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ORIGINAL ARTICLE

Naringin, a Natural Flavonone Glycoside attenuates N-Nitrosodiethylamine- induced Hepatocellular Carcinoma in Sprague-Dawley rats

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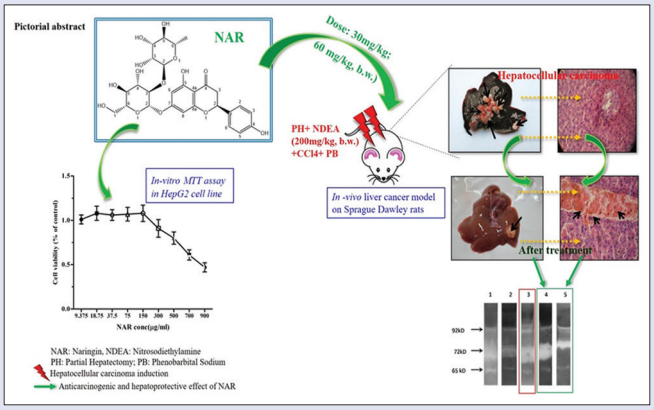
ABSTARCT

Background: Hepatocellular Carcinoma (HCC) being proclaimed as the world's fifth common cancer shows a high mortality rate due to delayed diagnosis. Regular day-to-day activities expose us to promotive factors that potentiate further to risk of liver damage, cirrhosis, and carcinogenesis. Matrix metalloproteinases (MMPs), a proteolytic enzyme, involved in cancer invasion are considered as a prognostic biomarker for HCC. Naringin (NAR), a flavanone glycoside, is recognized to have therapeutic efficacy in HCC. **Objectives:** The present research work focuses on evaluation of chemotherapeutic efficiency, NAR against HCC in Sprague–Dawley rats. **Materials and Methods:** *In vitro* studies were primarily carried out to estimate the cell viability of NAR in HepG2 cells followed by further justifications through *in vivo* studies in N-nitrosodiethylamine developed HCC. The efficacy of NAR at doses 30mg/kg/day and 60mg/kg/day was assessed through estimation of liver marker enzymes, antioxidant levels, glycoproteins, and level of MMP-2 and 9 to confirm the hypothesis. **Results:** While *in vitro* results revealed pronounced potency of NAR in inhibiting HepG2 cell viability, NAR concurrently stabilized the serum levels of liver marker enzymes, antioxidant levels, and serum glycoproteins. It also significantly reduced the levels of MMPs in treatment groups which was confirmed by zymography. **Conclusion:** With the support of these results, it can be concluded that NAR attenuates HCC by controlling the alterations in morphological, biochemical, and histopathological parameters that make it a suitable candidate as a potential chemotherapeutic agent against HCC.

Key words: Hepatocellular carcinoma, matrix metalloproteinases, Naringin, N-nitrosodiethylamine, zymography

SUMMARY

- Naringin (NAR), the flavanone glycoside derivative, shows hepatoprotective and chemotherapeutic action against hepatocellular carcinoma (HCC). *In vitro* analysis in HepG2 cell lines and *in vivo* studies in N-nitrosodiethylamine-induced HCC model in experimental animals were considered in the current study. While MTT assay demonstrated significant suppression of viability of HepG2 cells, NAR also revealed its potency in inhibiting development of HCC which was confirmed by various morphological, histological, and biochemical parameters. In addition, zymography technique depicted that matrix metalloproteinases that serve as a pivotal biomarker of cancer pathogenesis were found to be significantly attenuated after NAR treatment.



Abbreviations used: NAR: Naringin; NDEA: N-nitrosodiethylamine; HCC: Hepatocellular carcinoma; MMPs: Matrix metalloproteinases.

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INTRODUCTION

Hepatocellular Carcinoma (HCC) is considered as the fifth most predominant cancer in the world. Despite the curative therapies, i. e., liver transplantation, surgical resection, local ablation, etc., the disease shows very unsatisfactory prognosis due to its diagnosis at an advanced stage.[1] Alcoholism, food additives, viral infection, water pollution, and chemical toxicity are the major factors that promote hepatocarcinogenesis.[2] The multiple tumor invasion and metastasis stages include degradation of barriers like basement membrane and extracellular matrix (ECM) that is supported by several proteolytic enzymes, among which matrix metalloproteinases (MMPs) have a major role.[3] To proceed with the metastatic cascade of HCC, intravasation and extravasation of HCC cells are required through the basement membrane.[4] MMPs belong

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to a multigene zinc-dependent family of endopeptidases sharing a similar structure.[5] During cancer invasion, MMPs reduce the ECM components and basement membrane boundaries, hereby promoting cell migration.[6,7] Thus, enhanced MMPs level is directly correlated with poor prognosis and metastasis in HCC patients.[8,9] MMP-2 and 9 are type IV collagenases that promote the invasiveness of tumors.[10] Requirement of a biomarker for defining cancer malignancy, determining prognosis, predicting therapeutic efficacy, and therapeutic response monitoring is very essential.[11,12] Therefore, MMPs can be involved as a biomarker for tissue invasion and metastasis.[13] Zymography, a simple technique that was first described in 1980, is widely used for the determination of MMP activity in different systems. It is based on protein separation technique known as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). To detect the activity of MMP-2 and 9 (gelatinase A and B), zymography has been used by taking gelatin as a substrate.[14] Zymography is advantageous because it is a simple, sensitive, and quantifiable technique for determining MMPs and tissue inhibitors of metalloproteinases in biological samples.[15,16]

N-nitrosodiethylamine (NDEA), a potent hepatocarcinogen, is found in agricultural chemicals, cosmetics, water, tobacco smoke, some foods, etc., and has the potential to produce reproducible neoplasms of the liver by reactive oxygen species (ROS) production and lipid peroxidation mechanism. Induction of hepatocarcinogenesis has been successfully established using nitrosamine, partial hepatectomy, polychlorinated aromatic hydrocarbons as promoting agent, and CCl_4 as a hepatotoxin.[17] The modern therapy directs toward the use of natural plant-based materials for the treatment of HCC. Flavonoids are phytoconstituents that have reported *in vitro* and *in vivo* antitumor activities (by scavenging various oxidizing species) in animal experimental models.[18,19]

Naringin (NAR, 4′,5,7-trihydroxy-flavonone-7-rhamnoglucoside, a natural flavanone glycoside) consisting flavanone naringenin and disaccharide neohesperidose belongs to the family Rutaceae and is derived from *Citrus pomelo*[20] [Figure 1a]. This bioflavonoid is ubiquitously distributed in grapefruit and related citrus species such as orange and kino and serves as non-toxic healthy supplement.[21-24] NAR, a biologically active compound, has reported activity against HCC cell line (HepG2 cells) by the induction of apoptosis.[25] NAR also exhibits other pharmacological activities such as bone regeneration (*in vitro* and *in vivo*), anti-inflammatory, effects on metabolic syndrome and oxidative stress, activity against CNS disease, and effects on genetic damage (provides protection against peroxide induced chromosome damage).[26]

The present study hypothesized that NAR was a suitable drug for the treatment of HCC in rat model. Furthermore, varied expression of MMPs was determined by zymography technique to be considered as a relevant biomarker for HCC invasion. A comparative evaluation was done on five different groups of Sprague–Dawley (SD) rats. SDS-PAGE technique was involved in detecting and quantifying MMPs. The study was also supported by histopathological studies and biochemical estimation.

MATERIALS AND METHODS

Materials

All the chemical and solvents used were of the highest purity availed from commercial sources. NAR (M. W.: 580.53g/mol) was obtained from Sigma Aldrich, India, of analytical standard. NDEA (200 mg/kg b. w., i. p.) was obtained from Sigma Aldrich, India; phenobarbitone (0.05% w/v, in drinking water) from CDH (P) Ltd., New Delhi, India, CCl_4 (1 mL/kg b. w., s. c.) from CDH (P) Ltd., New Delhi, India; diethyl ether from RFCL Ltd., New Delhi, India; and formalin (10% v/v) from RFCL Ltd., New Delhi, India.

Cell lines and culture

HCC (HepG2) cells were maintained in continuous culture at 37°C temperature in Dulbecco's modification of Eagle's medium (DMEM)

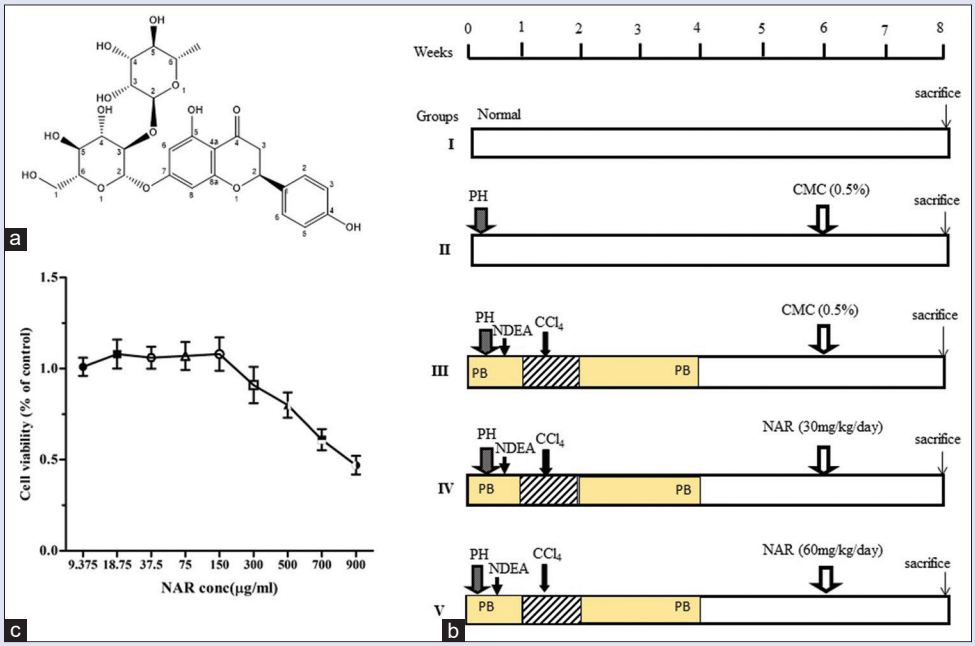

Figure 1: (a) Chemical structure of Naringin. (b) Study experimental design for NDEA-induced hepatocellular carcinoma in Sprague–Dawley rats. PH: Partial hepatectomy; PB: Phenobarbital sodium; CMC: Carboxy methyl cellulose (vehicle); NAR: Naringin (30 and 60 mg/kg/day); NDEA: N-nitrosodiethylamine. (c) Graphical representation of MTT assay depicting cytotoxic potential of NAR on HepG2 cell at different concentrations (9.375, 18.75, 37.5, 75, 150, 300, 500, 700, and 900 μg/ml). The data are represented as mean ± SEM

supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.5 µg/ml Fungizone.

In vitro studies

MTT assay

MTT assay was carried out as per the methods of Kumar *et al.*[27] HepG2 cells with a concentration of 10^4 cells/well were seeded in 96 well plates for 24 h and treated with NAR in different concentrations of six replicates each. After 96 h incubation, the cells were incubated with 0.5mg/ml solution of methylthiazoletetrazolium for 2 h at 37°C additionally. Then, DMSO (200µl) was added to the aspirated MTT containing medium to solubilize the formazan product formed. Absorbance (Abs) was recorded using a microplate reader at 570nm. The formula used to calculate competitive inhibition is as follows:

$(Abs_{control} - Abs_{NAR\text{-}control})/Abs_{control} \times 100\%)$

Experimental animals

50–55 days' old Sprague–Dawley rats were acquired from Central Animal Facility of Birla Institute of Technology, Mesra, Ranchi, Jharkhand, India (Reg. No. 621/02/ac/CPCSEA) for the study. The animals were provided with standard pellet diet and water was given *ad libitum*. The experiment was strictly carried out as per the CPCSEA norms with prior approval from Institutional Animals Ethics Committee, BIT Mesra, Ranchi, India.

In vivo experimental model

Following the methods of Pattanayak *et al.*,[28] HCC was induced in SD rats. Partial hepatectomy (PH) was surgically done on all the SD rats that followed a stabilization time of 24 h. After that, 200 mg/kg b. w NDEA was administered i. p. After the NDEA dose, the animals were given phenobarbital sodium (0.05% w/v) orally in drinking water for a continuation of 4 weeks. Furthermore, CCl_4 (1ml/kg b. w.) was injected subcutaneously twice a week for the initial two weeks. The study experimental design is depicted in Figure 1b.

Thirty SD rats were used for the study. Five groups of animals were being divided keeping six animals in each group. The groups taken were:

- Group I: (Normal control) received no treatment
- Group II: (PH control) Only partial hepatectomy was performed and animals were given 0.5% CMC (vehicle)
- Group III: (Induced control) PH + 200mg/kg b. w. NDEA (i. p.) and 0.5% CMC (vehicle)
- Group IV-V: (Treatment) PH + NDEA + NAR (30 and 60 mg/kg/day b. w. i. p. respectively) for a duration of 2 weeks

Body weight of experimental animals was monitored on a weekly basis. After 8 weeks of HCC induction and treatment, the animals were starved overnight and sacrificed by cervical dislocation.

Blood and tissue collection

After sacrificing, the livers of the animals were dissected surgically and washed twice with phosphate buffer saline having pH 7.2. Then, the hepatic tissues were weighed and examined for lesions, tumors, and foci. Cardiac puncture technique was applied for the collection of blood and the blood was centrifuged to obtain the serum. The blood serum was stored for estimating the biochemical parameters. Liver-to-body weight ratio was calculated following the method of Bose *et al.*[29] and was calculated using the formula: liver-to-body weight ratio = (Rat liver weight/Rat body weight) ×100%.

Biochemical estimation of blood serum

Estimation of aspartate dehydrogenase (AST) and alanine transaminase (ALT) was performed on blood serum following the method of King *et al.*[30] Estimation of alkaline phosphatase (ALP) was done following the changed method of Belfield and Goldberg *et al.*[31] and gamma-glutamyl transferase (γ-GT) estimation from serum was done following the standard method. Estimation of glycoproteins in the blood serum was done following the method of Morgan[32] for hexosamine estimation, method of Goodwin[33] for hexose estimation, and method of Aminoff[34] for sialic acid estimation. Lipid peroxidation (LPO) was expressed as mono-aldehyde (MDA) in nanomoles in liver and was determined by following the method of Lowry *et al.*[35] superoxide dismutase (SOD) activity was determined from serum following the method of Otting *et al.*,[36] whereas catalase (CAT) activity was rectified by the method given by Sinha *et al.*[37] Glutathione oxidation was measured at 420 nm spectrochemically following the method of Retruck *et al.*[38] Further, estimation of glutathione reductase (GR) and reduced glutathione (GSH) was done following the method of Habig *et al.*[39]

Histopathological studies

Liver tissues first fixed in 10% neutral-buffered formalin and then infixed in paraffin blocks were cut into thin sections (6–8 µm) using a rotary microtome and stained with hematoxylin and eosin dyes as per the protocol of Pattanayak *et al.*[40] Then, the tissues were mounted using DPX (Merck, India) and observed under Leica (Leica DME) microscope for determining the histopathological alterations.

Preparation of sample for zymography

Thirty milligrams of animal liver tissue was weighed and transferred to mortar and pestle. Liquid nitrogen was added to it cautiously followed by vigorous mixing of the tissue. When the ingredients were transformed to a fine powder, they were immediately transferred to Eppendorf tubes containing homogenizing buffer (Tris base, Triton 0.5%, ddH_2O, 1M HCl) and centrifuged. The supernatant was separated and stored at −20°C.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis technique for matrix metalloproteinases detection

The gelatinolytic property of MMPs is utilized to assess their activity through electrophoresis. It was carried out according to Laemmli protocol.[41] The expression of different MMPs in liver sample was detected by SDS-PAGE in 8% acrylamide gel copolymerized with gelatin A. After running of gel at 200V for 2 h, washing was done with 2.5% Triton X-100 to wash off the SDS. Gel was incubated for 20 h at 37°C in Tris buffer including 0.15 M NaCl, 10 mM $CaCl_2$, 0.1M glycine, and 1µM $ZnCl_2$. Following incubation, gel was subjected to staining and destaining with 0.05% Coomassie Brilliant Blue R-250, acetic acid, and methanol. Proteolytic activity of MMPs was foreseen as clear bands in front of the blue background. Gelatinolytic bands were measured densitometrically using imageJ analyzer.

Statistical analysis

The results obtained were statistically analyzed by comparing the data of normal control, partially hepatectomy control, induced control, and the treatment groups (mean ± SEM) with one-way ANOVA. This was further analyzed by Bonferroni's multiple comparison tests. The significance level denoted by *P* value was considered at $P < 0.05$.

RESULTS

Naringin showed marked cytotoxicity on the proliferation of HepG2 cells

To determine the cytotoxicity, HepG2 cells were treated with various concentration (9.375–900 µg/ml) of NAR. The cell viability was significantly suppressed by NAR in a concentration gradient manner. 300 µg/ml of NAR and its subsequently ascending concentrations

induced marked cytotoxicity on HepG2 cell proliferations, as depicted in Figure 1c.

Naringin restored the morphological alterations in livers isolated from cancer bearing animals

The macroscopic images of livers isolated from different animal groups under study are represented in Figure 2a. Normal and partially hepatectomized liver looked almost similar with normal hepatic lobules. No macroscopical architectural changes or hepatic lesions were observed [Figure 2a]. However, Group III animals showed brown swelled liver with irregular and uneven hepatic surface. Prominent nodule formations along with detectable tumor masses and granular appearance were visible as well [Figure 2a]. Following treatment with NAR, these morphological alterations of hepatic surface were significantly improved with the highest dose (60 mg/kg/day) of NAR exhibiting maximum protection and restorative potential [Figure 2a].

Naringin altered body and liver weight in N-nitrosodiethylamine induced hepatocellular carcinoma bearing Sprague–Dawley rats

Animals bearing HCC in Group III showed a considerable decline in body weight ($P < 0.001$) with respect to Group I and Group II animals. NAR treatment significantly ($P < 0.001$) increased the body weight of cancer bearing animals. In comparison to induced control rats in Group III, Group IV (receiving NAR, 30mg/kg/day) and V animals (receiving NAR, 60 mg/kg/day) demonstrated dose dependently improvement in body weight, as shown in Figure 2b. NDEA treatment in Group III animals caused considerable ($P < 0.001$) increment in liver weight when compared to Group I, as shown in Figure 2c. While treatment with NAR (30 mg/kg/day) in Group IV showed moderately significant ($P < 0.05$) decrement in liver weight with respect to Group III, its higher dose (60 mg/kg/day dose) in Group V resulted in gradual normalization of liver weight ($P < 0.001$).

The liver-to-body weight ratio as determined in each group is given in Table 1. The liver-to-body weight ratio elevated considerably ($P < 0.001$) in Group III with respect to Groups I and II. Treatment with NAR 30 mg/kg/day and 60 mg/kg/day (Groups IV and V) produced significantly ($P < 0.001$) reduced results in the ratio.

Naringin attenuated the serum levels of liver marker enzymes and glycoproteins

Although Groups II animals showed inconsiderable ($P > 0.5$) alterations in the levels of liver marker enzymes (AST, ALT, ALP, and γ-GT) in comparison to Group I (normal control) SD rats, cancer induction in Group III animals considerably ($P < 0.001$) elevated the levels of corresponding enzymes [Figure 3a]. However, administration of NAR restored the transmuted enzyme profile almost back to normalcy. While NAR in lower dose (30 mg/kg/day) in Group IV prevented escalation of liver marker enzymes, ($P < 0.5$) with respect to induced control group, its higher dose (60 mg/kg/day) in Group V showed maximum ($P < 0.001$) protection, thereby supporting the ameliorative potency of NAR in diminishing the levels of liver marker enzymes [Figure 3a].

Similarly, the levels of glycoproteins hexose, hexosamine, and sialic acid in serum were profoundly ($P < 0.001$) augmented in Group III animals following development of hepatocellular cancer, as depicted in Figure 3b. On the contrary, treatment with 30 mg/kg/day and 60 mg/kg/day NAR (Groups IV and V), respectively, conferred to highly considerable ($P < 0.001$) minimization in glycoprotein level with respect to Group III.

Naringin improved the serum antioxidant status of hepatocellular carcinoma-bearing animals

The antioxidant profile of the experimental animals was also analyzed to validate the hepatoprotective efficacy of NAR [Figure 4]. LPO expressed as MDA nanomoles, in contrast was found to be significantly increased ($P < 0.001$) in Group III rats bearing HCC which upon NAR treatment was remarkably ($P < 0.001$) depleted dose dependently, as observed in Figure 4a. CAT GPx, SOD, GSH, and GR in serum were also observed to be significantly decreased ($P < 0.001$) in Group III rats when analyzed with Groups I and II animals, as shown in Figure 4b-f. NAR reinstated

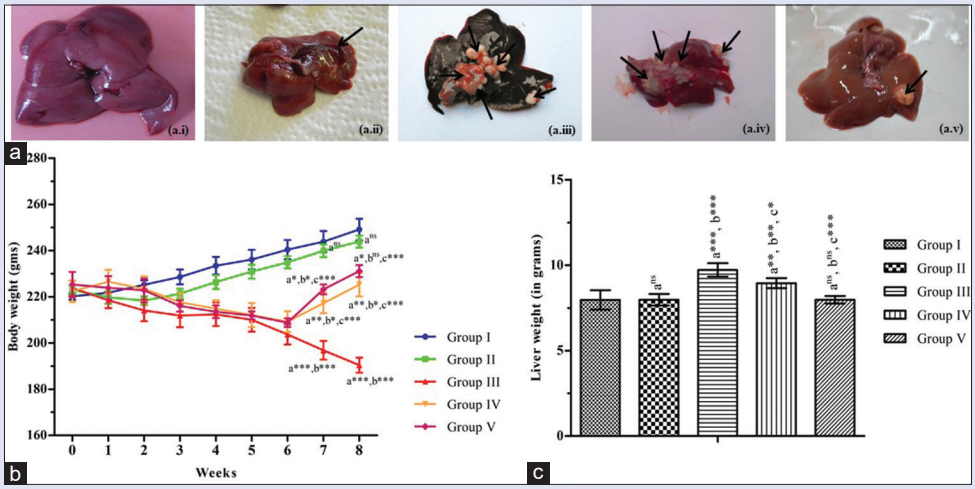

Figure 2: (a) Macroscopic representative pictorial views of liver isolated from animals belonging to individual groups under study depicting hepatic morphological characteristics (arrows indicate tumor and foci generation in liver). (b) Variations of mean body weight and (c) liver weights of animals from all experimental groups for the study period of 8 weeks. The values are represented as mean ± SEM; $n = 6$. Group I: Normal Control; Group II: PH Control; Group III: Induced Control (NDEA 200mg/kg b. w., i. p.); Group IV: NDEA + NAR (30mg/kg/day i. p.); Group V: NDEA + NAR (60mg/kg/day i. p.). Comparisons: a = Group II, III, IV, and V with Group I, b = Group III, IV, and V with Group II, c = Group IV and V with Group III. Significance: $^{ns}P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$;$^{***}P < 0.001$. PH: Partial hepatectomy; NDEA: N-nitrosoethylamine; NAR: Naringin

Table 1: Ratio between liver weight and body weight [(Rat liver weight (g)/Rat body weight (g)) x100] of the experimental animals from all groups

Groups	Number of animals	Body weight (g)	Liver weight (g)	Liver body weight ratio
Group I	6	249.092±1.91	7.967±0.23	3.192±0.079
Group II	6	245.87±0.76	7.975±0.138	3.237±0.062[a] (NS)
Group III	6	190.37±1.324	9.718±0.161	5.1±0.083[a,***, b,***]
Group IV	6	227.27±2.088	8.942±0.12	3.933±0.065[a,***, b,***, c,***]
Group V	6	206.98±1.069	7.97±0.088	3.848±0.027[a,***, b,***, c,***]

The values are represented as mean±SEM; *n*=6. Group I: Normal Control; Group II: PH Control; Group III: Induced Control (NDEA 200 mg/kg body weight i.p.); Group IV: NDEA+NAR (30 mg/kg/day i.p.); Group V: NDEA+NAR 60 mg/kg/day). Comparisons: [a]Group II, III, IV, and V with Group I; [b]Group III, IV, and V with Group II; [c]Group IV and V with Group III. **P*=*P* < 0.05; ***P*=*P* < 0.01; ****P*=*P* < 0.001. PH: Partial hepatectomy; NDEA: N-nitrosoethylamine; NAR: Naringin; NS: Not significant; SEM: Standard error of mean

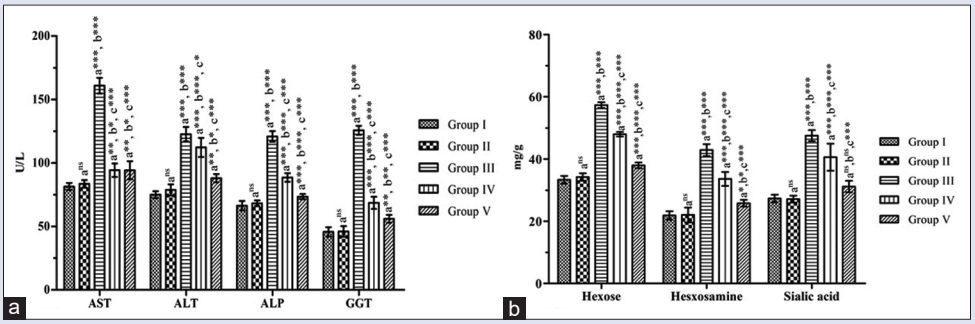

Figure 3: Effect of NAR on the (a) liver marker enzymes (AST, ALT, ALP, GGT) and (b) glycoprotein levels (hexose, hexosamine, sialic acid) in blood serum of experimental animals from all groups. The values are represented as mean ± SEM; *n* = 6. Group I: Normal Control; Group II: PH Control; Group III: Induced Control (NDEA 200mg/kg b. w. i. p.); Group IV: NDEA + NAR (30mg/kg/day i. p.); Group V: NDEA + NAR 60mg/kg/day i. p.). Comparisons: a = Group II, III, IV, and V with Group I, b = Group III, IV, and V with group II, c = Group IV and V with Group III. Significance: [ns]*P* > 0.05; **P* < 0.05; ***P* < 0.01; ****P* < 0.001. PH: Partial hepatectomy; NDEA: N-nitrosoethylamine; NAR: Naringin; AST: Aspartate dehydrogenase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase

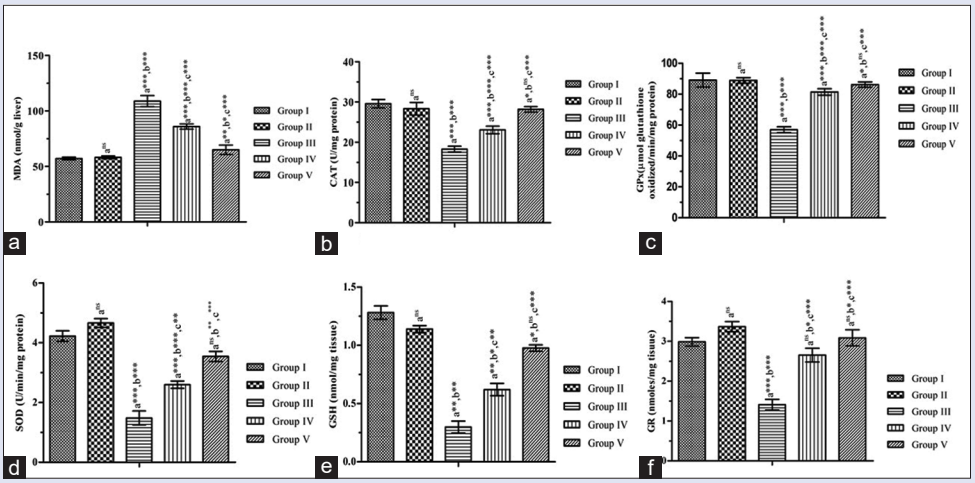

Figure 4: Effect of NAR on (a) lipid peroxidation and serum antioxidant status of (b) CAT (c) GPx, (d) SOD, (e) GSH, and (f) GR of experimental animals from all groups. The values are represented as mean ± SEM; *n* = 6. Group I: Normal Control; Group II: PH Control; Group III: Induced Control (NDEA 200mg/kg b. w. i. p.); Group IV: NDEA + NAR (30mg/kg/day i. p.); Group V: NDEA + NAR (60mg/kg/day i. p.). Comparisons: a = Group II, III, IV, and V with Group I, b = Group III, IV, and V with Group II, c = Group IV and V with Group III. Significance: [ns]*P* > 0.05; **P* < 0.05; ***P* < 0.01; ****P* < 0.001. PH: Partial Hepatectomy; NDEA: N-nitrosoethylamine; NAR: Naringin; SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; GR: glutathione reductase and GSH: reduced glutathione

the antioxidant status almost toward normalcy, with the higher dose of NAR (60 mg/kg/day) in Group V exhibiting profound upsurge in antioxidant levels ($P < 0.001$) when compared with Group III. Such outcomes validate the radical scavenging potency of NAR to revive the levels of antioxidant markers in liver cancer-induced animals.

Effect of Naringin on histopathology of N-nitrosodiethylamine induced hepatocellular carcinoma in rat liver

The liver tissues isolated from Groups I and II showed normal hepatic architecture and lobules, as shown in Figure 5. The nuclei

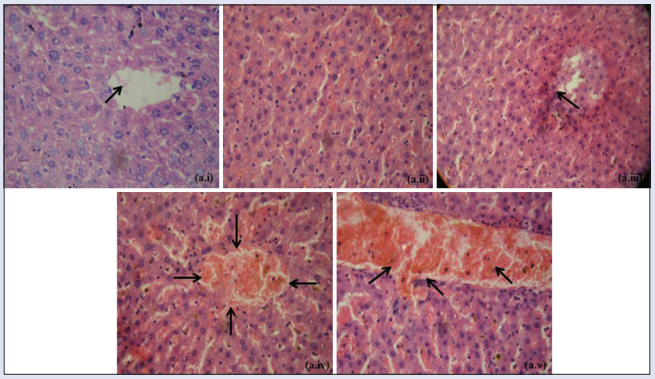

Figure 5: Histopathological characteristics of isolated liver tissues of experimental animals from all groups observed under a magnification of × 400. Group I: Normal Control; Group II: PH Control; Group III: Induced Control (NDEA 200mg/kg b. w.); Group IV: NDEA + NAR (30 mg/kg/day i. p.) and Group V: NDEA + NAR (60mg/kg/day i. p.). PH: Partial hepatectomy; NDEA: N-nitrosoethylamine; NAR: Naringin. Arrows represent the central vein in the liver in 5a. i, loss of architecture and hyperpigmentation of nuclei in 5a. iii and restoration of abnormal histopathological structures and lesser pyknotic nuclei in 5a. iv and 5a

observed in the hepatocytes were small and uniform. There was no presence of lipid droplets and blood congestion in the normal tissue histopathology [Figure 5a]. Group III liver tissues showed an abnormal architecture with larger and irregular hepatocytes having prominent nuclei. ECM was observed to be degraded by MMPs and rupture of matrix was elucidated along with the diluted sinusoids. Simultaneous cellular infiltration of inflammatory cells was prevalent with cytoplasmic leakage [Figure 5a]. Treatment with NAR 30 and 60 mg/kg/day (in Groups IV and V) reverted the abnormal hepatic architecture and lobules as fewer pyknotic nuclei were observed along with mild necrosis and focal degeneration [Figure 5a].

Naringin altered the level of matrix metalloproteinase-2 and matrix metalloproteinase-9 in hepatocellular carcinoma-bearing animals as evaluated by gelatin zymography

Liver tissue from all animal groups was tested to determine the level of MMP-2 and MMP-9 using gelatin zymography. Figure 6a shows zymogram of samples where lanes 1, 2, 3, 4, and 5 correspond to control, PH control, induced control, and treatment groups with doses of NAR, 30 mg/kg/day, and 60 mg/kg/day, respectively. Gelatinolytic zymography showed an enhanced expression of MMP-2 and MMP-9 in lane 3 which represented induced control group of animals. On treating with 30 and 60 mg/kg/day doses of NAR, the expression of MMPs was observed to be lessened in lanes 4 and 5.

Naringin attenuated the relative gelatinolytic activity quantitatively

After performing gelatin zymography, the gels holding the samples were verified with densitometric analysis for relative activity quantification of the two gelatinases. Figure 6b shows the average values of MMPs 2 and 9 in all the experimental animals. The densitometric values of MMPs were calculated to be highly significant when compared with Group I and III which upon treating with 30 and 60mg/kg/day doses of NAR were found to be attenuated in Groups IV and V. The band densities that demonstrate MMP-2 and MMP-9 in different groups are given in Table 2.

DISCUSSION

Natural products that are regularly consumed as components of human diets with minimum or negligible toxicity and high medical potential are attaining prominence in human health science.[42] The quest for new anticarcinogenic compounds in medicinal plants and traditional foods is a promising and realistic approach for cancer prevention.[43] One such potent bioflavonoid, NAR, has been reported to effectuate diversified pharmacological functions with promising hepatoprotective and inhibitory activity against proliferation of different cancer cell lines.[22,44] On the other hand, animal models on cancer are effective tools to understand pathogenesis of the disease and subsequent screening of drugs. Developing a model to mimic tumor heterogeneity and complex etiology of the disease similar to those in humans is a challenge.[45] NDEA (nitrosamines) is a well-established, genotoxic chemical carcinogen which causes hepatic injury by steatosis and death of hepatocytes which results in fibrosis and inflammation. NDEA causes primary multifocal HCC by inducing DNA damage and cell degeneration by alkylation of DNA structures and by ROS production activated by hepatic cytochrome P450.[46]

Carbon tetrachloride (CCl_4) and phenobarbital are used as promoters in NDEA-induced HCC in animals. The hepatotoxic potential of CCl_4 interferes with cell membrane integrity which results in progressive hepatocytic oxidative damage.[47] It also works by another mechanism of production of chemokines, cytokines, and pro-inflammatory parameters by inducing an inflammatory response by Kupffer and stellate cells. This activates lymphocytes, neutrophils, and monocytes which contributes to liver necrosis, fibrosis, and inflammation.[48-50] On the other hand, phenobarbital has been reported to increase the levels of tumor and angiogenesis markers, nitric oxide, lipid peroxidation, and pro-inflammatory cytokine-interleukin 6.[51] The current study was done to evaluate the potency of NAR (30mg/kg/day and 60mg/kg/day) on *in vivo* HCC cancer model and to verify the significance of MMPs as a prognostic biomarker in HCC through zymographic technique.

After carcinogenesis induction by NDEA, a significant decrease in body weight of animals was observed which was indicative of response related to stress due to hepatotoxicity and interference in metabolism which resulted in reduction of food intake.[52] The increase in liver weight in NDEA-treated animals was due to generation of tumor mass by rapidly proliferating cells in liver of the animals.[53] Treatment with NAR led to restoration of body and liver weight in animals. The effect of NAR was observed in morphological and histopathological studies where significant restoration of normal liver architecture and structure was observed. The glycoproteins hexose, hexosamine, and sialic acid are synthesized in liver and are circulated in blood. Sialic acid, an acylated neuraminic acid derivative, is a major glyco-component among glycoproteins. Glycoproteins are involved in various surface-related cell functions of different tissues; therefore, an increase in their levels was recorded in NDEA-treated animals which was significantly reverted to normal by treatment with NAR. Therefore, NAR administration can be effective against synthesis of glycoproteins in tumor cells.[54]

The extent of liver damage caused by the administration of NDEA and CCl_4 can be represented by the liver marker enzymes. Liver function and the extent of liver damage can be examined by elevated levels of ALP and γ-GT.[55] Likely, pathological hindrances in biliary flow and bilirubin discharge can be represented by elevated ALP activity. Similarly, elevation of γ-GT in serum, a plasma membrane enzyme in hepatocytes, indicates damage in the liver and shows high reliability as a hepatic damage marker.[52] NDEA-treated group of animals showed significant

Table 2: Peak areas of bands (band densities) representing matrix metalloproteinase-2 and matrix metalloproteinases-9 in different treatment groups

	Group I	Group II	Group III	Group IV	Group V
MMP-2 (AU)	1230±6.045	1620.84±4.37[a] (NS)	4372.59±10.24[a,***]	3943±1.022[a,**]	990.53±1.035[a,**]
MMP-9 (AU)	16865.51±23.12	16491±14.93[a] (NS)	19694±18.64[a,***]	21544.33±42.16[a,***]	17807±10.18[a,*]

Each value is represented as Mean±SEM. Group I: Normal Control; Group II: PH Control; Group III: Induced Control (NDEA 200 mg/kg body weight i.p.); Group IV: NDEA+NAR (30 mg/kg/day i.p.); Group V: NDEA+NAR 60 mg/kg/day). Comparisons: [a]Group II, III, IV, and V with group I; [b]Group III, IV, and V with Group II; [c]Group IV and V with Group III. Significance: NS=$P > 0.05$; *$P=P < 0.05$; **$P=P < 0.01$; ***$P=P < 0.001$. PH: Partial Hepatectomy; NDEA: N-nitrosoethylamine; NAR: Naringin; NS: Not significant; SEM: Standard error of mean; MMP: Matrix metalloproteinase; AU: Arbitrary units

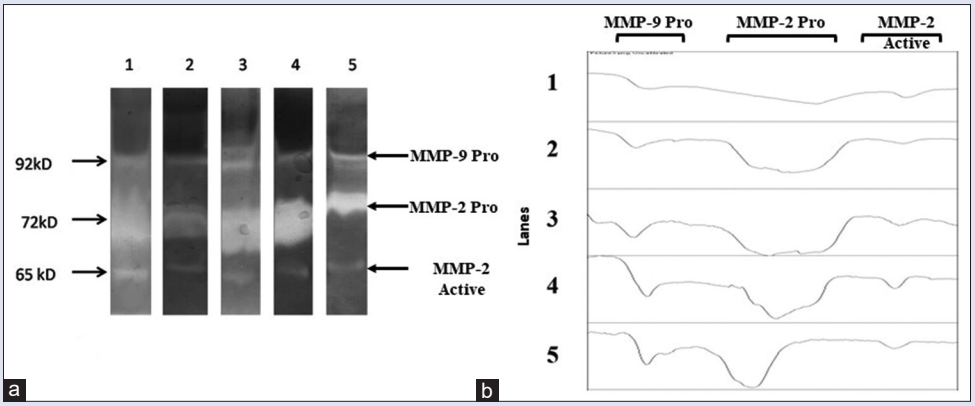

Figure 6: (a): Gelatin zymography of serum from experimental animals of all groups. Position of molecular weight markers is denoted by arrows on the left. Lanes 1, 2, 3, 4, and 5 denote gelatinolytic activities of groups: Normal control, PH control, induced control (NDEA 200mg/kg b. w.), NAR (30mg/kg/day i. p.), and NAR (60mg/kg/day i. p.), respectively. PH: Partial hepatectomy; NDEA: N-nitrosoethylamine; NAR: Naringin; MMPs: Matrix metalloproteinases. (b) Quantitative gelatinolytic densitometry profile of MMPs 2 and 9 in HCC-bearing experimental animals. 1, 2, 3, 4, and 5 denote the quantitative gelatinolytic analysis of Normal Control, PH Control, Induced Control (NDEA 200mg/kg b. w.), NAR (30 mg/kg/day i. p.), and NAR (60 mg/kg/day i. p.), respectively. PH: Partial hepatectomy; NDEA: N-nitrosoethylamine; NAR: Naringin; MMPs: Matrix metalloproteinases

elevation in liver marker enzyme levels which was significantly lowered by treatment with NAR, thereby concluding its hepatoprotective activity. NAR might be able to restore the membrane integrity which would have resulted in decrease in enzyme leakage and concurrent decline in serum enzymatic marker level.

Production of ROS causes cell death by oxidative degeneration of DNA and disruption of signal transduction pathways which regulates gene expression. NDEA functions by producing ROS because of which levels of CAT, GPx, SOD, GSH, and GR were found significantly reduced in induced control group.[56] Induced control group animals were subjected to oxidative stress due to metabolism of NDEA. As a result, membrane lipid peroxidation occurred which further resulted in generation of the end product, MDA which reach into the bloodstream. The level of MDA in NDEA-treated animals was elevated and hence considered as a biomarker for lipid peroxidation. Treatment with NAR led to reduction of MDA level in a dose-dependent manner which confirmed its free radical scavenging activity. SOD, a metalloenzyme, protects the cells from ROS insult.[57] It scavenges the superoxide anions by the formation of H_2O_2 which takes down free radical effect.[58] The induced control group showed a significant decline in SOD level in the study by the possible utilization of enzyme for scavenging off the H_2O_2 radicals. Catalase (CAT) is the first-line defense that is active against carcinogen oxidative degeneration. CAT decomposes the H_2O_2 produced in the tissues.[59] The NDEA-treated group of animals showed decreased CAT level which denoted the availability of CAT in the decomposition of H_2O_2 radical.[60] GSH, a nonprotein antioxidant present in the cell, plays a role in clearing off the superoxide free radicals and gives off electrons to GPx which is an enzyme involved in the reduction of H_2O_2 to H_2O.[61-64] NDEA-induced group showed decline in level of GSH which might be indicative of higher application of the enzyme for protection against free radicals.

Overexpression of MMP activities has been observed in many pathophysiological conditions such as arthritis, periodontal disease, multiple sclerosis, liver cirrhosis, and cancer. Reportedly, tumor-specific activation of pro MMP-2 results in invasion of certain cancers such as bladder, breast, and lung carcinomas along with tumor grade in case of HCC. MMP-2 overexpression has also been related to tumor dedifferentiation. Furthermore, MMP-9 is more expressed in cancerous tissues with respect to noncancerous tissues. Hereby, overproductivity of MMPs can be certainly related to cancer metastasis and invasion.[65] MMP-2 and 9 are type IV collagenases that degrade the basement membrane composed of type IV collagen. In HCC cases, the ongoing vascular invasion status can be reflected by plasma MMP9 levels.[66] MMP2 and MMP9 with molecular weights 66kDa and 76kDa can be predominantly seen where gelatin is employed as a substrate in zymography. The current study showed an elevated level of MMP2 and MMP9 in HCC bearing animal serum. Inactive form of MMP2 was found in normal as well as NDEA-treated liver sample but to variable extent. Zymogen form of MMP2 was detected only in the case of oncologic tissue. This suggests that MMP9 is found in well-developed HCC. Treatment with different doses of NAR was able to decrease the intensity of bands, thereby suggesting that the activity of MMPs has been suppressed but to different extents in each case. Treatment with 60mg/kg NAR showed better results. The results suggest that both forms of MMP2 get enhanced in the case of HCC as reported by Ogata *et al.*[65] Thus, it was concluded that both the MMPs were predominantly implicated in the case of HCC.

CONCLUSION

As the results of the study suggest, NAR was efficient in attenuating HCC induced by NDEA in SD rats. NAR stabilized the altered levels of liver

injury marker enzymes, antioxidant levels, cell surface glycoproteins, and had a profound effect on cell invasion and metastasis by lowering the enhanced expression of serum MMPs (MMP-2 and MMP-9) as was revealed by zymography technique. As a concluding remark, the role of NAR can be postulated as tumor static and an important adjuvant in existing chemotherapy.

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Conflicts of interest

There are no conflicts of interest.

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