Biochemical and Biophysical Research Communications 434 (2013) 203–209

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Inhibitory effect of naringenin (citrus flavonone) on *N*-nitrosodiethylamine induced hepatocarcinogenesis in rats

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ARTICLE INFO

Article history:
Received 4 March 2013
Available online 21 March 2013

Keywords:
Naringenin
N-Nitrosodiethylamine
Hepatocellular carcinoma
Lipid peroxidation
Antioxidants

ABSTRACT

We evaluated the effects of naringenin on *N*-nitrosodiethylamine (NDEA)-induced hepatocarcinogenesis in rats. Administration of NDEA induced hepatocellular carcinoma (HCC), as evidenced by changes in histopathological architecture, increased activity of cytochrome P450, decreased activity of glutathione *S*-transferase (GST) as well as decreased antioxidant status, enhanced lipid peroxidation and increased liver marker enzymes. Pre- and post-treatment with naringenin effectively suppressed NDEA-initiated hepatocarcinoma and the associated preneoplastic lesions by modulating xenobiotic-metabolizing enzymes (XMEs), alleviating lipid peroxidation (through both free radical scavenging and the enhanced antioxidant status), and decreased levels of liver marker enzymes. These results indicate that naringenin prevents lipid peroxidation and hepatic cell damage and also protects the antioxidant system in *N*-nitrosdithylamine-induced hepatocarcinogenesis.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignant tumor and the third leading cause of cancer mortality worldwide. Approximately 560,000 new cases and approximately 550,000 deaths occur each year, mostly in developing countries [1,2]. The major risk factors for HCC include viral infection with hepatitis B and C excessive alcohol consumption, and exposure to environmental or chemical carcinogens, such as aflatoxins [1–3]. Although various risk factors are associated with human HCC, chemicals in the environment are considered to be a significant health hazard to humans. In particular, the presence of nitroso compounds and their precursors in the environment and in products such as tobacco, cosmetics, and pharmaceuticals are known to induce liver cancer. Nitroso compound can also be formed in the human body from dietary products. Preneoplastic lesions, including hepatomegaly, hepatoportal sclerosis, fibrosis, and cirrhosis often occur after chronic exposure to the above chemicals [4,5].

N-Nitrosodiethylamine (NDEA), one of the most important environmental nitrosamines, is metabolized to its active ethyl radical metabolite ($CH_3CH_2^+$) by the cytochrome P-450-dependent monoxygenase system [6]. The reactive products and free radicals generated by P-450-dependent enzymes interact with DNA, producing mutations as well as an increase in the serum indices of liver function, such as alanine transaminase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), and total bilirubin and ultimately cause severe histopathological lesions in liver tissues followed by neoplastic transformation [7,8]. To maintain cellular health it is essential to have a specific and effective chemical and reactive oxygen species (ROS) scavenger to target both xenobiotic metabolizing enzymes and multiple types of radicals. In particular, antioxidants are known to protect cells from the deleterious effects of ROS and chemical carcinogens by preventing the metabolic activation of procarcinogens and thereby inactivating carcinogens, blocking DNA binding sites and enhancing DNA repair mechanisms [9,10]. Hence, antioxidants have emerged as promising chemopreventive agents for cancer.

Several epidemiological and animal studies have provided evidence that a high intake of natural products rich in antioxidant phytochemicals is associated with the decreased risk of many cancers [10,11]. Flavonoids, which are ubiquitous in common dietary fruits and vegetables, have diverse biological activities as a result of their anti-allergic, anti-inflammatory, antioxidant, and anticancer properties. They are potentially highly effective without significant systemic toxicity; therefore, therapeutic flavonoids are of great interest. Many recent studies have explored the role of flavonoids in improving health, in particular their ability to fight some cancers and cardiovascular diseases [12,13]. The flavonoid naringenin (4′,5,7-trihydroxyflavanone), predominantly found in citrus fruits, has been found to exhibit antioxidant, anti-mutagenic and anti-carcinogenic effects [14–16]. Moreover, naringenin acts as chemopreventive agent against colon carcinogenesis *in vitro* and *in vivo* [17,18]. Recently, we demonstrated that naringenin inhibits cell proliferation via downregulation of NF-kappa B, VEGF, and

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http://dx.doi.org/10.1016/j.bbrc.2013.03.039

MMPs and also induce apoptosis via Bcl-2, Bax and caspase in a rat model of hepatocarcinogenesis by *N*-nitrosodiethylamine (NDEA) [19]. The present study addresses the status of serum marker enzymes, liver tumor marker and antioxidant enzymes in the prevention of NDEA-induced hepatocarcinogenesis by naringenin.

2. Materials and methods

2.1. Drug and chemicals

Naringenin, NDEA, 2-thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), reduced glutathione (GSH), 2, 2′-dipyridyl, xylenol orange, 2,4-dinitrophenylhydrazine (DNPH), γ-glutamyl-p-nitroanilide, and 5, 5′-dithiobis-2-nitrobenzoic acid were obtained from Sigma Chemical Co. (St. Louis, Mo, USA). The rest of the chemicals utilized were obtained from a local firm (India) and were of analytical grade.

2.2. Animals

Adult male albino Wistar rats (150–170 g) were used for the experiment. The animals were housed in plastic cages and maintained in a 12 h light/12 h dark cycle, 50% humidity and 25 ± 3 °C. The animals had free access to a standard pellet diet (M/S. Pranav Agro Industries Ltd., Bangalore, India) and water *ad libitum*. This study was approved (Vide. No. 714, 2010) by the Institutional Animal Ethics Committee of Annamalai University and was conducted in accordance with the "Guidelines for the Care and Use of Laboratory Animals".

2.3. Experimental design

The animals were randomly divided into five groups, with six rats in each group. Group 1: control rats were given corn oil orally for 16 weeks. Group 2: rats received naringenin alone (200 mg/kg body weight, dissolved in corn oil) orally for 16 weeks [16]. Group 3: rats were induced with hepatocellular carcinoma with 0.01% NDEA through drinking water for 15 weeks [20,21]. Group 4: rats were pretreated with naringenin (200 mg/kg body weight) 1 week before the administration of 0.01% NDEA and administration of naringenin was continued until the end of the experiment (i.e., 16 weeks). Group 5: after the administration of NDEA for 10 weeks, rats were post-treated with naringenin orally (200 mg/kg body weight) for 5 weeks along with the carcinogen until the end of experimental period [16].

After the last treatment, the rats were fasted overnight, and all of the rats were anesthetized with pentobarbital sodium (35 mg/kg, i.p.) and euthanized by cervical decapitation. Blood samples were collected in tubes containing ethylenediaminetetra acetic acid (EDTA). The plasma was obtained after centrifugation (2000×*g* for 20 min at 4 °C) and used for various biochemical measurements. The livers were excised immediately from the animals, washed with ice-chilled physiological saline, and stored at −80 °C until analysis.

2.4. Histopathological studies

The liver samples were fixed for 48 h in 10% formalin, dehydrated in a mixture of ethyl alcohol–water, cleaned in xylene and embedded in paraffin. Sections of liver (5–6 μm thick) were prepared, stained with hematoxylin and eosin dye (H&E), and mounted in neutral DPX medium for microscopic analysis.

2.5. Biochemical assays

Cytochrome P450 content was assayed by the method of Omura and Sato [22]. The activities of GST were assayed by the methods of Habig et al. [23] using 1-chloro-2,4-dinitrobenzene as a substrate. Lipid peroxidation in the liver was estimated spectrophotometrically by measuring thiobarbituric acid reactive substances (TBARS) and hydroperoxides using the method of Niehius and Samuelson [24] and Jiang et al. respectively [25]. Superoxide dismutase (SOD) activity was determined by the method of Kakkar et al. [26]. The activity of catalase was determined by the method of Sinha [27]. Glutathione peroxidase was estimated by the method of Rotruck et al. [28]. A known amount of enzyme preparation was allowed to react with hydrogen peroxide in the presence of GSH for a specified time period of time. Then, the remaining GSH was measured using the method of Ellman [29]. Vitamin C concentration was measured by the method of Omaye et al. [30]. Vitamin E was estimated using the method of Desai [31].

The activities of serum aspartate aminotransferase (AST, E.C. 2.6.1.1), alanine aminotransferase (ALT, E.C. 2.6.1.2), alkaline phosphatase (ALP, E.C. 3.1.3.1), and lactate dehydrogenase (LDH, E.C. 3.1.3.1) were assayed spectrophotometrically according to standard procedures using commercially available diagnostic kits (Sigma diagnostics (I) Pvt. Ltd., Baroda, India). Gamma glutamyl transferase (GGT, E.C. 2.3.2.2) activity was determined by the method of Rosalki et al. [32] using γ-glutamyl-p-nitroanilide as a substrate. The quantitative estimation of the tumor marker α-fetoprotein (AFP) was based on a solid phase enzyme-linked immunosorbent assay (ELISA) using the UBI MAGIWELL (USA) enzyme immunoassay kit [33].

2.6. Statistical analysis

Data were analyzed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using a statistical software package (SPSS for Windows, V. 13.0, Chicago, USA). The results are presented as the means ± SD. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Body weight and liver weight of rats treated with NDEA and naringenin

Table 1 shows the body and liver weight of control and experimental animals from each group. The body weights were significantly decreased in NDEA-treated animals compared to the control. Pre- and post-treatment of NDEA-treated rats with naringenin significantly improved the body weight compared to animals in group 3 NDEA treatment increased the liver weight relative to the body weight compared to controls animals (group I). However, administration of naringenin to the group 4 and group 5 animals significantly reduced the relative liver weight compared to group 3 animals.

3.2. Development of liver nodules in NDEA treated animals

Table 2 shows the total number of nodules, the number of nodules per nodule-bearing liver, and the nodular sizes in millimeters in tumor-bearing animals. The naringenin-treated groups (4 and 5) showed a significant decrease in both the number of nodules and nodule size, when compared with group 3 animals.

Table 1
Effect of naringenin on body, liver and relative liver weights on NDEA induced hepatocarcinogenesis in rats.

	Group 1	Group 2	Group 3	Group 4	Group 5
Body weight final (g)	302 ± 19.09	307 ± 20.04	201 ± 12.58[a]	287 ± 17.83[b]	239 ± 15.55[b]
Liver weight (g)	7.92 ± 0.82	7.99 ± 0.87	11.62 ± 1.21[a]	9.02 ± 0.97[b]	9.51 ± 1.08[b]
Relative liver weight (g)	2.78 ± 0.09	2.79 ± 0.11	4.48 ± 0.13	3.46 ± 0.12	3.91 ± 0.10

1. Control, 2. Naringenin alone, 3. NDEA, 4. Naringenin pre treatment, 5. Naringenin post treatment.
[a] Significantly different from control $p < 0.001$ ANOVA followed by DMRT.
[b] Significantly different from group 3 $p < 0.001$ ANOVA followed by DMRT.

Table 2
Effect of naringenin on number and size of hepatocellular nodules on NDEA induced hepatocarcinogenesis in rats.

	Group 1	Group 2	Group 3	Group 4	Group 5
Tumor incidence	–	–	6/6	2/6	6/6
Total number no of nodules	–	–	107[a]	56[b]	93[b]
Average number of nodules/nodules bearing liver	–	–	17.83 ± 1.5[a]	9.33 ± 0.5[b]	15.5 ± 1.10[b]
<1 mm	–	–	54 (50.5)	31 (55.3)	39 (53.4)
>1 mm <3 mm	–	–	33 (30.8)	17 (30.3)	21 (28.76)
>3 mm	–	–	20 (18.7)	8 (14.3)	13 (17.8)

1. Control, 2. Naringenin alone, 3. NDEA, 4. Naringenin pre treatment, 5. Naringenin post treatment.
[a] Significantly different from control $p < 0.001$ ANOVA followed by DMRT.
[b] Significantly different from group 3 $p < 0.001$ ANOVA followed by DMRT.

3.3. Histopathological changes

The histological examinations shown in Fig. 1 support the results obtained from serum enzyme and tumor marker assays. Fig. 1A shows the normal architecture (group I) and cytoplasm of hepatic cells displaying granulated cytoplasm, a central vein, small uniform nuclei and nucleoli. Group 2 animals showed a normal architecture indicating the non-toxic nature of naringenin (Fig. 1B). NDEA treatment alone (Fig. 1C) resulted in the loss of normal architecture and the appearance of tumor cells which were smaller than normal cells and displayed granular cytoplasm with large hyperchromatic nuclei, whereas group 4 animals pretreated with naringenin showed few neoplastically transformed cells, and their hepatocytes maintained a near normal architecture (Fig. 1D). Group 5 animals post-treated with naringenin showed a loss of normal architecture and a comparably lesser tendency to spread via intrahepatic veins, both in hepatic and portal vessels (Fig. 1E).

3.4. Biochemical assays

Fig. 2 shows the status of phase I (cytochrome P450) and phase II (GST) detoxification enzymes in the liver of control and experimental animals in each group. The activity of GST was significantly decreased, whereas the status of cytochrome P450 was increased, in the livers of tumor-bearing animals (group 3) compared to

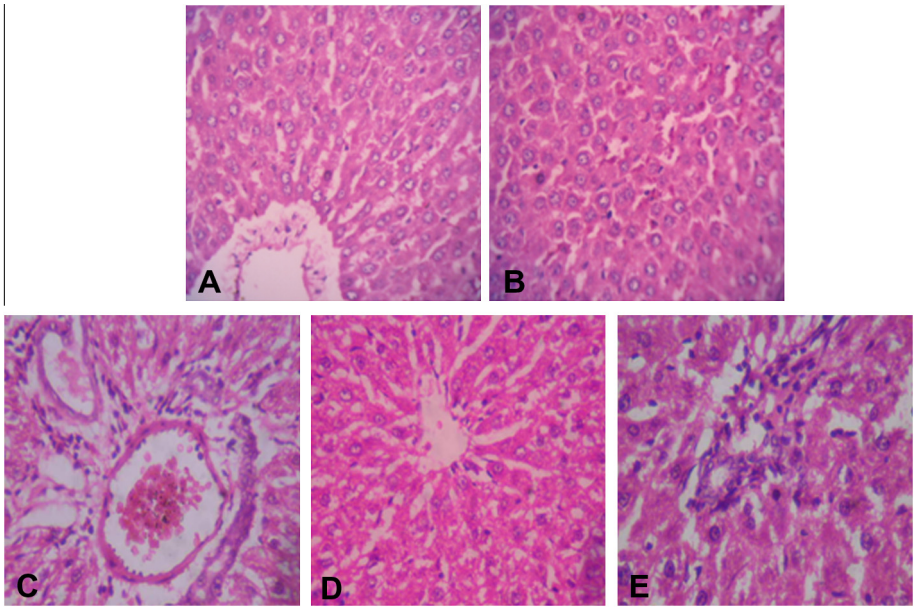

Fig. 1. Histopathological observation in the liver tissues of control animals and experimental animals. (A) Liver tissue from the normal group (control) showed hepatic lobules with normal architecture (40×, H&E). (B) Liver tissue of the naringenin-treated group showed hepatic lobules with normal architecture (40×, HE). (C) NDEA alone showed loss of architecture, mitotic, granular cytoplasm and neoplastic cells. (D) A small number of neoplastically transformed cells and hepatocytes maintaining near normal architecture were observed in naringenin pre-treated animals. (E) Naringenin post-treated animals showed loss of architecture, mitotic, granular cytoplasm and neoplastic cells.

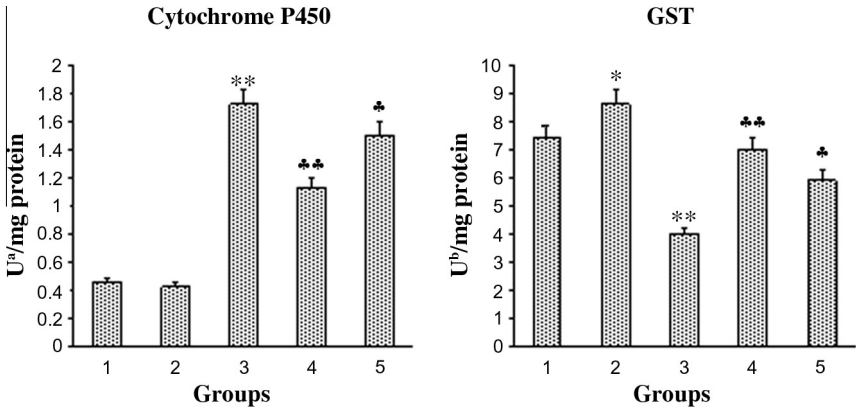

Fig. 2. Activities of phase II enzymes in the liver of experimental and control animals. *Significantly different from control $p < 0.01$, **Significantly different from control $p < 0.001$, ♣Significantly different from NDEA $p < 0.01$, ♣♣Significantly different from NDEA $p < 0.001$ ANOVA followed by DMRT, [a]μmoles of cytochrome P450, [b]μmoles of CDNB conjugated with GSH per minute.

control animals. Administration of naringenin to NDEA-treated animals significantly decreased phase I and elevated phase II enzyme activities compared to animals in group 3. Although treatment with naringenin alone (group 2) showed no significant difference in the activities of phase I enzymes, the activities of phase II enzymes in the liver were significantly increased compared to the control (group 1).

Changes in the levels of lipid peroxidation products in the control and experimental animals are shown in Fig. 3. In NDEA-treated rats, the levels of TBARS and lipid hydroperoxide were significantly increased compared to control rats. Administration of naringenin alone significantly decreased the levels of TBARS and LPO compared to the control.

Fig. 4 illustrates the levels of enzymatic and non-enzymatic antioxidants, namely SOD, CAT, GPx, vitamin C, vitamin E and GSH in the livers of control and experimental rats. A significant decrease in the activities of enzymatic antioxidants and non-enzymatic antioxidants was observed in rats from group 3. Pre- and post-treatment with naringenin significantly increased the levels of enzymatic and non-enzymatic antioxidants in the liver compared to NDEA-treated rats. Administration of naringenin alone significantly increased the level of enzymatic and non-enzymatic antioxidants compared to the controls.

Table 3 shows the levels of serum hepatic marker enzymes in control and experimental rats. NDEA-induced hepatocarcinogenesis caused abnormal liver function in all rats. Activities of serum hepatospecific enzymes such as AST, ALT, ALP, LDH, and GGT, along with the levels of bilirubin and AFP, were significantly increased ($P < 0.05$) in NDEA-treated animals when compared to control animals. However, in naringenin-treated groups 4 and 5, the values returned toward those observed in the untreated control animals. No significant changes were observed in rats treated with naringenin alone compared to the controls.

4. Discussion

Administration of naringenin prevented the development of NDEA-induced hepatic carcinomas by modulating xenobiotic metabolizing enzymes, oxidant and antioxidant status as well as decreasing the levels of liver marker enzymes. Our results agree with those obtained using other chemopreventive agents [7,8,20,21].

Metabolic biotransformation of NDEA by cytochrome P450 enzymes produces *O*6-ethyldeoxyguanosine as well as *O*4- and *O*6-ethyldeoxythymidine, active ethyl radical metabolites ($CH_3CH_2^+$) that are responsible for the initiation of carcinogenesis [34,35]. Subsequently, these reactive products of NDEA can be detoxified by phase II enzymes such as GST. Increased activity of cytochrome P450 and decreased activity of GST were observed in the present study, providing evidence for the development of HCC in

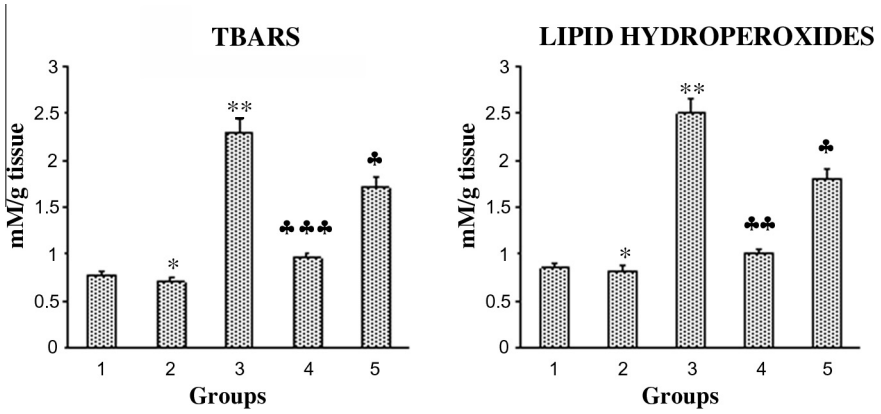

Fig. 3. Changes in the levels of lipid peroxidation in liver tissues of experimental rats. *Significantly different from control $p < 0.05$, **Significantly different from control $p < 0.01$, ♣Significantly different from NDEA $p < 0.05$, ♣♣Significantly different from NDEA $p < 0.01$, ♣♣♣Significantly different from NDEA $p < 0.001$ ANOVA followed by DMRT.

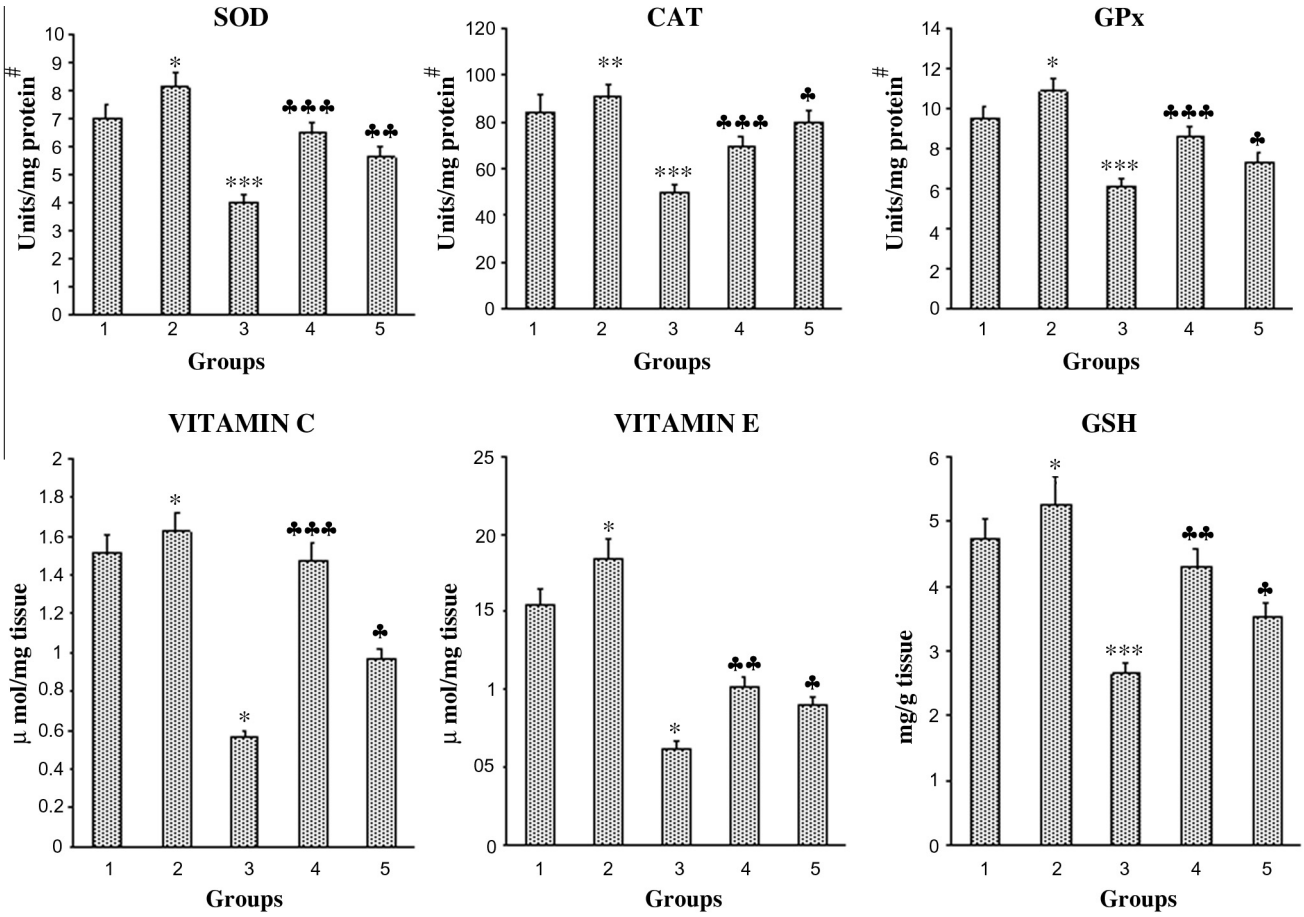

Fig. 4. Changes in the levels of enzymatic and non-enzymatic antioxidant status in control and experimental rats. *Significantly different from control $p < 0.05$, **Significantly different from control $p < 0.001$, ***Significantly different form control $p < 0.05$, ♣Significantly different from NDEA $p < 0.05$, ♣♣Significantly different from NDEA $p < 0.01$, ♣♣♣Significantly different from NDEA $p < 0.001$ ANOVA followed by DMRT. # Units of enzyme activities are expressed as: SOD – one unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in 1 min. CAT – μmol of hydrogen peroxide consumed per min. GPx – μg of glutathione consumed per min.

Table 3
Effect of naringenin on the activities of marker enzymes in the serum of control and experimental groups of rats.

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5
AST (IU/l)	118.32 ± 7.84	119.21 ± 7.54	252.64 ± 15.97*	132.46 ± 8.37♣♣♣	196.10 ± 12.40♣
ALT (IU/l)	39 ± 2.48	40.19 ± 2.54	94.23 ± 5.96*	46.19 ± 2.92♣♣♣	71.32 ± 4.51♣
ALP (IU/l)	42.12 ± 2.66	42.62 ± 2.69	84.39 ± 5.33*	46.56 ± 2.94♣♣	62.86 ± 3.97♣
LDH (IU/l)	91.25 ± 5.77	92.12 ± 5.82	146.66 ± 9.26*	98.93 ± 6.25♣♣♣	124.31 ± 7.86♣
GGT (IU/l)	6.13 ± 0.39	6.20 ± 0.39	12.17 ± 0.77*	6.62 ± 0.42♣♣	9.15 ± 0.58♣
Bilirubin (mg/dl)	0.82 ± 0.05	0.84 ± 0.05	1.52 ± 0.10*	0.97 ± 0.06♣♣	1.06 ± 0.07♣
AFP (ng/ml)	8.90 ± 0.56	8.80 ± 0.56	50.20 ± 3.17**	10.10 ± 0.64♣♣♣	22.20 ± 1.40♣

1. Control, 2. Naringenin alone, 3. NDEA, 4. Naringenin pre treatment, 5. Naringenin post treatment.
* Significant different from group 1 $p < 0.05$.
** Significant different from group 1 $p < 0.001$.
♣ Significant different from group 3 $p < 0.05$.
♣♣ Significant different from group 3 $p < 0.01$.
♣♣♣ Significant different from group 3 $p < 0.001$ ANOVA followed by DMRT.

NDEA-treated animals. Our results provide evidence that naringenin is a dual-acting agent that decreases phase I enzyme cytochrome P450 and increases phase II enzyme GST to block initiation of NDEA-induced hepatocarcinogenesis. It has been documented in several studies that dual-acting agents are ideal chemopreventive agents with high efficacy [36,37].

Modulation of XMEs by naringenin is correlated with the establishment of an equilibrium between oxidant and antioxidant which favors the antioxidant side. The reactive metabolites of NDEA and the free radicals generated by cytochrome P450-dependent enzymes disturb the antioxidant status and ultimately produce oxidative stress, which leads to carcinogenesis [38], lipid peroxidation plays an important role in carcinogenesis [39] and is the most studied biologically relevant free radical chain reaction. It is initiated by the attack of a free radical on a fatty acid or fatty acyl side chain of any chemical species that has sufficient reactivity to remove a hydrogen atom from a methylene carbon side chain. Lipid peroxidation may lead to the formation of several byproducts, such as malondialdehyde (MDA) and 4-hydroxynonenal. These products can attack cellular targets including DNA, thereby promoting mutagenicity and carcinogenicity [40]. The decrease in the activities of enzymatic and non-enzymatic antioxidants in NDEA-

induced HCC may be due to the over-utilization of these antioxidants to scavenge the products of lipid peroxidation and indicate the complete disruption of the antioxidant defense mechanism of the liver. Our results are consistent with other works reported in the literature [7,8,41].

Naringenin (pre- and post-treated) could protect cells through the inhibition of lipid peroxidation, as shown by the decreased the levels of tissue TBARS and lipid hydroperoxides. Our results suggest that the anti-lipid peroxidative role of naringenin is most likely mediated by its ability to scavenge free radicals because of the presence of a 4-hydroxyl group possessing electron donating properties in the β-ring [14]. In addition, naringenin could prevent the cell membrane from free radical attack and thus protect the membrane and inhibit lipid peroxidation. Furthermore, naringenin inhibits lipid peroxidation and generates free radicals by enhancing antioxidant status, as shown by increasing the levels of SOD, catalase, GPx, the non-enzymatic antioxidants vitamins E and D, and GSH. Our findings demonstrate that the modulation of the delicate balance between oxidants and antioxidants mediated by various natural phytochemicals such as naringenin, is a rational approach for blocking tumor progression. The antioxidant potential of naringenin has also been documented in cadmium-induced hepatotoxicity and MNNG-induced gastric carcinogenesis [14,16].

Generally, liver damage induced by NDEA is related to the disruption of liver cell metabolism and membrane instability and subsequently causes distinctive changes in the activities of serum enzyme activities. Upon liver injury, liver marker enzymes (AST, ALT, and ALP) enter into the circulatory system because of the altered permeability of the membrane [7,8].This observation correlates with our results, which show increased activities of marker enzymes in the serum of HCC-induced animals. Serum LDH, a cytoplasmic marker enzyme, and GGT, a membrane-bound enzyme, are also well-known indicators of liver damage during various physiological and pathological conditions [42,43]. The increased activities of AST, ALT, ALP and LDH observed in the present study are indicators for NDEA-induced liver damage and HCC. The significant increase in the concentration of serum bilirubin observed in NDEA-treated rats is also consistent with the presence of hepatic damage [8,38]. Alpha-fetoprotein is a tumor marker produced by regenerating hepatic tumors. Several studies have documented that exposure of rats to certain carcinogens such as NDEA causes an elevation of circulating AFP levels [7,8,43,44].

Administration of naringenin attenuated NDEA-induced hepatocarcinogenesis, as shown by the decreased activities of AST, ALT, ALP, LDH, bilirubin and α-fetoprotein. Our results suggest that naringenin aids in parenchymal cell regeneration in the liver, thus protecting liver cell and membrane integrity by scavenging free radicals and enhancing the antioxidant status thus decreasing enzyme leakage and hindering the process of carcinogenesis. Several studies provide evidence that naringenin decreases the levels of liver marker enzymes in serum during chemically induced hepatotoxicity by inhibiting free radicals and lipid peroxidation and enhancing antioxidants [14,44,45]. To conclude, administration of naringenin effectively suppressed NDEA-initiated hepatocarcinogenesis and the appearance of preneoplastic lesions by modulating XMEs, attenuating lipid peroxidation through the scavenging of free radicals, enhancing antioxidant status, and decreasing liver marker enzymes. Taken together, naringenin can markedly modulate oxidative stress by activation of the antioxidant defense system. Thus, naringenin may be an attractive candidate as an antioxidant supplement for anticancer therapy.

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