches, three times /week for 14weeks. Group B: (self-control) *thirty* animals were injected intra-peritoneally (i.p.) with old nanoparticles (GNPs), thymoquinone

(TQ), and thymoquinone loaded on gold nanoparticles (GNPs-TQ) by an insulin syringe 3 times/week for 3 and6w respectively. Group C: (chemotherapeutic group) thirty animals were painted with DMBA to left cheek pouches three times/week for 14weeks, followed by intra-peritoneal injection of gold nanoparticles (GNPs), thymoquinone (TQ), and thymoquinone loaded on gold nanoparticles (GNPs-TQ) three times/week for 3 and 6w respectively.

Blood and serum analysis: Before sacrificing, the animals were anesthetized and blood samples were taken from the inner canthus of eye, for total white blood cells, lymphocytes count, and lymphocytes percentage. To ensure safety of the used drugs, toxicity enzymes were measured through AST, ALT for liver tests, and CR, UR for kidney tests in each group. The blood samples were collected in heparinized tube, then immediately sent for blood counting, using fully automatic cell counter (Heco serc, Italy). A second blood sample was collected in sterile tubes without anticoagulant that was left to clot at room temperature, and then centrifuged at 3000 rpm for 15 mins using a bench top centrifuge (MSN Minor, England). After centrifugation, serum samples were separated into eppendorf tubes and stored in the refrigerator at -20oC until analysis. The analysis was carried out according to manufacturer's instructions. The results were statistically analyzed using ONE WAY ANOVA TEST with five replicate. CoStat software program, version 6.311 was used to perform the analysis, means, and compared them using LSD P≤ 0.05.

Histopathological evaluation (H&E): After hamsters sacrificing, the pouches were surgically removed, and processed for regular H&E stain, and then photographed by E-330 Olympus digital camera. Grading of epithelial dysplasia was carried out according to Banoczy and Sciba[10] and modified by El-Dakhakhny et al[11] due to thin nature of hamsters' epithelial lining. Grading of squamous cell carcinoma was carried out according to Broder's classification[12].

Results

Characterization of GNPs, and GNPs-TQ: Formation of GNPs was preliminarily confirmed by visual observation of color change from pale yellow to deep red color. TEM micrographs of GNPs showed spherical and well dispersed particles without agglomeration (Fig. 1-A). Most of particles were between 25-30nm in size (average size of 27 nm, Fig. 1-B). The produced solutions of GNPs, TQ, and GNPs-TQ were subjected to characterization by UV-visible spectroscopy. Sharp peak was given by UV-visible spectrum for GNPs at λmax=526 nm which confirmed the nanoparticles formation. The maximum absorption peak for TQ was recognized at λmax= 316nm, while GNPs-TQ gave maximum absorption peak λmax =532nm (Fig. 2). This deviation supported the loading of TQ on GNPs.

Liver and Kidney enzymes results: Group A1 (negative control) and group B (self-control): There was no statistically significant difference between all groups in WBCs, lymphocytes counts. However, there was a statistically significant difference in lymphocytes% (different letters, histogram 1). Increase in lymphocytes% was statistically significant in groups treated with GNPs-TQ for 3 & 6 weeks, respectively when compared to other groups. Group A_2 (positive control 14w) and group C (treated groups): The results of WBCs, lymphocytes (count, %) showed statistically significant difference between groups (different letters). Increases in WBCs and lymphocytes count with decrease in lymphocytes % were statistically significant in group A_2 (DMBA-

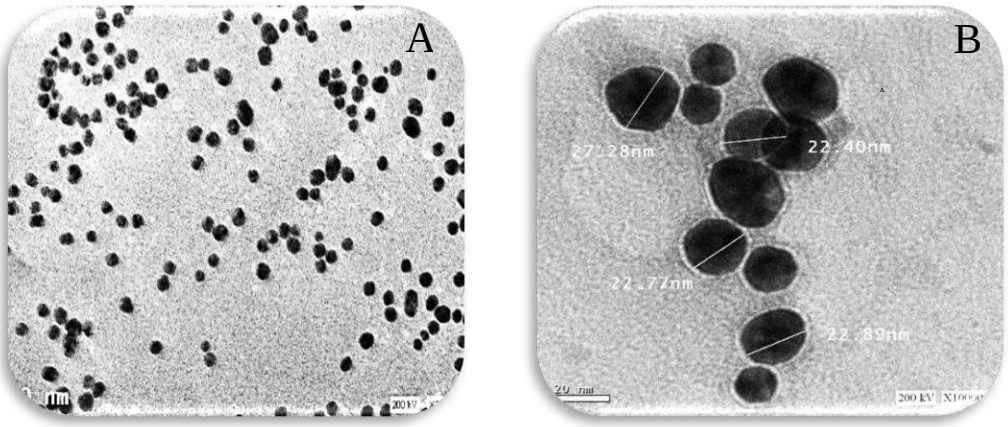


Figure 1: TEM micrographs of GNPs at magnification of 1:30.000 (A), 1:100.000 (B).

treated for 14w) when compared to negative control group. There was statistically significant decrease in total number of WBCs & lymphocytes count with increase in lymphocytes% in groups B5 & B6 (i.p of GNPs-TQ for 3 & 6w) in comparison to positive control group (histograms 2, 3). During studying the parameters of kidney, there is no statistically significant difference between all groups in CR and UR. Significant increase in ALT, and AST was statistically significant (different symbols) in group A_2 (DMBA 14w) compared to all other groups but after treatment there was significant reduction in all groups and seemed like to be normal (histogram 4).

Discussion

This study aimed to combine the antitumor effect of thymoquinone (TQ) with the efficient penetration of gold nanoparticles (GNPs) to cells and nuclei[13'14]. This combined formulation was used to investigate a possible therapeutic effect of TQ loaded on GNPs when given through intra-periton-

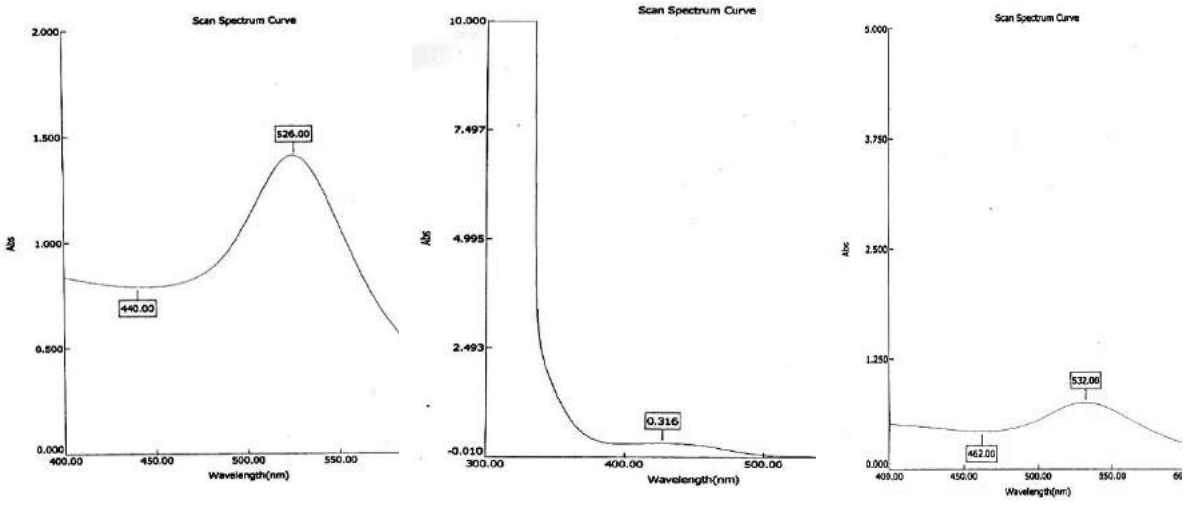
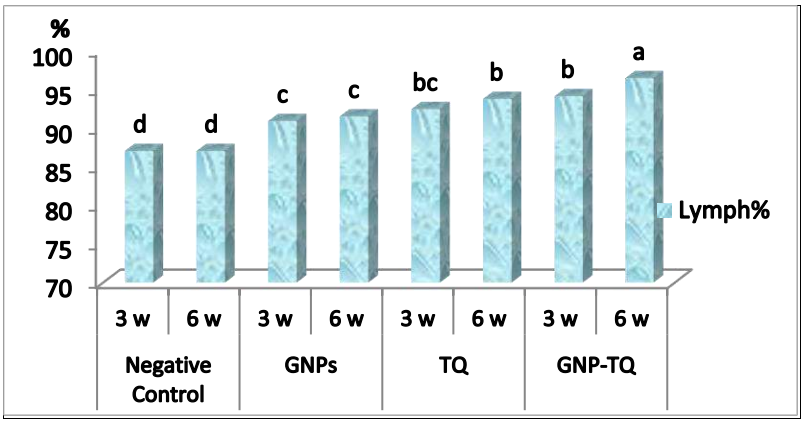
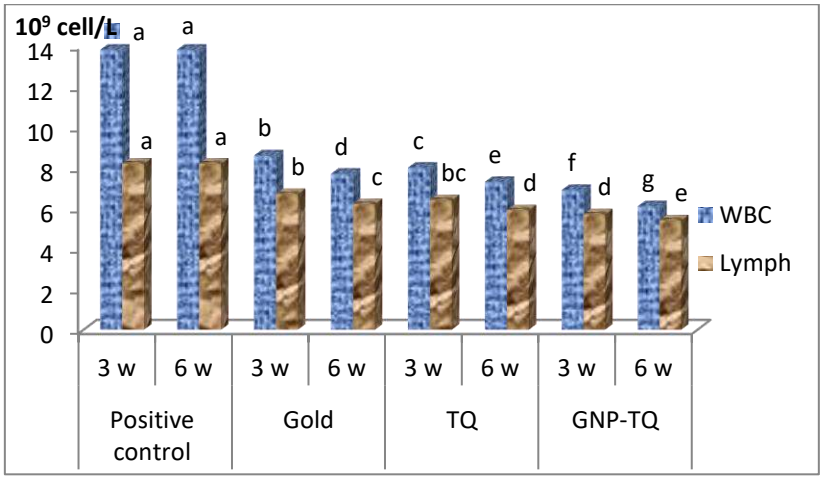


Figure 2: Photograph showed sharp peak of UV-visible spectrum for GNPs at 526 nm (right), for TQ at 316 nm (middle), and for GNPs-TQ at 532 nm (left).



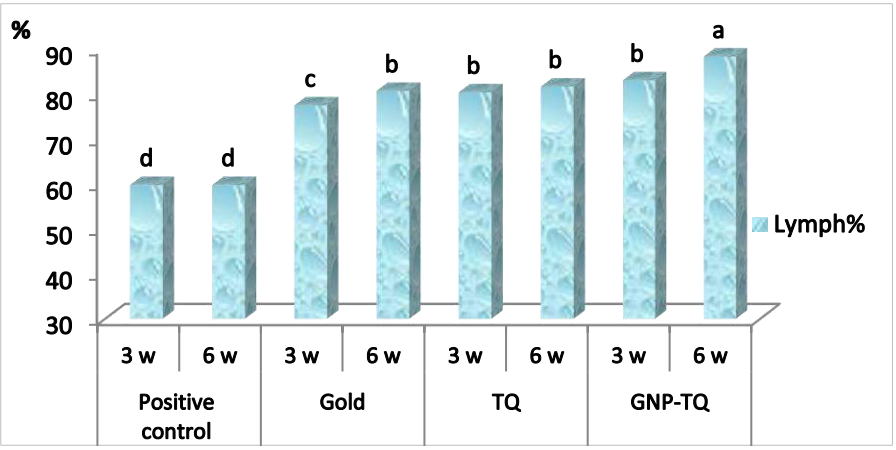
Histogram 1: The mean score of lymphocytes percentage in negative and self-control groups at 3&6 w. Increase in lymphocytes% was statistically significant in groups treated with GNP-TQ for 3&6w, respectively.



Histogram 2: The mean score of WBCs and lymphocytes count in positive control and treated groups at 3&6 weeks. A statistically significance increase in the +ve control, while after treatment with different working solutions for 3&6weeks, respectively, significant decrease with GNP-TQ was recorded.

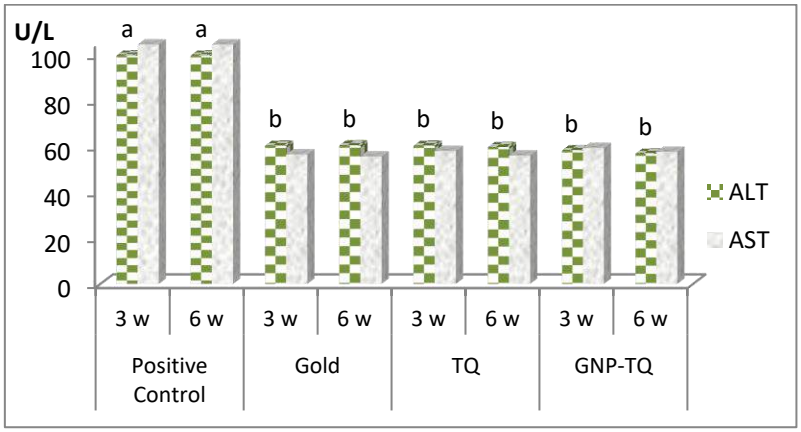
eal injection in the hamster buccal pouch carcinogenesis (HBP/DMBA) model. The best result was associated with GNPs-TQ when compared to either GNPs or TQ alone. Significant regression of malignancy, improvement in general health of the experimental animals and most interesting is elongation of the shortened cheek pouches with muscle regeneration. Gold nanoparticles were used as a drug carrier due to its ability to cross cellular membranes[15]. Based on its ease of fabrication, having high surface area and binding capacity to targeted cancer cells is higher than other nanoparticles[8]. The use of GNPs as anticancer agent was attempted by several ways as antibody conjugation[16], intra-tumor injection[17], and even when painted topically alone[13] In this work, the technique used to prepare GNPs had resulted in spherical, well dispersed, without agglomeration; with particle sizes between 25-30 nm (average size was 27 nm). This result was in consistent with Afifi et al[17] who used GNPs to treat DMBA-induced HBPs carcinoma with average size (30 nm) through intra-lesional injection, without any sign of toxicity. Also, the authors were on line with the present results who reported the maximum peak at

530nm. In the current study, it was necessary to ensure safety of the used drugs, through analysis of liver (AST-ALT) & kidney (UR-CR) enzymes, white blood cells (WBCs), lymphocyte count and lymphocyte%. Comparing the results between negative and self-control groups, there were no statistically significant differences up to 6 weeks, indicating the safety of the used drugs. These results were consistent with Hassan et al[18] who used the same material in different concentrations for 14w through topical application to HBP/DMBA model.



Histogram 3: The mean score of lymphocytes percent in the positive control) and treated groups. Statistically significant decrease in +ve control was noted. While after treatment with different working solutions for 3&6weeks, there was a significant increase with GNP-TQ treatment.

These results could be attributed to the results of Lipka et al[19] when reported that GNPs were excreted in urine 100 times more than polyethylene glycol (PEG)-modified GNPs after systemic application of different GNPs formulations or concentrations. They suggested that renal clearance is the expected excretion pathway due to its lowest diameter and highest surface polarity. Hsieh et al[20] had shown that there were no significant differences between serum levels of creatinine, blood urea, bilirubin, alkaline phosphatase, ALT, or AST in mice-treated with EGCG-GNPs, or GNPs for 7 constitutive days when compared to untreated mice.



Histogram 4: The mean score of alanine aminotransferase and aspartate aminotransferase in the positive control, and treated groups. Different letters indicate statistically significant difference between groups especially in positive control.

It seems that GNPs' size would affect these values as, Abdelhalim and Moussa (2013)[21] reported that urea and creatinine values had no significant change after the systemic administration of GNPs when compared with the negative control rats. Results of the present work showed that in the positive control group elevation of the liver enzymes (AST-ALT) as compared to negative control group, confirming the toxic effect of DMBA even when given topically. This result could be explained according to Jagatheesh et al (2010)[22] who suggested the increase of liver enzymes, in DMBA-treated group, might be due to altered membrane permeability and leakage of enzymes from the tissue. In the current work, after DMBA treatment followed by i.p injection with the different preparations, there was significant decrease in liver enzymes. These results were comparable to Singh et al (2013)[23] findings of significant decrease in the serum biomarker enzymes (AST & ALT) after oral administration of THQ-SLNs (thymoquinone-solid lipid nanoparticles) as compared to positive control (paracetamol-induced liver cirrhosis) that resulted in significant increase in liver enzymes. Moreover, Omayma et al (2015)[24] showed prominent increase in liver enzymes in DMBA-induced mammary carcinoma. After treatment with N-acetyl nano-composite, significant improvement was noted as compared to the DMBA-treated group. Results of the present work regarding selective blood count, there was no statistically significant difference between negative and self-control groups in WBCs and lymphocytes' count. There was significant improvement in lymphocytes% in self-control group given GNPs-TQ for 6w. This finding would be in favor to the immune enhancement when TQ loaded on GNPs when given for extended period (6w). After DMBA painting for 14w, there was a statistically significant increase in WBCs and lymphocytes' count, with significant decrease in lymphocytes' percentage. Here, the known immune suppressive effect of DMBA is evident, as significant decrease in lymphocyte% and significant increase in WBC counts especially lymphocyte count (as a defense mechanism against the inflammatory reaction of DMBA) as well as its systemic toxicity[22]. Of interest, significant improvement was noted in the group treated with GNPs-TQ for 6w after DMBA-painting, stressing the immune-enhancing effect of GNPs-TQ with extension time of treatment (6w). On contrary to the present findings, Al Jawfi et al (2008)[25] observed significant decrease in total WBC and lymphocyte counts in DMBA-painted group as compared to untreated group. Whereas the group received *Nigella sativa* oil (*NSO*)-only showed no statistical difference when compared to untreated group. The authors emphasized that DMBA, a potent carcinogen, induces immune-suppres-sion, through prevention of B and T-cell mitogenesis, and decrease natural killer cell activity as neutrophils & lymphocytes levels. A comparable finding was noted by Jagatheesh et al (2010)[22] who studied the anti-tumor, and antioxidant activities of *Morphophallus paeonifolius*, in DMBA-induced mammary tumor in rats. The authors reported significant increase in WBC counting in DMBA-treated group, with significant increase in neutrophil counting, indicating an inflammatory response in animals with large tumors. In another study carried out by Hidayati *et al* (2015)[26], reported a statistical significant decrease in lymphocyte's percentage in DMBA-treated mice, and after *Nigella Sativa* oil treatment, significant increase in lymphocyte % was observed. Other study supported the hypothesis of great association between high levels of WBCs count, tumor progression, cancer mortality, and rate of metastasis[27]. A recent study was

carried out by Hassan et al (2016)[18] confirming the safety of GNPs-TQ topical application in different concentrations, and documented the best concentration was 0.001mg/ 100gm. In DMBA-treated group, there was significant increase in WBCs and lymphocytes' counts with decreased lymphocytes' percent. Following GNPs-TQ treatment, there was significant improvement in these readings in comparison to the DMBA-treated group.

Conclusions

Systemic application of TQ loaded on GNPs at a concentration of (0.001mg/ 100gm body weight) could be a promising treatment of that malignancy model, without toxic effect on liver and kidney enzymes.

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