Exploring the Potential Role of Chemopreventive Agent, Hesperetin Conjugated Pegylated Gold Nanoparticles in Diethylnitrosamine-Induced Hepatocellular Carcinoma in Male Wistar Albino Rats

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Abstract Liver cancer is the fifth most common cancer and is still one of the leading causes of death world wide, due to food additives, alcohol, fungal toxins, air, toxic industrial chemicals, and water pollutants. Chemopreventive drugs play a potential role in liver cancer treatment. Obviously in the production of anticancer drugs, the factors like poor solubility, bioavailability, biocompatibility, limited chemical stability, large amount of dose etc., plays a major role. Against this backdrop, the idea of designing the chemopreventive nature of bio flavanoid hesperetin (HP) drug conjugated with pegylated gold nanoparticles to increasing the solubility, improve bioavailability and enhance the targeting capabilities of the drug during diethylnitrosamine (DEN) induced liver cancer in male wistar albino rats. The dose fixation studies and the toxicity of pure HP and HP conjugated gold nanoparticles (Au-mPEG₍₅₀₀₀₎-S-HP) were analysed. After concluded the dose fixation and toxicity studies the experimental design were segregated in six groups for the anticancer analysis of DEN induced HCC for 16 weeks. After the experimental period the body weight, relative liver weight, number of nodules and size of nodules, the levels of tumor markers like CEA, AFP and the level of lipid peroxidation, lipid hydroperoxides and the activities of antioxidant enzymes were assessed. The administration of DEN to rats resulted in increased relative liver weight and serum marker enzymes aspartate transaminase, alanine transaminase, alkaline phosphatase, lactate dehydrogenase, and gamma glutamyl transpeptidase. The levels of lipid peroxides

elevated (in both serum and tissue) with subsequent decrease in the final body weight and tissue antioxidants like superoxide dismutase, catalase, reduced glutathione, glutathione peroxidase, and glutathione reductase. HP supplementation (20 mg/kg b.wt) significantly attenuated these alterations, thereby showing potent anticancer effect in liver cancer and the HP loaded gold nanoparticles (Au-mPEG₍₅₀₀₀₎-S-HP) treated animals shows the better treatment than the pure HP due to the solubility of drug, bioavailability and the target drug delivery of the biodegradable polymer. Histological observations were also carried out, which added supports to the chemopreventive action of the pure HP and HP loaded gold nanoparticles (Au-mPEG₍₅₀₀₀₎-S-HP) against DEN induction during liver cancer progression. These findings suggest that HP loaded gold nanoparticles (Au-mPEG₍₅₀₀₀₎-S-HP) shows better efficacy than the pure HP against lipid peroxidation, hepatic cell damage and protects the antioxidant system in DEN induced hepatocellular carcinogenesis.

Keywords Hepatocellular carcinoma · Diethylnitrosamine · Hesperetin · Gold nanoparticles · Chemoprevention · Antioxidants

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the fourth leading cause of cancer death, with an estimated death rate of more than 500,000 per year [1]. Liver cancer incidence has been increasing in recent years in India. The major risk factors of HCC may be age, gender, hepatitis B and C, alcohol consumption, hormone exposure, haemochromatosis, vinyl chloride, arsenic poisoning, aflatoxin B1, obesity, diabetes, renal

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transplant patients, tobacco smoking and parasitic infections such as clonorchiasis and schistosomiasis [2] which results in liver damage such as cirrhosis [3]. Diethylnitrosamine (DEN) or N-Nitrosodiethylamine (NDEA) is a powerful environmental hepatocarcinogen that has been used as an initiating agent for hepatocarcinogenic activities. N-nitroso compounds are considered to be a tragic health hazards to man, and these compounds were present in tobacco products, cheddar cheese, cured and fried meals, occupational settings, cosmetics, agricultural chemicals and pharmaceutical agents [4]. The biotransformation of DEN resulting in oxidative stress alters the structure of DNA and forming alkyl DNA adducts [5]. It has been proven that metabolic bioactivation of cytochrome p450 enzymes to reactive electrophiles is required for initiation of their cytotoxic, mutagenic and carcinogenic activity by the help of nitrosamines with hepatocyte proliferation following cell necrosis [6], hyperplasia, hyperbasophilia and tumour. The treatment of HCC is still a big challenge in medicine. Several chemicals are known to possess chemopreventive property against a broad spectrum of cancers. Chemoprevention serves as an attractive alternative to control malignancy [7]. Several herbal drugs have been evaluated for their potential as liver protectant against DEN-induced hepatotoxicity in rats [8]. The recent approach to control liver cancer is chemoprevention, it is entirely prevented, slowed or reversed substantially by the administration of one or more nontoxic naturally occurring and/or synthetic agent, otherwise called as anticarcinogen [9].

According to the World Health Organization (WHO) primary health care is still relied upon the natural products and approximately 80 % of the residents of developing countries in the world use natural products as foods medicines. An example of the dominant role of natural products can be seen in the last 25 years, where the 77.8 % of the cancer therapeutic of approved drugs are either natural products or based on natural products, or mimics of natural products [10]. Hesperetin (HP) (3', 5, 7-Trihydroxy-4-methoxy flavanone) with molecular weight about 302.3; it is a flavonoid that exists widely in plants, fruits, flowers, foods of plant origin and abundant in citrus fruits [11]. The

drug HP is an important bioactive compound in medicinal herbs and it also has biological and pharmacological activities, such as anticarcinogenic effect, antihypertensive, anti-inflammatory, antioxidant and lipid-lowering efficacy [12]. Since many antioxidants exhibit antiplatelet and anticarcinogenic effects [13] it is possible that HP can also function in a similar way but HP is poor bioavailability and poor solubility in water and shows a slow dissolution rate from solid oral forms, thus restricting its use in therapy [14]. Most current anticancer drugs do not greatly differentiate between cancerous and normal cells, leading to systemic toxicity and adverse effects. In addition, many natural chemopreventive drugs have a poor solubility, low bioavailability and they can be quickly cleared in the body by the reticuloendothelial system and it is the major drawback associated with the failure of many natural chemopreventive agents in clinical settings. In this condition we need an effective drug delivery system with the help of various biomaterials such as biodegradable nanoparticles (NPs). To enhance the efficacy of the cancer therapeutic agent, the use of nanoparticle-based drug formulation is an important aspect of nanomedicine [15]. In particular, gold NPs possess biological activities like antioxidant, anti-inflammatory, anti-angiogenesis and anticancer properties and therefore it has been used for the delivery of drugs, proteins, peptides and oligonucleotides etc. [16].

The design of suitable molecular carriers for flavonoids is an area of research that is in rapid progress, but efficient solutions are still far from being developed and applied to humans. In our previous study we introduced a new method for effective drug delivery system to improve the drug efficacy, solubility and bioavailability with the help of nanomaterials by synthesizing gold (Au) NPs stabilized and reduced with polymer *O*-[2-(3-mercaptopropionylamino)ethyl]-*O'*-methyl polyethylene glycol (mPEG₍₅₀₀₀₎-SH). Further, it is capped with anticancer drug-HP for effective drug delivery to treat HCC in vitro [17]. From the continuation of the above study, here we aim to divulge the chemopreventive nature of HP loaded gold NPs (Au-mPEG₍₅₀₀₀₎-S-HP) and pure HP (Fig. 1) during DEN-induced liver cancer in male wistar albino rats.

Fig. 1 Structure of pure hesperetin (HP) and gold nanoparticles polymer coated hesperetin drug

Materials and Methods

Sources of Chemicals

HP and DEN were purchased from Sigma chemical Ltd. (USA). All other chemicals and reagents used were of analytical grade.

Animals

Male, wistar strain albino rats weighing about 150–180 g were obtained from Tamil Nadu Veterinary and Animal Science University (TANUVAS), Madhavaram, Chennai, India. The animals were housed in cages under proper environmental conditions and were fed with a commercial pelleted diet (M/S Hindustan foods Ltd, Bangalore, India). The animals had free access to water. All the experiments were designed and conducted according to the ethical norms approved by Institutional Animal Ethics Committee guidelines (IAEC No: 12/02/2012).

Set 1: Dose Fixation Studies

The suitable optimum dosage schedule for Au-mPEG₍₅₀₀₀₎-S-HP NPs was identified by administering the drug at different dosage (0.1, 0.3, 0.5, 1 and 1.5 ml of nanoparticles) at different time intervals (viz; 0, 7, 14, and 21 days). At the end of the experimental period the animals were sacrificed and the serum was used to assess the biochemical changes. The dosage and the duration of dosage, which gave the maximum protective efficacy (as elicited by the activities of marker enzymes in the serum) was fixed as the optimum dosage for the Au-mPEG₍₅₀₀₀₎-S-HP NPs as shown in Fig. 2a, b as compared with the DEN induced animal. Based on this dosage fixation assessment, the optimum dosage for Au-mPEG₍₅₀₀₀₎-S-HP NPs was found to be 0.5 ml via intraperitoneally twice a week. It shows the half the amount of pure HP dose.

Set 2: Toxicity Study of Pure Hesperetin (HP) and Au-mPEG₍₅₀₀₀₎-S-HP Nanoparticles

After fixation of optimum dosage, the toxicity of pure HP and Au-mPEG₍₅₀₀₀₎-S-HP NPs was analysed in rats by giving 20 mg/kg b.wt of pure HP and 0.5 ml of Au-mPEG₍₅₀₀₀₎-S-HP NPs were given by intraperitoneally for 21 days, the rats were sacrificed by cervical decapitation and the tissues of liver samples and kidney samples were investigated by histopathological examination as shown in Fig. 3.

Fig. 2 **a** Serum aspartate transaminase activity in experimental groups of rats treated with different doses of Au-mPEG₍₅₀₀₀₎-S-HP nanoparticles for different time intervals. Results are expressed as mean $\pm$ S.D for six rats in each group. Statistical significance at $P < 0.05$ compared with ^a0.1 ml, ^b0.3 ml, ^c0.5 ml, ^d1 ml, ^e1.5 ml and ^{NS} Non significant compared with 0.1 ml. Units are expressed as μ moles of pyruvate liberated/min/mg of protein. **b** Serum alanine transaminase activity in experimental groups of rats treated with different doses of Au-mPEG₍₅₀₀₀₎-S-HP nanoparticles for different time intervals. Results are expressed as mean $\pm$ S.D for six rats in each group. Statistical significance at $P < 0.05$ compared with ^a0.1 ml, ^b0.3 ml, ^c0.5 ml, ^d1 ml, ^e1.5 ml and ^{NS} Non significant compared with 0.1 ml. Units are expressed as μ moles of pyruvate liberated/min/mg of protein

Experimental Design

- Group 1: Normal control animals fed with standard diet and pure drinking for 16 weeks.
- Group 2: Animals were administered with DEN (0.01 %) in drinking water for 16 weeks to induce liver cancer.
- Group 3: Animals were treated with HP alone by intraperitoneally; twice a week at a dose of 20 mg/kg b.wt (based on effective dose fixation Studies) for 16 weeks to study the toxicity (if any) induced by HP.
- Group 4: Animals were treated with pure HP (20 mg/kg b.wt by intraperitoneally) twice a week for one week prior to the first dose of 0.01 % DEN administration and continued for 15 weeks.
- Group 5: Animals were treated with 0.5 ml of Au-mPEG₍₅₀₀₀₎-S-HP NPs (i.e. 1.5 mg/0.5 ml) intraperitoneally twice a week for 16 weeks to study the cytotoxicity (if any) induced by gold nanoparticles.

Fig. 3 Histological alterations in the liver and kidney at 21 days after administration of pure HP and Au-mPEG₍₅₀₀₀₎-S-HP nanoparticles (H&E staining, $\times 40$). Both **a** photomicrograph of liver and kidney section of control rat demonstrated normal cellular structure. Both

b sections of liver and kidney of pure HP drug at 20 mg/kg b.wt show normal cellular architecture and both **c** section of liver and kidney of Au-mPEG₍₅₀₀₀₎-S-HP nanoparticles drug at 0.5 ml depicted normal cellular architecture

Group 6: Animals received 0.01 % DEN along with 0.5 ml of Au-mPEG₍₅₀₀₀₎-S-HP NPs (i.e. 1.5 mg/0.5 ml) intraperitoneally for twice a week. Au-mPEG₍₅₀₀₀₎-S-HP NPs was started one week prior to the first dose of DEN administration and continued for 16 weeks.

After the experimental period, the animals were fasted overnight, anaesthetized with diethyl ether and then sacrificed by cervical decapitation.

Evaluation of Biochemical Estimations

After the cervical decapitation, the blood samples were collected from the experimental animals and liver tissue was removed and washed in ice-cold saline. The total protein in the liver was estimated by the method of Lowry et al. Quantitative estimation of tumor markers of carcinoembryonic antigen (CEA) and α -feto protein (AFP) were carried out by solid phase enzyme linked immunosorbent assay (ELISA) using UBI MAGICWELL (USA) enzyme

immune assay kit. The levels of lipid peroxidation (LPO) products were estimated in serum and liver tissue homogenate [18]. The level of lipid hydroperoxides (OH) in liver homogenate was assayed by the method of Jiang et al. [19]. The activities of marker enzymes such as Aspartate transaminase (AST), alanine transaminase (ALT), Alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and γ -glutamyl transpeptidase (γ GT), were assayed in the serum [20, 21]. The activities of antioxidant enzymes-superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione (GSH) were estimated in the liver tissue homogenate [22–24]. The level of membrane bound ATPase like total ATPase [25], Na^+/K^+ ATPase [26], Mg^{2+} ATPase [27], Ca^{2+} ATPase [28] were assayed. The Activities of mitochondrial enzymes such as isocitrate dehydrogenase [29], α -ketoglutarate dehydrogenase [30], succinate dehydrogenase [31], malate dehydrogenase [32], NADH-dehydrogenase [33] and cytochrome C-oxidase [34] were assessed according to the published papers.

Liver Histopathology

Histological evaluation was performed on a portion of liver specimen fixed in 10 % formalin and embedded in paraffin wax. Sections were cut at 4 μ m in thickness, stained with hematoxylin and eosin and viewed under light microscope for histological changes.

Statistical Analysis

All the grouped data were significantly evaluated with SPSS/10 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. P values of less than 0.05 were considered to indicate statistical significance. All these results were expressed as mean $\pm$ S.D for six animals in each group.

Results

General Observations

During this study no evidence of mortality was observed and all the animals survived during the entire period of the experiment. The rats tolerated quite well the intraperitoneal injection of pure HP and Au-mPEG₍₅₀₀₀₎-S-HP NPs treatment. No clinical signs of toxicity were present in any group in this study. The morphological study and biochemical estimation was carried out in liver and kidney. There was no change in morphology and no change in concentration was observed in marker enzymes and in antioxidants. Similarly histopathological study of liver and kidney tissues of Fig. 3 shows no cellular architectural changes were observed in (a) control, (b) pure HP and (c) Au-mPEG₍₅₀₀₀₎-S-HP NPs. Both liver tissue and kidney tissue have similar architecture and these clearly concluded that no toxicity was observed in pure HP and Au-mPEG₍₅₀₀₀₎-S-HP NPs.

Figure 4 depicts the body weight of control and experimental group of animals. In DEN induced group 2 animals there is a significant decrease in the final body weight when compared with group 1 animals. The HP treated and Au-mPEG₍₅₀₀₀₎-S-HP treated groups 4 and 6 showed a significant increase when compared with group 2 animals. Here the all animals show better tolerance to treatment with Au-mPEG₍₅₀₀₀₎-S-HP when compared with pure HP. Figure 5 shows the liver weight and relative liver weight of control and experimental group of animals. In group 2 animals, the relative liver weight was significantly increased when compared with group 1 animals and there was a significant decrease in the liver weight in group 4 and group 6 animals when compared with group 2 animals. No

Fig. 4 Body weight changes in control and experimental groups of rats. Results are expressed as mean $\pm$ S.D for six rats in each group. Statistical significance at $P < 0.05$ compared with ^agroup 1, ^bgroup 2, and ^cgroup 4. Body weight change is expressed in grams

Fig. 5 Liver weights and relative liver weight of control and experimental groups of rats. Results are expressed as mean $\pm$ S.D for six rats in each group. Statistical significance at $P < 0.05$ compared with ^agroup 1, ^bgroup 2, and ^cgroup 4. Liver weight is expressed in grams. Relative liver weight is the average of liver weight at final body weight multiplied by 100

physical observation was observed in Au-mPEG₍₅₀₀₀₎-S-HP alone treated groups which is an indicative of nontoxic nature of gold encapsulated HP NPs.

Effect of Pure Hesperetin (HP) and Au-mPEG₍₅₀₀₀₎-S-HP Nanoparticles on Macroscopic Morphology of Liver

The normal macroscopic appearance of liver morphology was seen in control group 1 animals (Fig. 6a). Group 2 DEN induced animals shows heavy enlargement and covered with several grey-white nodules in various sizes and foci on the peripheral surface of the liver (Fig. 6b). Liver cells shows normal morphology in pure HP alone treated group 3 animals (Fig. 6c). Significant reduction in liver enlargement, nodule incidence and average nodule numbers per nodule-bearing liver was observed in HP 20 mg/kg b.wt+DEN treated rats showing the effect of chemoprevention in group 4 animals (Fig. 6d). Liver cells showing normal morphology in Au-mPEG₍₅₀₀₀₎-S-HP, indicating

Fig. 6 Macroscopic gross appearance of liver tumor and nodule phenotypes in DEN induced rats and the chemopreventive effect of HP and Au-mPEG₍₅₀₀₀₎-S-HP nanoparticles at week 16. Representative livers are taken from several groups: **a** liver of a control animal showing normal morphology (Group 1); **b** DEN-induced group showing enlarged liver and maximum number of nodules and the liver is covered by large number of grey-white nodules in various size at the same time there were clear necrosis regions in liver as the features of HCC (Group 2); **c** liver showing normal morphology in hesperetin (20 mg/kg b.wt) alone treated group of rats (Group 3); **d** significant

reduction in liver enlargement, nodule incidence and average nodule numbers per nodule-bearing liver was observed in hesperetin 20 mg/kg b.wt+DEN treated rats showing the effect of chemoprevention (Group 4); **e** liver showing normal morphology in hesperetin loaded gold nanoparticles, this clearly showing no cytotoxicity were seen in Au-mPEG₍₅₀₀₀₎-S-HP nanoparticles (Group 5); **f** most of the nodules disappeared and more reduction in liver enlargement was observed in Au-mPEG₍₅₀₀₀₎-S-HP nanoparticles + DEN treated rats showing much more effect of chemoprevention and targeted drug delivery (Group 6)

that there is no cytotoxicity were seen in gold NPs encapsulated HP in group 5 animals (Fig. 6e). Most of the nodules disappeared and more reduction in liver enlargement was observed in Au-mPEG₍₅₀₀₀₎-S-HP+DEN treated rats showing much more effect of chemoprevention and targeted delivery in group 6 animals (Fig. 6f).

Incidence and Multiplicity of Hepatocellular Nodules

The incidence and multiplicity of hepatocellular total number of nodules and number of nodules per nodule-bearing liver and nodular sizes in millimetre in tumor-bearing animals is shown in Table 1, respectively. The HP treated and Au-mPEG₍₅₀₀₀₎-S-HP NPs treated groups 4 and 6 showed a significant decrease in the number of nodules when compared with group 2 animals. No nodules were observed in group 3 and group 5 animals. This

clearly pointed that the gold NPs is biocompatible and it proves the anti-carcinogenic effect of HP encapsulated gold NPs.

Effect of Pure Hesperetin (HP) and Au-mPEG₍₅₀₀₀₎-S-HP Nanoparticles of CEA (Carcinoembryonic Antigen) and AFP (α -Feto Protein)

The Fig. 7a, b depicts the levels of tumor markers CEA and AFP in the serum of control and experimental groups of animals. CEA and AFP were found to be significantly increased in DEN induced group 2 liver cancer bearing animals when compared with control group 1 animals. Whereas their levels were significantly decreased in pure HP and Au-mPEG₍₅₀₀₀₎-S-HP treated groups 4 and 6 animals. Which reveals the anti-tumor effect of HP loaded gold NPs.

Table 1 Effect of hesperetin loaded gold nanoparticles (Au-mPEG₍₅₀₀₀₎-S-HP) and pure hesperetin (HP) on number and size of hepatocellular nodules during DEN induced hepatocarcinogenesis

Groups	No of rats examined (n)	Total number of nodules	Average number of nodules/nodule bearing liver	Relative size (% of total number)			
				<1 mm	>1 mm <3 mm	>3 mm	
Group 1 (Control)	6	0	0	0	0	0	
Group 2 (DEN)	6	108	18 ± 1.85 ^a	54 (50.0)	33 (30.6)	21 (19.4)	
Group 3 (HP)	6	0	0	0	0	0	
Group 4 DEN+HP	6	88	14.66 ± 1.74 ^b	46 (52.2)	25 (37.5)	17 (10.3)	
Group 5 (Au-mPEG ₍₅₀₀₀₎ -S-HP)	6	0	0	0	0	0	
Group 6 DEN+Au-mPEG ₍₅₀₀₀₎ -S-HP	6	30	5.83 ± 1.19 ^{b,c}	15 (51.0)	9 (34.1)	6 (14.9)	

Results are expressed as mean ± S.D for six rats in each group. Statistical significance at $P < 0.05$ compared with ^agroup 1, ^bgroup 2, and ^cgroup 4

Fig. 7 a and b Effect of hesperetin loaded gold nanoparticles (Au-mPEG₍₅₀₀₀₎-S-HP) and pure hesperetin (HP) on the level of tumor markers in the serum of control and experimental groups of rats. Results are expressed as mean ± S.D for six rats in each group.

Statistical significance at $P < 0.05$ compared with ^agroup 1, ^bgroup 2, and ^cgroup 4. Units are expressed as ng/ml for CEA and ng/ml for AFP

Effect of Pure Hesperetin (HP) and Au-mPEG₍₅₀₀₀₎-S-HP Nanoparticles of Lipid Peroxidation (LPO) and Lipid Hydroperoxides (OH)

The Fig. 8 shows the levels of LPO in the serum and liver and Fig. 9 shows the levels of OH in the liver of control and experimental groups of animals. In DEN induced group 2 animals, there was a significant increase in the levels of lipid peroxides and hydroperoxides when compared with group 1 normal control animals. Whereas in pure HP and Au-mPEG₍₅₀₀₀₎-S-HP treated groups 4 and 6 animals, there is a significant decrease in the level of lipid peroxides and hydroperoxides when compared with group 2 tumor bearing animals.

Effect of Pure hesperetin (HP) and Au-mPEG₍₅₀₀₀₎-S-HP Nanoparticles on the Activities of Marker Enzymes

Table 2 represents the effect of HP and Au-mPEG₍₅₀₀₀₎-S-HP NPs on the activities of marker enzymes in the serum of

Fig. 8 Effect of Au-mPEG₍₅₀₀₀₎-S-HP nanoparticles and pure hesperetin on the levels of lipid peroxides in the serum and liver of control and experimental groups of rats. Results are expressed as mean ± S.D for six rats in each group. Statistical significance at $P < 0.05$ compared with ^agroup 1, ^bgroup 2, and ^cgroup 4. LPO levels are expressed as nmol of MDA formed/min/mg protein

control and experimental group of rats. The activities of AST, ALT, ALP, LDH and γ GT are significantly ($P < 0.05$) increased in DEN induced group 2 animals when compared with group 1 normal control animals. HP

Fig. 9 Effect of hesperetin loaded gold nanoparticles (Au-mPEG₍₅₀₀₀₎-S-HP) and pure hesperetin (HP) on the level of OHs in the liver of control and experimental groups of rats. Results are expressed as mean $\pm$ S.D for six rats in each group. Statistical significance at $P < 0.05$ compared with ^agroup 1, ^bgroup 2, and ^cgroup 4. Units are expressed as nmoles of OH/mg of protein for OH

and Au-mPEG₍₅₀₀₀₎-S-HP NPs treated group 4 and 6 animals showed a significant decrease in the activities of these enzymes when compared with group 2 animals.

Evaluation of Antioxidant Status in Liver

Table 3 portrays the effect of antioxidant status in the liver of control and experimental group of animals. The activities of SOD, CAT, GSH, GPx and GR, in the DEN-induced group 2 animals were significantly decreased ($P < 0.05$) as compared to the normal control group 1 animals. Where as

in HP (HP) and Au-mPEG₍₅₀₀₀₎-S-HP NPs treated group 4 and 6 showed a significant increase in the activities of SOD, CAT, GSH, GPx and GR when compared with group 2 animals.

Effect of Au-mPEG₍₅₀₀₀₎-S-HP Nanoparticles on ATPase

Table 4 represents the activities of ATPase in liver tissues of control and experimental group of animals. Significant decrease ($P < 0.05$) in the activities of total ATPase, Na⁺/K⁺ ATPase, Mg²⁺ ATPase and Ca²⁺ ATPase activity were seen in cancer bearing group 2 animals when compared with group 1 normal control animals. The above alterations were significantly ($P < 0.05$) normalized more in Au-mPEG₍₅₀₀₀₎-S-HP NPs than pure HP when compared with group 2 animals. There was no significant difference in the activities of ATPase between hesperetin alone and gold NPs alone treated group 3 and group 5 animals.

Assessment of Mitochondrial Enzymes and Respiratory Chain Enzymes Status

Table 5 depicts the activities of mitochondrial TCA cycle enzymes and respiratory chain enzymes in liver of control and experimental groups of rats. The activities of TCA

Table 2 Effect of hesperetin loaded gold nanoparticles (Au-mPEG₍₅₀₀₀₎-S-HP) and pure hesperetin (HP) on the activities of marker enzymes in the serum of control and experimental groups of rats

Groups	AST	ALT	ALP	LDH	γ GT
Group 1 (Control)	116.12 $\pm$ 9.17	38.01 $\pm$ 3.57	36.11 $\pm$ 3.19	81.02 $\pm$ 4.62	7.21 $\pm$ 0.62
Group 2 (DEN)	248.10 $\pm$ 18.8 ^a	86.33 $\pm$ 6.01 ^a	75.39 $\pm$ 4.62 ^a	124.35 $\pm$ 6.83 ^a	12.43 $\pm$ 1.66 ^a
Group 3 (HP)	116.15 $\pm$ 9.39	38.04 $\pm$ 3.66	36.14 $\pm$ 3.22	81.04 $\pm$ 4.62	7.24 $\pm$ 0.67
Group 4 DEN+HP	189.22 $\pm$ 9.46 ^b	68.32 $\pm$ 5.62 ^b	58.98 $\pm$ 5.68 ^b	105.62 $\pm$ 5.92 ^b	10.78 $\pm$ 0.53 ^b
Group 5 (Au-mPEG ₍₅₀₀₀₎ -S-HP)	116.13 $\pm$ 9.23	38.02 $\pm$ 3.60	36.13 $\pm$ 3.22	81.03 $\pm$ 4.60	7.22 $\pm$ 0.62
Group 6 DEN+Au-mPEG ₍₅₀₀₀₎ -S-HP	140.73 $\pm$ 9.06 ^{b,c}	46.81 $\pm$ 4.02 ^{b,c}	40.82 $\pm$ 3.86 ^{b,c}	88.92 $\pm$ 4.90 ^{b,c}	9.23 $\pm$ 0.48 ^{b,c}

Results are expressed as mean $\pm$ S.D for six rats in each group. Statistical significance at $P < 0.05$ compared with ^agroup 1, ^bgroup 2, and ^cgroup 4. Activity is expressed as IU/L for AST, ALT, ALP, LDH, and γ GT

Table 3 Effect of hesperetin loaded gold nanoparticles (Au-mPEG₍₅₀₀₀₎-S-HP) and pure hesperetin (HP) on liver antioxidant status in control and experimental groups of rats

Groups	SOD	CAT	GSH	GPx	GR
Group 1 (Control)	9.73 $\pm$ 0.71	73.01 $\pm$ 5.02	46.27 $\pm$ 3.39	104.22 $\pm$ 6.09	168.0 $\pm$ 9.01
Group 2 (DEN)	4.99 $\pm$ 0.47 ^a	43.11 $\pm$ 4.01 ^a	13.72 $\pm$ 1.99 ^a	60.22 $\pm$ 5.78 ^a	104.77 $\pm$ 6.22 ^a
Group 3 (HP)	9.75 $\pm$ 0.72	73.03 $\pm$ 5.03	46.29 $\pm$ 3.40	104.27 $\pm$ 6.10	168.04 $\pm$ 9.07
Group 4 DEN+HP	5.71 $\pm$ 0.42 ^b	50.0 $\pm$ 3.72 ^b	27.28 $\pm$ 2.91 ^b	72.39 $\pm$ 4.53 ^b	122.52 $\pm$ 7.32 ^b
Group 5 (Au-mPEG ₍₅₀₀₀₎ -S-HP)	9.74 $\pm$ 0.71	73.01 $\pm$ 5.02	46.28 $\pm$ 3.39	104.23 $\pm$ 6.09	168.01 $\pm$ 9.05
Group 6 DEN+Au-mPEG ₍₅₀₀₀₎ -S-HP	7.35 $\pm$ 0.44 ^{b,c}	63.79 $\pm$ 3.39 ^{b,c}	35.12 $\pm$ 2.72 ^{b,c}	91.79 $\pm$ 5.11 ^{b,c}	145.39 $\pm$ 7.20 ^{b,c}

Results are expressed as mean $\pm$ S.D for six rats in each group. Statistical significance $P < 0.05$ compared with ^agroup 1, ^bgroup 2, and ^cgroup 4. Units: SOD superoxide dismutase in units/mg protein, CAT catalase in μ mol of H₂O₂ decomposed/min/mg protein, GPx glutathione peroxidase in μ mol of GSH utilized/min/mg protein, GR glutathione reductase in μ mol of NADPH oxidized/min/mg protein, GSH glutathione in μ g/mg protein

Table 4 Effect of hesperetin loaded gold nanoparticles (Au-mPEG₍₅₀₀₀₎-S-HP) and pure hesperetin (HP) on the activities of membrane bound ATPases in the liver of control and experimental groups of rats

Groups	Total ATPase	Na ⁺ K ⁺ ATPase	Mg ²⁺ ATPase	Ca ²⁺ ATPase
Group 1 (Control)	3.89 ± 0.29	1.77 ± 0.11	2.51 ± 0.19	1.59 ± 0.15
Group 2 (DEN)	1.95 ± 0.15 ^a	1.08 ± 0.05 ^a	1.95 ± 0.09 ^a	1.03 ± 0.05 ^a
Group 3 (HP)	3.91 ± 0.29	1.76 ± 0.13	2.50 ± 0.18	1.58 ± 0.14
Group 4 DEN+HP	2.29 ± 0.20 ^b	1.46 ± 0.07 ^b	2.12 ± 0.12 ^b	1.29 ± 0.09 ^b
Group 5 (Au-mPEG ₍₅₀₀₀₎ -S-HP)	3.91 ± 0.28	1.77 ± 0.12	2.50 ± 0.19	1.58 ± 0.15
Group 6 DEN+Au-mPEG ₍₅₀₀₀₎ -S-HP	3.09 ± 0.23 ^{bc}	1.61 ± 0.09 ^{bc}	2.38 ± 0.16 ^{bc}	1.41 ± 0.12 ^{bc}

Results are expressed as mean ± S.D for six rats in each group. Statistical significance at $P < 0.05$ compared with ^agroup 1, ^bgroup 2, and ^cgroup 4. Units are expressed as μ moles of phosphorous liberated/min/mg protein for total ATPase, Na⁺K⁺ ATPase, Mg²⁺ ATPase and Ca²⁺ ATPase

Table 5 Effect of hesperetin loaded gold nanoparticles (Au-mPEG₍₅₀₀₀₎-S-HP) and pure hesperetin (HP) on the activities of mitochondrial enzymes in the liver of control and experimental groups of rats

Particulars	ICDH	α -KGDH	SDH	MDH	NADH dehydrogenase	Cytochrome C-oxidase
Group 1 (Control)	820.32 ± 75.1	91.33 ± 6.11	22.54 ± 1.55	346.66 ± 33.21	30.73 ± 2.77	5.66 ± 0.41
Group 2 (DEN)	512.26 ± 45.23 ^a	51.61 ± 5.1 ^a	10.72 ± 1.03 ^a	210.76 ± 18.91 ^a	12.91 ± 1.47 ^a	2.88 ± 0.19 ^a
Group 3 (HP)	820.41 ± 75.18	91.22 ± 5.92	22.66 ± 1.61	346.22 ± 32.76	31.41 ± 2.91	5.58 ± 0.39
Group 4 DEN+HP	631.98 ± 57.43 ^b	65.89 ± 4.77 ^b	15.29 ± 1.39 ^b	255.41 ± 28.06 ^b	19.22 ± 1.77 ^b	3.91 ± 0.44 ^b
Group 5 (Au-mPEG ₍₅₀₀₀₎ -S-HP)	821.23 ± 75.81	91.28 ± 6.09	22.36 ± 1.46	345.81 ± 33.31	31.60 ± 2.56	5.61 ± 0.36
Group 6 DEN+Au-mPEG ₍₅₀₀₀₎ -S-HP	749.43 ± 68.24 ^{bc}	81.87 ± 5.19 ^{bc}	19.28 ± 1.66 ^{bc}	310.13 ± 28.99 ^{bc}	25.65 ± 2.31 ^{bc}	4.63 ± 0.31 ^{bc}

Results are expressed as mean ± S.D for six rats in each group. Statistical significance at $P < 0.05$ compared with with ^agroup 1, ^bgroup 2, and ^cgroup 4. Units: ICDH, nmoles of α -ketoglutarate liberated/min/mg protein; α -KGDH, nmoles of ferrocyanide liberated/min/mg protein; SDH, nmoles of succinate oxidized/min/mg protein; MDH, nmoles of NADH oxidized/min/mg protein; NADH dehydrogenase, nmoles of NADH oxidized/min/mg protein; cytochrome C oxidase, nmoles of cytochrome C oxidized/min/mg protein

cycle enzymes (ICDH, α -KGDH, SDH and MDH) and respiratory chain enzymes (NADH dehydrogenase and Cytochrome c-oxidase) were significantly decreased ($P < 0.05$) in DEN-induced group 2 animals when compared to group 1 normal control animals. This could be because of oxidative stress due to DEN induction leads to imbalance between oxidant production and the antioxidant capacity of the cell. Where as in Au-mPEG₍₅₀₀₀₎-S-HP NPs and HP administration the activities of these mitochondrial enzymes were markedly ($P < 0.05$) sustained near normal status in group 6 and group 4 animals when compared to DEN-induced group 2 animals.

Histopathological Examination

In the histopathological examination of liver tissue at week 16, the control group 1 animal's revealed normal lobular architecture. Hepatocytes were polyhedral in shape and their cytoplasm was granulated with small uniform nuclei (Fig. 10a). Liver sections from group 2 animals (Fig. 10b) revealed loss of normal architecture and irregular shaped hepatocytes with fatty changes, both hepatic and portal with significant tumor thrombi within portal vessels and moreover

presence of hyperchromatic with numerous mitotic figures. The cytological patterns were varied and the necrotic cells were small with basophilic nuclei and dark cytoplasm this shows the features of HCC. Group 3 animals showed normal architecture indicating the non-toxic nature of HP (Fig. 10c). In group 4 animals treated with pure HP showed few neoplastically transformed cells and a mild degeneration of hepatocytes maintaining near normal architecture (Fig. 10d). Group 5 animals showed normal architecture and uniform nuclei which shows the non-toxic and the biocompatibility of control Au-mPEG₍₅₀₀₀₎-S-HP NPs (Fig. 10e). Where as in group 6 animals (Fig. 10f) treated with Au-mPEG₍₅₀₀₀₎-S-HP NPs showed near liver architecture and few neoplastically transformed cells, with no evidence of necrosis or cirrhosis. This reveals that HP encapsulated gold NPs have better bioavailability and therapeutic effects.

Discussion

Hesperedin is a flavanone glycoside; its glycone form is called HP. It has been recognized to have stronger activity than hesperedin [35]. Its shows various clinical

Fig. 10 Representative photographs for the histopathological examination of liver tissue in control and experimental groups of rats at 16 weeks. **a** control group 1 animals revealed normal liver architecture ($\times 40$, HE). **b** Group 2 DEN alone slide showing loss of abnormal architecture, and irregular shaped hepatocytes with fatty changes, both hepatic and portal with significant tumor thrombi within portal vessels and moreover presence of hyperchromatic with numerous mitotic Figs. ($\times 40$, HE). **c** Group 3 hesperetin alone slide showing the

normal liver architecture ($\times 40$, HE). **d** Group 4 hesperetin treated slide showing loss of architecture, and comparatively less tendency to spread by intrahepatic veins, both hepatic and portal vessels ($\times 40$, HE). **e** Group 5 gold nanoparticles conjugated hesperetin alone slide shows the normal liver architecture ($\times 40$, HE). **f** Group 6 gold nanoparticles conjugated hesperetin treated slide showing few neoplastically transformed cells and the image highlights the hepatocytes maintaining near normal architecture ($\times 40$, HE)

implications for example, HP possess vitamin-like activity and can decrease capillary permeability (vitamin P) leakiness and fragility. HP shows a wide spectrum of pharmacological effects such as antioxidant, anti-inflammatory, anticarcinogenic, anti-inflammatory, antimutagenic, anti-hypertensive and anti allergic properties [36]. HP is reported to be a powerful radical scavenger and a promoter of cellular antioxidant defense-related enzyme activities [35]. This drug was already reported that it shows anticarcinogenic effects caused in cancers (colon, breast,) which is strongly supported by an in vitro and in vivo studies [37]. Numerous experimental models have been developed to define the pathogenesis of HCC and to test novel drug candidates but still the death of HCC is leading in world wide because of the drug having poor solubility, bioavailability, biocompatibility, limited chemical stability and loss of target drug delivery [38]. HP has the similar problem of poor bioavailability and solubility in water and thus restricting its use in chemotherapy.

The present study investigated more solubility and protective chemopreventive effects of HP conjugated gold

NPs during DEN induced liver cancer in male wistar albino rats. During the period of study the animals did not show any significant changes in the behaviour indicating that there was no toxic effect due to administration of pure HP and Au-mPEG₍₅₀₀₀₎-S-HP. In our preparation the size of Au-mPEG₍₅₀₀₀₎-S-HP NPs is 110 nm diameter so it does not cause any oxidative stress in animals and more over the dose of Au-mPEG₍₅₀₀₀₎-S-HP nanoparticles is half the dose of pure HP because of the NPs having the maximum solubility, sustained drug release at the target site. The toxicity effect of pure HP and Au-mPEG₍₅₀₀₀₎-S-HP nanoparticles alone treatment showed no side effects, and does not disrupt the normal cellular architecture of liver and kidney in histopathological examination, suggesting that Au-mPEG₍₅₀₀₀₎-S-HP NPs is non-toxic and shows the gold nanoparticles is biocompatible. The histopathological patterns of liver damage observed in animals treated with DEN is found to be more pronounced than normal animal tissue, indicating HCC during DEN administration. In contrast to this, liver section of animals treated with Au-mPEG₍₅₀₀₀₎-S-HP nanoparticles show much improved

hepatocellular architecture than pure HP. Signs of recovery, indicating the protective effect of gold nano particles.

Tumor markers are molecules that can be detected in higher or lower than normal amounts in the blood, urine, or body tissues of patients with certain types of cancer. CEA is a protein found in many types of cells but associated with tumors and developing fetus. Elevated CEA levels are found in serum of patients having various cancers such as breast, lung, pancreas, stomach, liver and ovary [39]. Elevation of serum AFP is higher in HCC compared to other liver diseases. AFP along with CEA is most extensively used in the diagnosis of HCC [40]. In this present study, increased levels of CEA and AFP confirms the incidence of HCC in DEN induced group 2 animals and Au-mPEG₍₅₀₀₀₎-S-HP NPs reduced the level of CEA and AFP, which revealed the antitumor effect of Au-mPEG₍₅₀₀₀₎-S-HP NPs against DEN-induced HCC. This indicates a positive prognosis.

Oxidative stress is usually implicated during all the stages of cancer development as well as in the genesis of other diseases. Reactive oxygen species (ROS) is formed from endogenous or exogenous sources and are highly reactive, toxic, and mutagenic. DEN have the potential to generate free radicals and these free radicals generation in the liver overwhelms the antioxidant status and ultimately proceeds to oxidative stress paving way to carcinogenesis [41]. The LPO and OH was significantly enhanced in the liver of DEN treated animals, which could be attributed to DEN induced oxidative stress. This oxidative stress may be the reason for the elevated LPO level in the liver of DEN treated animals. In line with this findings the animals treated with HP (pure HP and Au-mPEG₍₅₀₀₀₎-S-HP NPs) significantly lowered the levels of LPO both in serum and liver, when compared with animals induced with DEN. The Au-mPEG₍₅₀₀₀₎-S-HP nanoparticles treated drug shows much lower level of LPO compared with treated HP drug. This shows the antilipid peroxidative role of HP that is probably mediated by its ability to scavenge free radical generation.

During carcinogenesis, some enzymes can be used as a biochemical indicator of tumor response to therapy. Intracellular enzymes, such as AST, ALT, ALP, LDH, and γ GT are useful indicators for liver function and their increased levels are indicators of liver damage. AST and ALT are reliable marker enzymes of liver and they are the first enzymes to be used in diagnostic enzymology when liver damage has occurred [42] and these enzymes are remarkable increase in HCC and liver damage. The result showed that the increase in the AST, ALT activities in DEN induced animals serves as an index of hepatotoxicity and carcinogenesis with the development of preneoplastic changes, thus shows the advanced stage of liver carcinoma. The treatment with Au-mPEG₍₅₀₀₀₎-S-HP nanoparticles

decrease the activities of these enzymes and suggest that the HP encapsulated gold nanoparticles should be a hepatoprotective effect. LDH is a familiar sensitive marker of solid neoplasm and many studies revealed increased LDH activity in various types of tumor [43]. γ GT is an enzyme embedded in the hepatocyte plasma membrane, mainly in the canalicular domain; again the liberation of this enzyme into serum indicates damage to the cell and thus injury to the liver. It is pointing out that serum γ GT activity is considered to be one of the best indicators of liver damage [44]. γ GT has been shown to play an important role in the metabolism of foreign substances and also during cell growth and differentiation [45] and it is over expressed in tumor cells [46]. This elevation reflects the progress of carcinogenesis, since its activity correlates with tumor growth rate, differentiation and survival of the host. In concurrent with above findings there is an increase in the levels of LDH and γ GT in the serum and liver of animals bearing HCC. This elevation indicates the basis tumor burden, and treatment with Au-mPEG₍₅₀₀₀₎-S-HP NPs decreases the elevation of the level of this enzyme.

Antioxidants possess a variety of biological activities, including the induction of drug metabolizing enzymes, inhibition of prostaglandin synthesis, inhibition of carcinogen-induced mutagenesis, and scavenging of free radicals [47]. The powerful antioxidant defense mechanism of our body plays a crucial role against the toxic effects of ROSs. Among them, SOD and CAT are important antioxidant enzymes. These antioxidants are linked to the availability to develop the life threatening disease like cancer [48]. Exposure to carcinogens or tumor promoters usually decreases the activities of SOD and CAT [49]. Glutathione (GSH) is a major non-enzymatic antioxidant, which functions as the second line of defense against free radical damage in the body. GSH donates an electron during the peroxide reduction catalysed by GPx, which is a component of the enzyme system containing GSH oxidase and reductase. The dysfunction of GSH and its related antioxidant enzymes such as GPx and GR is reported to be involved in the initiation of cancer [50]. In the presence of xenobiotics GST and GPx generally help in detoxification by conjugating GSH with toxic electrophiles conferring a selective growth advantage to cancer cells. Induction of these enzymes has been become essential determining the potency of many anti-carcinogenic substances. Decrease in the activities of SOD, CAT, GPx, GR, and GSH are seen in tumor cells. The compounds that can scavenge excessive free radicals in the body are suggested to hinder the process of carcinogenesis [51]. In this study, we found that the DEN induced animals shows significant decrease in the activities of antioxidant enzymes in both serum and liver of animals treated with carcinogen when compared with normal animals. On the other hand, Au-mPEG₍₅₀₀₀₎-S-HP

NPs supplementation significantly increased in the activities of antioxidant enzymes in both serum and liver of the animals when compared with animals administered carcinogen alone which may be due to the better anticancer and antioxidant properties of HP conjugated gold NPs.

Na^+/K^+ ATPase and Mg^{2+} ATPase are membrane bound enzymes responsible for transport of ions by utilizing ATP [52]. Active transport of Na^+/K^+ is directly depends on the active calcium transport and resultant low calcium concentration. Mg^{2+} is one of the intracellular ions and has a key role in the intermediately metabolism. Inhibition the activity of these ATPase leads to impairment in intracellular signalling and functions. Calcium is a second messenger and important regulator for proliferation, apoptosis and progression. During chemically induced malignancy the activities of Ca^{2+} ATPase decreases which confirms the calcium concentration increased inside the cells. Au-mPEG₍₅₀₀₀₎-S-HP NPs treatment significantly reverses the activities of Na^+/K^+ ATPase and Mg^{2+} ATPase and also restores the Ca^{2+} ATPase to near normal in liver tissues. This result reported that HP conjugated gold NPs involved in protecting the ion pump ATPases and restoring the membrane integrity thus exhibits much more antitumor effect against DEN induced HCC.

Mitochondria are dynamic intracellular organelles that play a central role in oxidative metabolism and apoptosis for generating adenosine triphosphate (ATP) as they are the power house of the cells. ICDH and α -KGDH are the major regulators for the controlling flux in entire TCA cycle. During tumor proliferation impairments in functioning of mitochondrial enzymes such as ICDH, α -KGDH, SDH and MDH leads to alterations causes defection in several steps of TCA cycle [53]. Interestingly analysis the activities of mitochondrial enzymes shows sensitive targets in different pathological status. In this investigations Au-mPEG₍₅₀₀₀₎-S-HP NPs administration to DEN induced liver cancer animals significantly much more increase the activities of TCA cycle enzymes than the pure HP and thereby reveals its effective role on mitochondrial energy production and also Au-mPEG₍₅₀₀₀₎-S-HP NPs exhibits protective role on initiation phases of carcinogenesis.

In conclusion many current studies largely concentrate on only a few compounds such as EGCG and quercetin but there is a number of promising flavonoid antitumor agents that have poor in vivo parameters. In view from the above findings together with supporting literatures, it is clearly evidence that the HP conjugated pegylated gold NPs have shown better anticancer activity than the pure HP in DEN induced hepatocarcinoma in male wistar albino rats. The biochemical estimation and haematological parameters shows that the anticancer activity of Au-mPEG₍₅₀₀₀₎-S-HP NPs are higher than the anticancer activity of pure HP. The anticancer activity of Au-mPEG₍₅₀₀₀₎-S-HP NPs is higher

than pure HP at minimum dose of chemotherapy drug may be because of more solubility, enhance the targeting capabilities of pegylated gold NPs and sustained delivery to the cancer cells. The statement is proved in earlier study, based on the results of sustained drug delivery, cellular uptake and cytotoxicity of both drugs in Hep3B cells [17]. The Au-mPEG₍₅₀₀₀₎-S-HP NPs possesses more potent free radical scavenging and antioxidant activities. We have observed a significant decrease in the incidence and occurrence of liver cancer in animals treated with Au-mPEG₍₅₀₀₀₎-S-HP NPs as compared to those animals treated with pure HP of carcinogenesis. Taken together, HP conjugated pegylated gold NPs can markedly modulate oxidative stress by activating the antioxidant defence system. The over all results concluded that pegylated gold NPs should be a better bioavailability, solubility and more chemopreventive agent as compared with pure HP. The precise molecular mechanisms involved to prove Au-mPEG₍₅₀₀₀₎-S-HP NPs against DEN induced HCC is taken up in future.

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Compliance with Ethical Standards

Conflict of interest We are relevantly related to the work in this manuscript. We have no relationship, or any other financial source and this manuscript has not been published in any other publication.

Informed consent The purpose of this work is to produce a new effective nanotechnology based chemotherapy treatment and ensure the better prevention from liver cancer.

Research involving Human Participants and/or Animals This work is fully relevant to the animal ethical. We have the ethical certificate to do this work in male wistar albino rats and we have the ethical number: IAEC No. 12/02/2012 for your consideration we have enclosed the scan copy of ethical certificate.

References

1. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology*. 2007;132: 2557–76.
2. Harsh M. Text book of pathology. 5th ed. New Delhi: Anshan Publishers; 2005.
3. Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology*. 2004;127:35–50.
4. Sullivan BP, Meyer TJ, Stershic MT, Keefer LK. Acceleration of N-nitrosation reactions electrophiles. *IARC Sci Publ*. 1991;105: 370–4.
5. Yadav AS, Bhatnagar D. Chemo-preventive effect of star anise in N-nitrosodiethylamine initiated and phenobarbital promoted hepatocarcinogenesis. *Chem Biol Interact*. 2007;169:207–14.

6. Barbisan LF, Miyamoto M, Scolastici C, Salvadori DMF, Ribeiro LR, Eira AF, de Camargo JLV. Influence of aqueous extract of *Agaricus blazei* on rat liver toxicity induced by different doses of diethylnitrosamine. *J Ethnopharmacol.* 2002;83:25–32.
7. Kapadia GJ, Azuine MA, Takayasu J, Konoshima T, Takasaki M, Nishino H, Tokuda H. Inhibition of Epstein-Barr virus early antigen activation promoted by 12-*O*-tetradecanoylphorbol-13-acetate by the non-steroidal anti-inflammatory drugs. *Cancer Lett.* 2006;161:221–9.
8. Jayakumar S, Madankumar A, Asokkumar S, Raghunandhukumar S, Gokuladhas K, Kamaraj S, Divya MG, Devaki T. Potential preventive effect of carvacrol against diethylnitrosamine induced hepatocellular carcinoma in rats. *Mol Cell Biochem.* 2011;360:51–60.
9. Wattenberg LW. Inhibition of carcinogenesis by minor dietary constituents. *Cancer Res.* 1992;52:2085s–91s.
10. Formica JV, Regelson W. Review of the biology of quercetin and related bioflavonoids. *Food Chem Toxicol.* 1995;33:1061–80.
11. Wilcox LJ, Borradaile NM, Huff MW. Antiatherogenic properties of naringenin, a citrus flavonoid. *Cardiovasc Drug Rev.* 1999;17:160–78.
12. Karkabounas S, Sofis G, Evangelou A. Implication of free radicals in platelet aggregation: antiplatelet effects of free radical scavengers ex vivo. *Epith Klin Farmacol Farmakokin.* 1996;10:84–91.
13. Horvathova E, Sramkova M, Labaj J, Slamenova D. Study of cytotoxic, genotoxic and DNA protective effects of selected plant volatiles on human cells cultured in vitro. *Neuro Endocrinol Lett.* 2006;27:44–7.
14. Kanaze FI, Kokkalou E, Niopas I, Georgarakis M, Stergiou A, Bikiaris D. Dissolution enhancement of flavonoids by solid dispersion in PVP and PEG matrixes: a comparative study. *J Appl Polym Sci.* 2006;102:460–71.
15. Wang J, Zhou N, Zhu Z, Huang J, Li G. Detection of flavonoids and assay for their antioxidant activity based on enlargement of gold nanoparticles. *Anal Bioanal Chem.* 2007;388:1199–205.
16. Paciotti GF, Kingston DGI, Tamarkin L. Colloidal gold nanoparticles: a novel nanoparticle platform for developing multifunctional tumor-targeted drug delivery vectors. *Drug Dev Res.* 2006;67:47–54.
17. Gokuladhas K, Jayakumar S, Madankumar A, Rajan B, Elamaram R, Pramila S, Devaki T. Synthesis and characterization of biocompatible gold nanoparticles stabilized with hydrophilic polymer coated hesperetin drug for sustained drug delivery to treat hepatocellular carcinoma-derived cancer cells. *Int J Pharm Res.* 2014;8(2):98–105.
18. King J. The transferase-alanine and aspartate transaminase. In: King J, editor. *Practical clinical enzymology*. London: D Van Nostrand Company Ltd; 1965. p. 121–38.
19. King J. The dehydrogenases or oxidoreductase-lactate dehydrogenase. In: King J, editor. *Practical clinical enzymology*. London: D Van Nostrand Company Ltd; 1965. p. 83–93.
20. Rosalki SB, Rau D. Serum gamma-glutamyl transpeptidase activity in alcoholism. *Clin Chim Acta.* 1972;39:41–7.
21. Misra HP, Fridovich I. The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem.* 1972;247:3170–5.
22. Sinha AK. Colorimetric assay of catalase. *Anal Biochem.* 1972;47:380–95.
23. Moron MS, Depierre JW, Mannervik B. Levels of glutathione, glutathione reductase and glutathione-S-transferase activities in rat lung and liver. *Biochem Biophys Acta.* 1979;582:67–78.
24. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem.* 1979;95:351–8.
25. Keith C, Jamison DVM, George A, Padgett DVM, Thomas G, Bell DVM. Studies of adenine nucleotide biochemistry in the chediak-higashi syndrome. *Exp Mol Pathol.* 1993;58:40–52.
26. Bonting SL. In: Bittar EE, editor. *Membranes and ion transport*. London: Willey; 1970. p. 257.
27. Ohnishi T, Suzuki T, Suzuki Y, Ozawa K. A comparative study of plasma membrane Mg^{2+} ATPase activities in normal, regenerating and malignant cells. *Biochim Biophys Acta.* 1982;684:67–74.
28. Hjerten S, Pan H. Purification and characterization of two forms of a low affinity Ca^{2+} ATPase from erythrocyte membranes. *Biochim Biophys Acta.* 1983;728:281–8.
29. King J. The dehydrogenase of oxidoreductase-lactodehydrogenase. In: King JC, editor. *Practical clinical enzymology*. New Jersey: van Nostrand Company; 1965. p. 263.
30. Reed LJ, Mukherjee BB. α -Ketoglutarate dehydrogenase complex from *Escherichia coli*. *Methods Enzymol.* 1969;13:55–61.
31. Slater EC, Bonner WD. Effect fluoride on succinate oxidase system. *Biochem J.* 1952;52:185–96.
32. Mehler AH, Kornberg A, Grisolia S, Ochoa S. The enzymatic mechanism of oxidation reductions between malate or isocitrate or pyruvate. *J Biol Chem.* 1948;174:961–77.
33. Minakami S, Ringer RL, Singer TJP. Studies on respiratory chain linked dihydrodiphosphopyridine nucleotide dehydrogenase. *J Biol Chem.* 1962;237:569–76.
34. Pearl W, Cancercio J, Zweifach BW. Microdetermination of cytochrome oxidase in rat tissues by the oxidation of *N*-phenyl-*p*-phenylene diamine of ascorbic acid. *J Histochem Cytochem.* 1963;11:102–4.
35. Kim JY, Jung KJ, Choi JS, Chong HY. Hesperetin: potent antioxidant against peroxynitrite. *Free Radic Res.* 2004;38:761–9.
36. Garg A, Garg S, Zaneveld LJD, Singla AK. Chemistry and pharmacology of the citrus bioflavonoid hesperidin. *Phytother Res.* 2001;15:655–69.
37. Aranganathan S, Nalini N. Efficacy of the potential chemopreventive agent, Hesperetin (citrus flavanone), on 1, 2-dimethylhydrazine induced colon carcinogenesis. *Food Chem Toxicol.* 2009;47:2594–600.
38. Yalkowsky SH. *Techniques of solubilization of drugs*. New York: Marcel Dekker; 1981.
39. Shibayama T, Ueoka H, Nishii K. Complementary roles of pro-gastrin-releasing peptide (ProGRP) and neuron specific enolase (NSE) in diagnosis and prognosis of small-cell lung cancer (SCLC). *Lung Cancer.* 2001;32:61–9.
40. Banker DD. Viral hepatitis IV. *Indian J Med Sci.* 2003;57:511–7.
41. Gey KF. Prospects for the prevention of free radicals disease, regarding cancer and cardiovascular disease. *Br Med Bull.* 1993;49:679–99.
42. Whitby LG, Perey-Robb IW, Smith AT. *Enzymes tests indigagnosis*. 3rd ed., lecture notes in clinical chemistry London: Black Well Scientific Publications; 1984. p. 138–69.
43. Hirose M, Imaida K, Tamano S. Cancer chemoprevention by antioxidants. In: Ho CT, editor. *Food phytochemicals: teas, spices and herbs*. Washington: American Chemical Society Press; 1994. p. 122–32.
44. Sun Y. Free radicals, antioxidant enzymes, and carcinogenesis. *Free Radic Biol Med.* 1990;8:583–99.
45. Gutteridge JM. Antioxidants, nutritional supplement and life threatening diseases. *Br J Biomed Sci.* 1994;51:288–95.
46. Rajeshkumar NV, Ramadasan K. Modulation of carcinogenic response and antioxidant enzymes of rats administered with 1, 2-dimethylhydrazine by Picroliv. *Cancer Lett.* 2003;191:137–43.
47. Ramakrishnan G, Augustine TA, Jagan S, Vinodkumar R, Devaki T. Effect of silymarin on *N*-Nitrosodiethylamine induced hepatocarcinogenesis in rats. *Exp Oncol.* 2007;29:39–44.

48. Haridas V, Higuchi M, Jayatilake GS, Bailey D, Mujoo K, Blake ME, Arntzen CJ, Gutterman JU. Avicins: triterpenoid saponins from *Acacia victoriae* (Benth) induce apoptosis by mitochondrial perturbation. *PNAS USA*. 2001;98:5821–6.
49. Vanisree AJ, Shyamala Devi CS. Status of lipid peroxidation and antioxidant enzymes in malignant (bronchogenic carcinoma) and non-malignant pleural effusions. *Indian J Cancer*. 1999;36:127–34.
50. Singh RP, Banerjee S, Kumar PVS, Raveesha KA, Rao AR. *Tinospora cordifolia* induces enzymes of carcinogen drug metabolism and antioxidant system, and inhibits lipid peroxidation in mice. *Phytomedicine*. 2006;13:74–84.
51. Sumathi R, Baskaran G, Varalakshmi P. Effect of DL-lipoic acid on tissue redox state in acute cadmium-challenged tissues. *J Nutr Biochem*. 1996;7:85–92.
52. Jesberger JA, Richardson JS. Oxygen free radicals and brain dysfunction. *Int J Neurosci*. 1991;57:1–17.
53. Venkatraman M, Konga D, Peramaiyan R, Ganapathy E, Dhanapal S. Reduction of mitochondrial oxidative damage and improved mitochondrial efficiency by administration of crocetin against benzo[a]pyrene induced experimental animals. *Biol Pharm Bull*. 2008;31:1639–45.