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Effect of diosmin on apoptotic signaling molecules in *N*-nitrosodiethylamine-induced hepatocellular carcinoma in experimental rats

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Abstract
The aim of the present study was to evaluate the antioxidant and chemopreventive efficiency of diosmin against N-nitrosodiethylamine (NDEA)-induced hepatocarcinogenesis in adult male rats. Rats were classified into four groups as follows: Group I: Control, Group II: NDEA-induced hepatocellular carcinogenic rats, Group III: Cancer-bearing animals treated with diosmin (200 mg/kg/body weight/day) orally for 28 days, Group IV: Control animals treated with diosmin (200 mg/kg/body weight/day) alone for 28 days. The model of NDEA-induced HCC rats elicited significant increases in alpha-fetoprotein (AFP), lipid peroxidation (LPO) and increase in anti-apoptotic proteins (Bcl-2, Bcl-xL and Mcl-1) with a concomitant significant decline in liver antioxidant enzymes, pro-apoptotic (Bax and Bad) and caspase-3 &-9 proteins. The oral administration of diosmin as a protective agent normalized the altered levels of AFP, LPO, antioxidant enzymes, pro- and anti-apoptotic proteins as well as caspase-3 and -9 proteins. Transmission electron microscopical studies also revealed that treatment of diosmin has a perspective anti-cancer activity by rearranging hepatic cell structure and its integrity. Results of this study suggest that diosmin may be one of a pharmacological and therapeutic representative against hepatocellular carcinoma.

Keywords Hepatocellular carcinoma (HCC) · Diosmin · Apoptotic signaling · Antioxidant enzymes

Introduction

Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver and accounts for as many as one million deaths worldwide in a year. HCC is a global health problem and fourth leading cause of cancer-related deaths [1]. Chronic infection with hepatitis B virus (HBV), hepatitis C virus (HCV) are the major risk factors of hepatocellular carcinoma and it is also associated with exposure to aflatoxins, alcohol abuse and non-alcoholic fatty liver [2].

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One approach to control liver cancer is chemoprevention. The disease is prevented, slowed or reversed substantially by the administration of one or more non-toxic naturally occurring or synthetic agents. In this regard, recently naturally occurring polyphenols are receiving increased attention because of their promising efficacy in several cancer models [2, 3].

Flavonoids are polyphenolic bioactive compounds known universally over a billion of years that occur naturally in almost all dietary plants like fruits and vegetables. Since flavonoids are distributed in several medicinal plants and in folk medicine, flavonoids containing herbal remedies are practiced worldwide [4]. Numerous epidemiological studies have suggested that the consumption of fruits reduces the risk of cancer due to the polyphenolic compounds they contain. Plant-derived dietary polyphenolic compounds, such as flavonoids, with cancer cell-specific pro-apoptotic activity and chemopreventive potential are thought to be promising anti-cancer agents [4].

Diosmin is a well-known flavonoid having a broad spectrum of biological activities, including antioxidant,

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modulator of capillary permeability and anti-carcinogenic [5–7] widely used in medicine. Diosmin formulations are used for the treatment of chronic venous insufficiency, hemorrhoids, venous ulcers (especially of the lower limbs) and the prevention of postoperative thromboembolism [8–10]. It is widely used as prescription medicine in Europe mainly for its phlebotropic properties, while in USA is employed as a dietary supplement.

It is well known for boosting venous tone and lymphatic drainage with suppression of capillary hyperpermeability [11]. Interestingly, diosmin has exerted versatile beneficial effects against experimental diabetes mellitus [12], hepatic and renal injuries [13, 14], myocardial infarction [15] along with hepatocarcinogenesis [16, 17]. Lewinska et al. [18] reported that administration of three selected flavonoid glycosides (naringin, diosmin and hesperidin) in DU145 prostate cancer cell line, diosmin was found the most potent genotoxic agent in DU145 cells which in turn resulted in its pro-apoptotic activity.

Arab et al. [19] explained the gastroprotective actions of diosmin in ethanol gastric injury which were mediated via concerted multi-pronged actions, including suppression of oxidative stress, gastric inflammation and apoptosis besides boosting of the antioxidant and the cytoprotective defenses.The report stated that administration of diosmin to NDEA-induced hepatocellular carcinogenesis normalized the altered levels of changes in the body and organ weight, levels of the tumor marker enzymes (AST, ALT, ALP, LDH, γ-GT and 5′ND), xenobiotic enzymes (Phase I & II) and histopathological changes of the liver and kidney tissues suggesting that chemotherapeutic efficacy of diosmin [20]. However, molecular mechanisms underlying the protective effects of diosmin on pro- and anti-apoptotic and tumor suppressor proteins is obscure. Therefore, the present study was aimed at assessing potentials of diosmin on apoptotic signaling molecules in N-Nitrosodiethylamine-induced hepatocellular carcinoma in animal model.

Materials and methods

Chemicals

Diosmin and *N*-nitrosodiethylamine and mouse monoclonal anti-β-actin antibody were purchased from Sigma Chemical Company, St Louis, MO, U.S.A. All other chemicals and reagents used in this study were of molecular and analytical grade; and they were purchased from Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom; and Sisco Research Laboratories, Mumbai. Primary antibodies against Bcl-2, Bcl-xL, Mcl-1, Bax, Bad, caspase-9, caspase-3 and p53 were purchased from Cell Signaling (Danvers, Massachusetts, United States) and Santa Cruz Biotechnology (Texas, United States). The secondary antibodies, Horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG and goat-anti-rabbit IgG were obtained from Santa Cruz Biotechnology (Texas, United States).

Animals

Animals were maintained as per the National Guidelines and Protocols approved by the Institutional Animal Ethics Committee (IAEC No: 07/018/08). Healthy male albino rats of Wistar strain (*Rattus norvegicus*) weighing 180–210 g (150–180 days old) were used in this study. Animals were obtained and maintained in clean polypropylene cages under specific humidity (65 ± 5%) and temperature (27 ± 2 °C) with constant 12 h light and 12 h dark schedule at the Central animal house facility, University of Madras (Taramani campus). They were fed with standard rat pelleted diet (Lipton India, Mumbai, India), and clean drinking water was made available *ad libitum*.

Experimental design

Healthy adult male albino rats were divided into four groups consisting of six animals each. In the present study, diosmin dose (200 mg/kg body weight) was selected based on the study from our laboratory [20].

Group I—Normal control (vehicle treated; DMSO: 1 ml/kg body weight). Group II—Hepatocellular carcinogenic-induced rats (0.01% NDEA orally for 16 weeks).Group III—Cancer-bearing rats were treated with diosmin (200 mg/kg/body weight/day) orally for 28 days. Group IV—control rats treated with diosmin (200 mg/kg/body weight/day) alone for 28 days. At the end of the experimental period, animals were subjected to ether anesthesia; blood was collected from retro orbital plexus and serum was separated by centrifugation. Animals were sacrificed by cervical decapitation and liver tissues from control and treated animals were excised, washed in ice-cold saline and blotted to dryness. A 10% homogenate of the tissue was prepared in 0.1 M Tris–HCl buffer (pH 7.4), centrifuged and the clear supernatant was used for further analysis.

Biochemical analysis

Estimation of alpha-fetoprotein (AFP)

In this present study, level of AFP was estimated by solid phase enzyme-linked immunosorbent assay (ELISA) and the result was expressed in IU/ml.

Estimation of lipid peroxidation

In the present study, the lipid peroxidation levels in both serum and tissue were measured at the levels of basal, H_2O_2, ascorbate and $FeSO_4$-induced lipid peroxidation as per the standard methods [21, 22]. Results for same are expressed as n moles of TBARS formed/mg protein/min.

Measurement of enzymatic and non-enzymatic antioxidants

Superoxide dismutase (SOD) was estimated as per the methods of Marklund and Marklund [23] and the results are expressed as IU/mg protein/min. Activity of catalase was measured as per the previous method [24] and the results of catalase (CAT) activity are expressed as μmol of H_2O_2 consumed/mg protein/min. Glutathione peroxidase (GPx) was estimated by standard method [25] and results are expressed as μg of GSH utilized/mg protein/min. Reduced glutathione (GSH) was determined [26] and the results are expressed as μg of GSH/mg protein/min. Ascorbic acid (Vitamin C) level was measured by Omaye et al. [27] and Vitamin E was estimated as per the method [28] and results of the same are expressed as mg/dl.

Protein expression analysis by western blotting

Preparation of tissue lysate

Tissues from control and experimental animals were homogenized in RIPA buffer containing and protease inhibitor cocktail (Sigma) using a polytron-equipped homogenizer at a precise low setting on ice. The homogenate was centrifuged at 13,000×*g* for 10 min at 4 °C. The supernatant was centrifuged at 12,000×*g* for 15 min at 4 °C. The resultant supernatant was sampled as the total proteins for the Western blot analysis of p53, Bcl-2, Bcl-xl, Mcl-1, Bax, Bad, caspase-9 and caspase-3. Then, protein concentration was estimated as per the standard method [29] using bovine serum albumin (BSA) as a standard.

Western blot analysis of proteins

The sample (50 μg) was subjected to heat denaturation at 96 °C for 5 min with Laemmli buffer. Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gels and then transferred to PVDF membrane (Amersham Biosciences, UK). The membrane was blocked with 5% blocking buffer (Amersham Biosciences, UK) in TBS-T (Tris buffered saline and Tween 20), for 1 h at room temperature followed by incubation with primary antibody to Bcl-2, Bcl-xl, Mcl-1, Bax, Bad, caspase-9 and caspase-3 at a dilution of 1:1000. The membrane was subjected to repeated wash for three times with TBS-T and then incubated for 1 h in horseradish peroxidase (HRP)-conjugated mouse/rabbit secondary antibody by 1:7500 dilutions in TBS-T. The membrane was again subjected to repeated wash for three times with TBS and TBS-T. Protein bands were visualized in chemidoc using enhanced chemiluminescence reagents (ECL; Amersham Biosciences, UK). The detected bands were quantified by Quantity Software (Bio-Rad). Later, the membranes were incubated in stripping buffer [50 ml, containing 62.5 mM of Tris–HCl (pH 6.7) and 1 g of SDS and 0.34 ml of β-mercapto ethanol] at 55 °C for 40 min. Following this, the membranes were re-probed using β-actin antibody (1:5000). In this study, β-actin was used as the loading control.

Transmission electron microscopy (TEM)

Ultra-structural changes of the liver tissues were investigated by transmission electron microscopical studies as described [30]. Approximately, 1 mm of fresh liver samples or tissues were fixed immediately in glutaraldehyde (primary fixatives) for 4 to 6 h at 4 °C. Then, tissue was rinsed in wash buffer twice for 15 min each, and post-fixed in 1% buffered osmium tetroxide (secondary fixative) for 2 h at 4 °C. Subsequently, the tissue was washed thoroughly in wash buffer twice for 15 min each, to remove excess osmium tetroxide. Then, the tissue was dehydrated gradually in increasing concentration of ethyl alcohol (30, 50, 70, 80 and 90%) each for 10 min and finally with 100% alcohol twice. The alcohol was cleared using propylene oxide, the tissue was then infiltrated with propylene oxide and Epon 812 EMBED resin mixture (electron microscopy sciences, USA) at increasing concentration (25, 50, 75 and 100% resin) each for 2 h at room temperature using a slow speed rotary shaker. The infiltrate tissue was then embedded in epoxy resin (Epon 812 resin mixture) for 48 h at 60 °C. The blocks thus obtained, were then trimmed and sectioned using ultra-microtome (Leica ultra cut R ultra microtome) with a diamond knife. Initially, the semi-thin Sections (1) were stained with freshly filtered toulidine blue O solution (TBO) and were screened under high microscopy (binocular microscopy) for histopathological changes. Areas were chosen from semi-thin sections and ultra-thin sections were cut on copper grids and were stained in uranyl acetate and lead citrate, then screened under JEOL JEM 100 S transmission electron microscopy at 80 kV. The images of ultra-thin sections of TEM were photographed for subsequent evaluation.

Statistical analysis

Data were presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison method was used to compare the

means of different groups using SPSS 12.5 student's versions. $p < 0.05$ was considerable statistically significant in all cases.

Results

Effect of diosmin on alpha-fetoprotein (AFP)

AFP is reported as a specific marker for HCC. The level of AFP in control and experimental animals are presented in Fig. 1. In hepatocarcinogenic-induced rat, the levels of AFP was significantly elevated ($p < 0.05$) when compared to control animals. Conversely, these levels was considerably ($p < 0.05$) retrieved back to near normal in group III diosmin-treated animals. Whereas no noteworthy changes were observed in group IV diosmin alone-treated animals, when compared to group I.

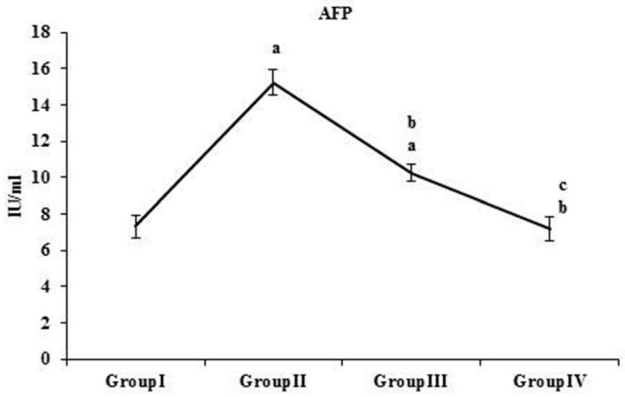

Fig. 1 Effect of diosmin on alpha-fetoprotein levels in serum of NDEA-induced animals. Each value represents mean ± SD of six animals. Significance at $p < 0.05$, (*a*) compared with control; (*b*) NDEA-induced cancer-bearing animals; (*c*) NDEA + Diosmin-treated animals

Effect of diosmin on the levels of LPO

The role of diosmin on LPO in the serum and liver of control and experimental animals are presented in Fig. 2a, b. The levels of LPO were found to be significantly increased ($p < 0.05$) in group II animals when compared to group I. On the contrary, the administration of diosmin significantly reduces ($p < 0.05$) the peroxidation in group III animals.

Effect of diosmin on the levels of enzymatic and non-enzymatic antioxidants

Enzymatic and non-enzymatic antioxidants are essential in downhill burden of cancer. In this connection, the effects of diosmin on the levels of antioxidant enzymes in serum are shown in Fig. 3a, b. NDEA-induced cancer-bearing animals (Group II) showed a significant decrease in SOD, CAT and GPx enzyme levels (Fig. 3a) compared to control ($p < 0.05$). Similarly GSH, vitamin E and C enzyme levels (Fig. 3b) were also found to be significantly decreased ($p < 0.05$) in cancer-bearing animals compared to control. Ironically, the levels of these antioxidants were increased significantly ($p < 0.05$) in diosmin-administered group III animals, compared to group II cancer-bearing animals. No remarkable changes were observed in group IV diosmin alone-treated animals when compared to group I control animals.

Effect of diosmin on the ultra-structural analysis of the hepatocytes in NDEA-induced hepatocarcinogenic rats

Hepatocyte ultra-structural alterations of experimental and control animals were demonstrated in Fig. 4. Group I control

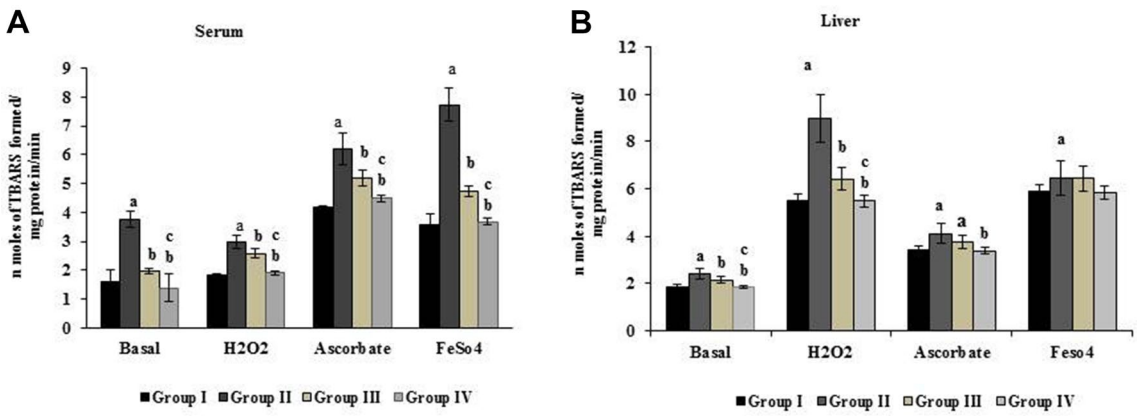

Fig. 2 Effect of diosmin on lipid peroxidation in serum and tissue of NDEA-induced animals. Each value represents mean ± SD of six animals. Significance at $p < 0.05$, (*a*) compared with control; (*b*) NDEA-induced cancer-bearing animals; (*c*) NDEA + Diosmin-treated animals

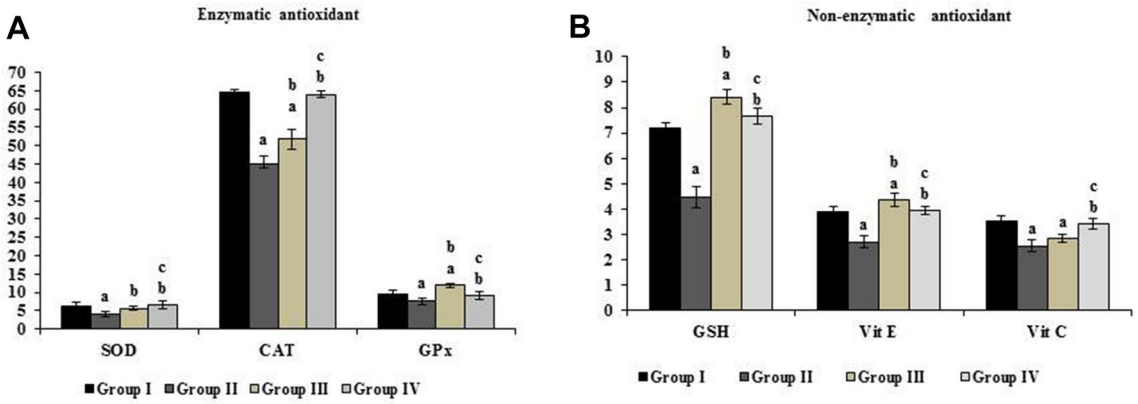

Fig. 3 Effect of diosmin on enzymatic and non-enzymatic antioxidants in liver of NDEA-induced animals. Each value represents mean ± SD of six animals. Significance at $p < 0.05$, (*a*) compared with control; (*b*) NDEA-induced cancer-bearing animals; (*c*) NDEA + Diosmin-treated animals. Units: SOD—units/mg protein; CAT—μ moles of H_2O_2 consumed/mg protein/min; GPX—μg of GSH utilized/mg protein/min; GSH—μg of GSH/mg protein/min; Vit C—mg/dL ; Vit E—mg/dL

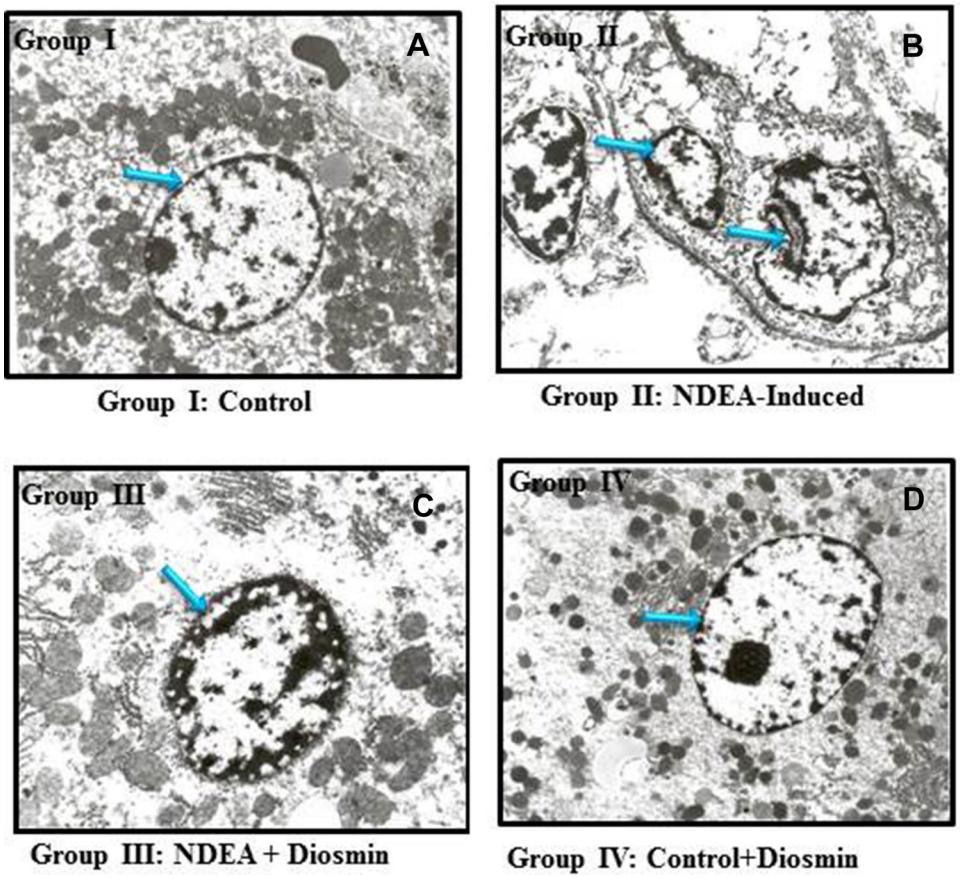

Fig. 4 Effect of diosmin on transmission electron micrographs of liver tissue of control and NDEA-induced animals. **a** Group I-control, showing normal hepatocytes and normal in shape; **b** Group II-NDEA-induced cancer-bearing animals, showing abnormal cells, disturbed mitochondria, irregular nucleus and nucleolus variation, mitotic cell edema; **c** Group III-NDEA + Diosmin-treated animals showing reduced DNA fragment, hepatocytes are retrieved to normal shape through apoptosis; **d** Group IV drug control animals showing normal nucleus and nucleolus, ring-shaped DNA, normal shape in mitochondria and free ribosomes

and group IV drug control rats showed habitual architecture of the liver cells with regular cytoplasm and normal nucleus. NDEA-induced hepatocytes exhibit irregular morphology, amorphous nucleus and reduced cytoplasmic content. Diosmin treatment brought back all the abnormality to near normal and put forward its medicinal property.

Effect of diosmin on the expression of Bcl-2 family members

To examine the status of intracellular signaling molecules in the diosmin-treated cancer-bearing animals, protein expression analysis was performed. In cancer-bearing animals

(Group II), pro-apoptotic protein such as Bad and Bax levels were found to be significantly reduced ($p < 0.05$) (Fig. 5a, b) compared to control (Group I). Conversely, anti-apoptotic proteins (Bcl-2, Bcl-xl and Mcl-1) were found to be significantly increased in cancer-bearing animals (Fig. 6a–c). Treatment with diosmin normalized the altered levels of pro- and anti-apoptotic proteins (Group III) caused by NDEA. No significant change was observed group between I and IV.

Effect of diosmin on the expression of p53 protein

The level of p53 protein in cancer-bearing animals was found to be significantly ($p < 0.05$) decreased (Group II) compared to Group I control animals (Fig. 7). However, treatment with diosmin significantly increased the same and brought back to the normal levels (Group III). No significant change was observed in diosmin alone-treated control animals (Group IV).

Effect of diosmin on the expression of caspase-9 and -3 proteins

To determine whether caspases are involved in the diosmin-induced apoptosis, protein expression analysis was examined. In NDEA-induced animals caspase-9 and -3 protein levels were significantly ($p < 0.05$) decreased (Group II), whereas diosmin treatment, the same were increased significantly (Fig. 8a, b). No significant change was observed in diosmin alone-treated control animals (Group IV). This shows that cell death is mediated by intrinsic apoptotic pathway.

Discussion

The goal of cancer chemoprevention is to slow, block or reverse the process of carcinogenesis through the use of natural or synthetic compounds [31]. Dietary compounds are reported to have the innate ability to modify the deregulated intracellular pathways thereby delaying the process of carcinogenesis [32]. Chemoprevention serves as an attractive alternative to control malignancy. Several chemicals are known to possess chemopreventive properties against a broad spectrum of cancers [33]. Numerous herbal drugs have been evaluated for their potential as liver protection against NDEA-induced hepatotoxicity in rats [34]. Recently, identification of bioactive ingredients from medicinal plants to inhibit tumorogenesis in a variety of experimental carcinogenesis, involving target of organ, such as the skin, lungs, oral cavity, Oesophagus, stomach, liver, pancreas, small intestine, colon, and prostrate is gaining considerable attention [35]. A number of non-nutrients including polyphenolic compounds have chemopreventive role in cancer through the induction of enzymes affecting carcinogen metabolism and inhibiting various activities of tumor promoters which are involved in the process of carcinogenesis [35].

Alpha-feto proteins is a unique immune-modulatory glycoprotein (65 kDa) normally made by the immature liver cells in the fetus and progressively lost during development, and it is virtually absent from healthy adult [36]. AFP is most extensively used tumor marker enzyme for diagnosis of HCC because AFP levels increase

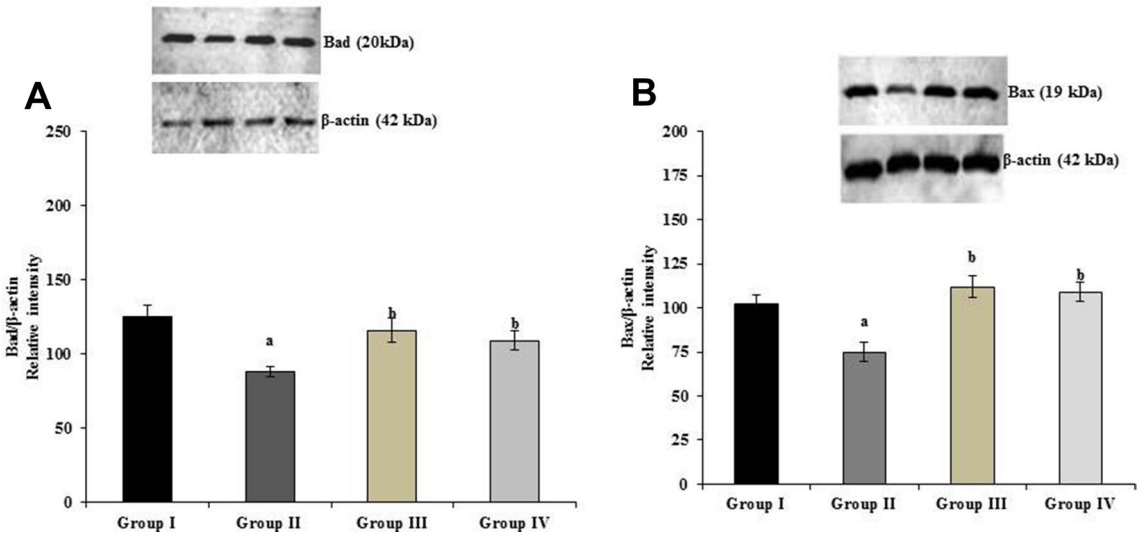

Fig. 5 Effect of diosmin on the expression of pro-apoptotic proteins. **a** Bad protein; **b** Bax protein. Each bar represents mean ± SD of three observations representing six animals. Significance at $p < 0.05$, (*a*) compared with group I control; (*b*) compared with group II NDEA-induced cancer-bearing animals

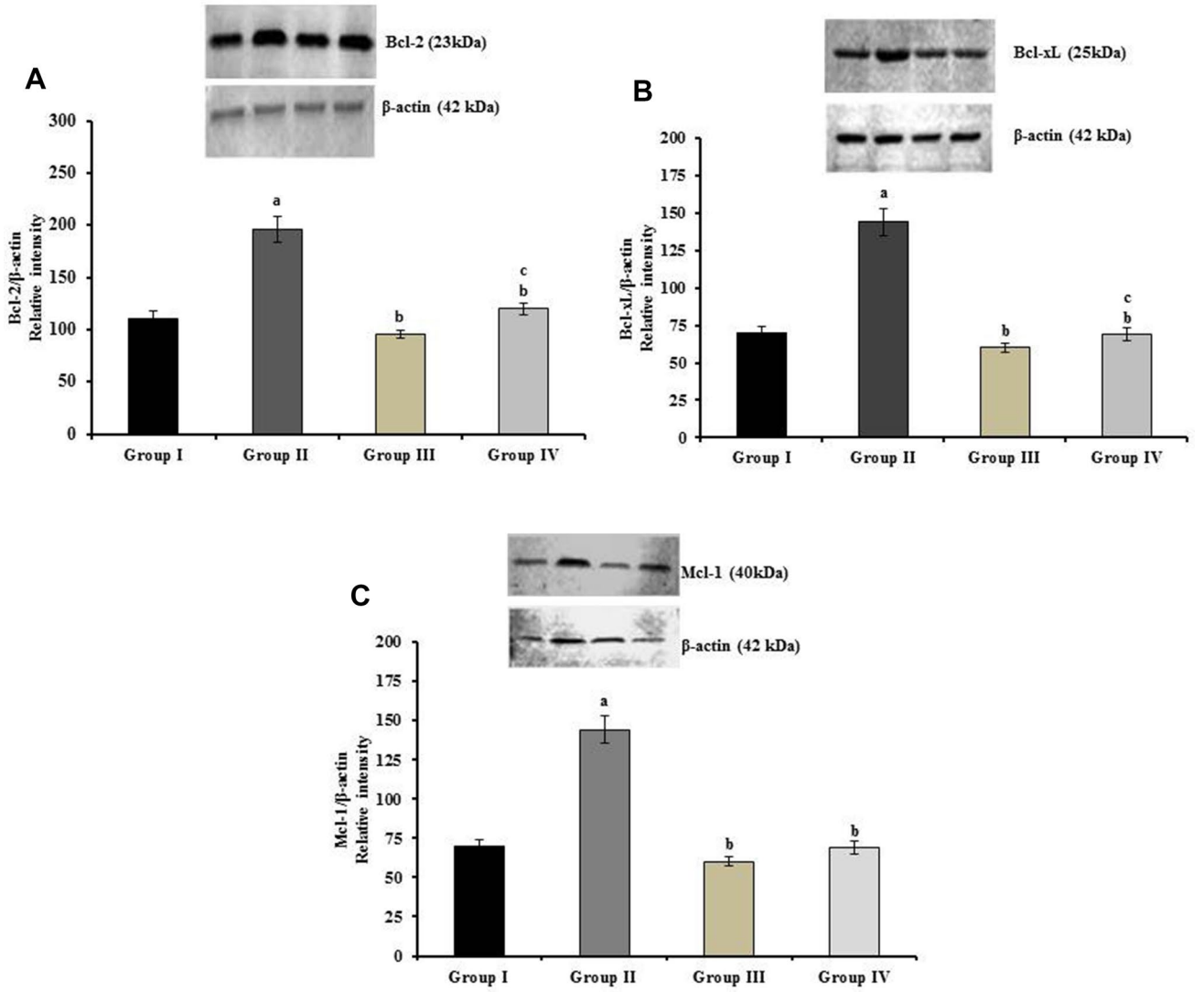

Fig. 6 Effect of diosmin on the expression of anti-apoptotic proteins. **a** Bcl-2 protein; **b** Bcl-xL protein; **c** Mcl-1 protein. Each bar represents mean ± SD of three observations representing six animals. Significance at $p < 0.05$, (*a*) compared with group I control; (*b*) compared with group II NDEA-induced cancer bearing animals; (*c*) compared with group III diosmin-treated animals

significantly in HCC patients. However, in animal model, it is one of the useful markers to analyze HCC, serum level of AFP which is dependent on regimen and in general AFP level in liver is related to growth rate and size [37]. The detection of AFP during HCC treatment is well accepted in patients with increased AFP levels prior to therapy and it is recommended by the European Association for the Study of the Liver (EASL). It has long been recognized that exposure of rats to certain carcinogens like NDEA increases the circulating AFP levels. This corroborates the results, this investigation showing significant rise in levels of AFP obtained in NDEA-induced group II rats. In group III, diosmin-treated animals shows the decreased level of AFP, this may be due to the strong counteracting property exhibited by the drug diosmin.

Generally, it is guaranteed that the NDEA is genotoxic, and is primarily associated to an excessive production of free radicals in the liver. As a consequence, reactive electrophilic intermediates are formed, which overwhelms the antioxidant defenses and ultimately proceeds to oxidative stress paving a way to liver damage [38]. NDEA induces a post-necrotic hepatocellular proliferation that contributes to enhance the number of initiated cells and it is accepted as a model to study the relations among liver necrosis, cancer initiation and replication [39].

It has been reported that elevated levels of LPO was observed in diethylamine-induced experimental hepatocarcinogenesis. There is disequilibrium between oxidant and antioxidant balance which is tilted towards oxidant side [40]. This oxidative stress may be the reason for the elevated lipid

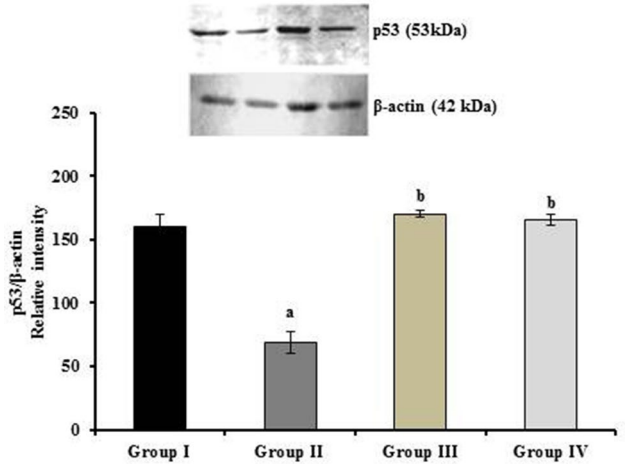

Fig. 7 Effect of diosmin on the expression of p53 protein. Each bar represents mean ± SD of three observations representing six animals. Significance at $p < 0.05$, (*a*) compared with group I control; (*b*) compared with group II NDEA-induced cancer-bearing animals

peroxidation levels in the liver of NDEA-induced animals. In this investigation, it was found that, there is a significant increase in the levels of lipid peroxidation in the liver of the animals induced with NDEA as a consequence of oxidative stress. However, in animals treated with diosmin exhibited significant dwindled levels of lipid peroxidation in the liver when compared with animals induced with NDEA. This may be due to the anti-lipid peroxidative function of diosmin and is probably mediated by its ability to inhibit free radical generation. Flavonoids can exert their antioxidant activity by various mechanisms through various ways such as free radicals scavenging or quenching, metal ions chelating or inhibiting enzymatic systems responsible for free radical generation [41].

Antioxidant defense mechanisms like enzymatic and non-enzymatic are gifted in living tissues. SOD is the first line of defense against superoxide radical-mediated oxidative damage in tumor cells [42].SOD engaged in decomposing of superoxide radicals ($O2^{\cdot-}$) and generated H_2O_2. In the peroxisomes, H_2O_2 is consequently detached to water by CAT, or in the cytosol GPx oxidizing GSH [42]. Therefore, the activities of these enzymes have been used to assess oxidative stress in cells. In our study, administration of NDEA significantly decreased these antioxidants in the liver tissues of experimental rats, whereas the enzyme levels were retrieved to near normal in diosmin-treated animals indicates the antioxidant potency of the drug and preventing the inactivity of these enzymes by ROS. Ahmed et al. 2016 [43] have also observed that decreased level of SOD, CAT, GSH were significantly restored after 28 days of diosmin treatment to alloxan-induced diabetic rat. The ability of diosmin to protect the enzymatic antioxidant system can be explained by the following mechanisms: (1) The compound itself may scavenge free radicals and/or prevent the loss of antioxidants by ROS and (2) additionally the compound can also act by upregulating endogenous antioxidant defenses [43].

Non-enzymatic scavenger's such as glutathione, ascorbic acid, and α-tocopherol are known to be second line of defense mechanism that scavenge residual-free radicals and defend antioxidant enzyme decomposition [44]. Glutathione is a major non-protein thiol in living organism that plays a fundamental role in coordinating antioxidant defense

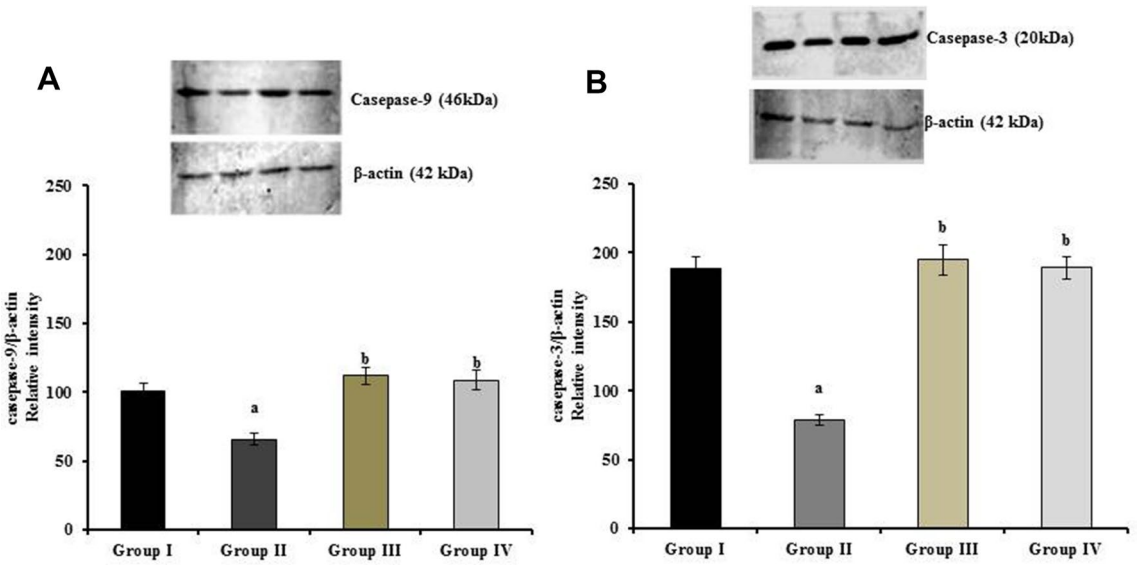

Fig. 8 Effect of diosmin on the expression of caspase-9 and -3 proteins. **a** Caspase-9; **b** Caspase-3 protein. Each bar represents mean ± SD of three observations representing six animals. Significance at $p < 0.05$, (*a*) compared with group I control; (*b*) compared with group II NDEA-induced cancer-bearing animals

process. It protects the essential thiol group from oxidation through straightforward acting in response to reactive oxygen species and electrophilic metabolites. In NDEA-induced rats, as an effect of oxidative stress, lowered glutathione symbolize the increased utilization of glutathione. Perturbation in the glutathione redox condition impairs cellular defense against toxic compounds and also results in oxidative injury [45].

Along with non-enzymatic antioxidant glutathione, another two biological defender ascorbic acid and α-tocopherol are significant scavengers of free radicals which protect cell membrane against toxic agents [46]. In this study, Vitamin C and E were found to be significantly decreased in cancer-induced condition when compared to that of control animals suggesting a decrease in antioxidant defense mechanism. In contrast, diosmin treatment upon their levels tends to become normal. Based on the results of our study, it may be possible to attribute the value of the diosmin to the anti-radical and antioxidant. Devaki et al. [47] elucidated that a diosmin treated restored the altered levels of enzymatic antioxidants such as SOD, CAT, GPx, GST and non-enzymatic antioxidants (vitamin C, vitamin E and glutathione) in high carbohydrate diet-induced hepatic steatosis.

The diagnosis of cancer cells is based on a variety of factors related to nuclear atypia, such as increase in the nuclear to cytoplasmic ratio, variation in nuclear size, irregular distribution of chromatin and increases in the amounts of DNA, RNA and protein [48]. In this present investigation, transmission electron microscopic studies discovered that DEN-induced hepatocyes showed multiple uncharacteristic nuclei with metastatic characteristic and asymmetrical cytoplasm. In addition, carcinogen-affected cell zone exhibit fragmentation, degranulation of the granular endoplasmic reticulum and a multiplicity of mitochondrial abnormalities. It was reported that DEN administration cause damage structure of the cell likely cell membrane injury, unbalanced cytoplasm and dysplastic nuclei. Diosmin management recovers architecture of hepatocytes with typical cell outline, balanced cytoplasm and shaped nucleus. Abdel-Daim et al. [49] also stated that diosmin (100 mg/kg) treatment showed a remarkable improvement of methotrexate MTX- induced histopathological changes in mice. It may be due to healing property of the drug and protection given to macromolecules from environmental carcinogen such as NDEA. It also maintains nutritional state of cell and proves its potential anti-carcinogenic perspective.

Apoptosis is a key process in cancer development [50]. Intrinsic pathway of apoptosis is one of the pathways activated by many cytotoxic drugs. Bcl-2 family members play a major role in the intrinsic pathway and this family contains 25 pro (e.g., Bax, Bok, Hrk, and Bad) and anti-apoptotic (e.g., Bcl-2, Bcl-XL, and Mcl-1) members which interact to maintain the balance between newly forming and old dying cells [50]. In the present study, NDEA-induced cancer-bearing animals showed increase in anti-apoptotic protein levels (Bcl-2, Bcl-xl and Mcl-1), while pro-apoptotic (Bax and Bad), p53, caspase-3 & -9 proteins levels were decreased. However, diosmin treatment significantly retrieved the altered levels of the signaling molecules. In this regard, it has been reported that diosmin induces genotoxicity and apoptotic cell death in DU145 prostate cancer cell line. In this study, also diosmin treatment could have contributed in the same manner. Thus, present data elucidate the antioxidant and antitumour effect of diosmin in NDEN-induced hepatocarcinogenic rat model via enhancing antioxidant defense and apoptotic cell death. This study confirms the potential efficacy of diosmin and suggests the use of diosmin as potential chemo-preventive drug.

Conclusion

Diosmin treatment powerfully attenuates lipid peroxidation reactions, regularize pathophysiological marker enzyme AFP equivalently promotes antioxidants proficiency of the cells. It safeguards cellular morphology from NDEA-induced damage and maintains customary occupation of the hepatocytes. It downregulated the anti-apoptotic proteins (Bcl-2, Bcl-xL and Mcl-1) and upregulated the pro-apoptotic proteins (Bax and Bad) and thereby paved the way to cell death. The p53, caspase-9 and -3 activation proved that diosmin acts through intrinsic apoptotic pathway. To conclude, the present study clearly shows that diosmin can be used as one of a therapeutic phytomedicines for the treatment of hepatocellular carcinoma.

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Compliance with ethical standards

Conflict of interest The authors declared that there is no conflict of interest from any of the authors.

References

1. Mittal S, El-Serag HB (2013) Epidemiology of HCC: Consider the Population. J Clin Gastroenterol 47:S2-S6. https://doi.org/10.1097/MCG.0b013e3182872f29
2. Caillot F, Derambure C, Bioulac-Sage P, Francois A, Goria O, Hiron M, Daveau M, Salier JP (2009) Transient and etiology-related transcription regulation in cirrhosis prior to hepatocellular carcinoma occurrence. World J Gastroenterol 15:300–309. https://doi.org/10.3748/wjg.15.300
3. Gurtsevitch VE (2008) Human oncogenic viruses: hepatitis B and hepatitis C viruses and their role in hepatocarcinogenesis.

Biochem (Mosc) 73:504–513. https://doi.org/10.1134/S0006297908050039
4. Lewinska A, Siwak J, Rzeszutek I, Wnuk M (2015) Diosmin induces genotoxicity and apoptosis in DU145 prostate cancer cell line. Toxicol In Vitro 29:417–425. https://doi.org/10.1016/j.tiv.2014.12.005
5. Bouskela E, Cyrino FZ, Lerond L (1997) Summary of Product Characteristics. Daflon 500 mg film-coated tablets. Micronized purified flavonoidfraction. Br J Pharmacol 122:1611–1616
6. Firuzi O, Miri R, Tavakkoli M, Saso L (2011) Antioxidant therapy: current status and future prospects. Curr Med Chem 18:3871–3888. https://doi.org/10.2174/092986711803414368
7. Silvestro L, Tarcomnicu I, Dulea C, Attili NR, Ciuca V, Peru D, Savu SR (2013) Confirmation of diosmetin 3-O-glucuronide as major metabolite of diosmin in humans, using micro-liquidchromatography-mass spectrometry and ion mobility mass spectrometry. Anal Bioanal Chem 405:8295–8310. https://doi.org/10.1007/s00216-013-7237-y
8. Tanaka T, Makita H, Kawabata K, Mori H, Kakumoto M, Satoh K, Hara A, Sumida T, Tanaka T, Ogawa H (1997) Chemoprevention of azoxymethane-induced rat colon carcinogenesis by the naturally occurring flavonoids, diosmin and hesperidin. Carcinogenesis 18:957–965
9. Katsenis K (2005) Micronized purified flavonoid fraction (MPFF): a review of its pharmacological effects, therapeutic efficacy and benefits in the management of chronic venous insufficiency. Curr Vasc Pharmacol 3:1–9. https://doi.org/10.2174/1570161052773870
10. Bogucka-Kocka A, Woźniak M, Feldo M, Kockic J, Szewczyk K (2013) Diosmin-isolation techniques, determination in plant material and pharmaceutical formulations, and clinical use. Nat Prod Commun 8:545–550
11. No Author (2004) Diosmin monograph. Altern Med Rev 9:308–311
12. Lyseng-Williamson KA, Perry CM (2003) Micronised purified flavonoid fraction: a review of its use in chronic venous insufficiency, venous ulcers and haemorrhoids. Drugs 63:71–100
13. Srinivasan S, Pari L (2012) Ameliorative effect of diosmin, a citrus flavonoid against streptozotocin-nicotinamide generated oxidative stress induced diabetic rats. Chem Biol Interact 195:43–51. https://doi.org/10.1016/j.cbi.2011.10.003
14. Rehman MU, Tahir M, Quaiyoom Khan A, Khan R, Lateef A, Hamiza OO (2013) Diosmin protects against trichloroethylene-induced renal injury in Wistar rats: plausible role of p53, Bax and caspases. Br J Nutr 110:699–710. https://doi.org/10.1017/S0007114512005752
15. Tahir M, Rehman MU, Lateef A, Khan R, Khan AQ, Qamar W (2013) Diosmin protects againstethanol-induced hepatic injury via alleviation of inflammation and regulation of TNF- alpha and NF-kappa B activation. Alcohol 47:131–139. https://doi.org/10.1016/j.alcohol.12.010
16. Queenthy SS, John B (2013) Diosmin exhibits anti-hyperlipidemic effects in isoproterenol induced myocardial infarcted rats. Eur J Pharmacol 718:213–218. https://doi.org/10.1016/j.ejphar.2013.08.031
17. Tahir M, Rehman MU, Lateef A, Khan AQ, Khan R, Qamar W (2013) Diosmin abrogates chemically induced hepatocarcinogenesis via alleviation of oxidative stress, hyperproliferative and inflammatory markers in murine model. Toxic Lett 220:205–218. https://doi.org/10.1016/j.toxlet.2013.04.004
18. Lewinska A, Siwak J, Rzeszutek I, Wnuk M (2015) Diosmin induces genotoxicity and apoptosis in DU145 prostate cancer cell line. ToxicolIn Vitro 29:417–425. https://doi.org/10.1016/j.tiv.2014.12.005
19. Arab HH, Salama SA, Omar HA, Arafa ES, Maghrabi IA (2015) Diosmin protects against ethanol- induced gastric injury in rats: novel anti-ulcer actions. PLoS ONE 10:e0122417. https://doi.org/10.1371/journal.pone.0122417
20. Perumal S, HariBabu L, Langeswaran K, Gowthamkumar S, Balasubramanian MP (2015) Pharmacological potential of natural flavonoid diosmin against n-nitrosodiethylamine- induced hepatocellular carcinogenesis in wistar albino rats. Int J Res Biosci 4:25–36
21. Hogberg J, Larson RE, Kristoferson A, Orrenius S (1974) NADPH-dependent reductase solubilised from microsomes by peroxidation and its activity. Biochem Biophys Res Commun 56:836–842. https://doi.org/10.1016/0006-291X(74)90681-0
22. Devasagayam TPA, Tarachand U (1987) Decreased lipid peroxidation in the rat kidney during gestation. Biochem Biophys Res Commun 145:134–138. https://doi.org/10.1016/0006-291X(87)91297-6
23. Marklund G, Marklund (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 47:469–474. https://doi.org/10.1111/j.1432-1033.1974.tb03714.x
24. Sinha AK (1972) Colorimetric assay of catalase. Anal Biochem 47(2):389–394. https://doi.org/10.1016/0003-2697(72)90132-7
25. Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hekstra WG (1973) Selenium biochemical role as a component of glutathione peroxidase purification and assay. Science 179:588–590. https://doi.org/10.1126/science.179.4073.588
26. Moron MS, Depierre JW, Mannervik B (1979) Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochem Biophys Acta 582:67–78. https://doi.org/10.1016/0304-4165(79)90289-7
27. Omaye ST, Turnbull JD, Sauberlich HE (1979) Selected methods for the determination of ascorbic acid in animal cells, tissues and fluids. Methods Enzymol 62:3–11. https://doi.org/10.1016/0076-6879(79)62181-X
28. Desai ID (1984) Vitamin E analysis methods for animal tissues. Methods Enzymol 105:138–147
29. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275
30. Robinson G, Gray T (1996) Electron microscopy 2: practical procedures. In: Bancroft JD, Stevens A (eds) Theory and practice of histological techniques, 4th edn. Churchill Livingstone, Edinburgh, pp. 586
31. Dimitrios S, Grigoris A, Antonios M, Dimitrios K (2012) Chemoprevention of liver cancer by plant polyphenols. Food ChemToxicol 50:2155–2170. https://doi.org/10.1016/j.fct.2012.04.002
32. Glauert HP, Calfee-Mason K, Stemm DN, Tharappel JC, Spear BT (2010) Dietary antioxidants in the prevention of hepatocarcinogenesis: a review. Nutr Food Res 54:875–896. https://doi.org/10.1002/mnfr.200900482
33. Shahjahan M, Vani G, Shyamaladevi CS (2005) Effect of Solanumtrilobatum on theantioxidant status during diethyl nitrosamine induced and phenobarbital promoted hepatocarcinogenesis in rat. Chem Biol Interact 156:113–123
34. Yang CS, Lambert JD, Ju J, Gang Lu, Sang S (2007) Tea and cancer prevention: molecular mechanisms and human relevance. Toxicol Appl Pharmacol 224:265–273. https://doi.org/10.1016/j.taap.2006.11.024
35. Plochmann K, Korte G, Koutsilieri E, Richling E, Riederer P, Rethwilm A, Schreier P, Scheller C (2007) Structure activity relationships of flavonoid-induced cytotoxicity on human leukemia cells. Arch Biochem Biophys 460:1–9. https://doi.org/10.1016/j.abb.2007.02.003
36. Sell S (2008) Alpha-fetoprotein, stem cells and cancer: how study of the production of alpha- fetoprotein during chemical hepatocarcinogenesis led to reaffirmation of the stem cell theory of cancer. Tumour Biol 29:161–180. https://doi.org/10.1159/000143402

37. Singhal A, Jayaraman M, Dhanasekaran DN, Kohli V (2012) Molecular and serum markers in hepatocellular carcinoma: predictive tools for prognosis and recurrence. Crit Rev Oncol Hematol 82:116–140. https://doi.org/10.1016/j.critrevonc.2011.05.005
38. Shabnam M, Shilpa B, Naveen C, Deepshikha K, Jain SK (2012) Den+ 2-AAF-induced multistep hepatotumorigenesis in Wistarrats:suppoertie evidence and insights. Protoplasma 250:175–183. https://doi.org/10.1007/s00709-012-0392-8
39. Nickerson J (2001) Experimental observations of a nuclear matrix. J Cell Sci 4:463–474
40. Rajkapoor B, Murugesh N, Chodon D, Sakthisekaran D (2005) Chemoprevention of N-nitrosodiethylamine induced phenobarbitol promoted liver tumors in rat by extract of *Indigofera aspalathoides*. Biol Pharm Bull 28:364–366. https://doi.org/10.1248/bpb.28.364
41. Lansky EP, Newman RA (2007) Punicagranatum (pomegranate) and its potential for prevention and treatment of inflammation and cancer. J Ethnopharmacol 109:177–206. https://doi.org/10.1016/j.jep.2006.09.006
42. Subash S, Essa MM, Al-Asmi A, Al-Adawi S, Vaishnav R, Braidy N (2014) Pomegranate from Oman alleviates the brain oxidative damage in transgenic mouse model of Alzheimer's disease. J Tradit Complement Med 4:232–238. https://doi.org/10.4103/2225-4110.139107
43. Ahmed S, Mundhe N, Borgohain M, Chowdhury L, Kwatra M, Bolshette N, Ahmed A, Lahkar M (2016) Diosmin modulates the NF-kB signal transduction pathways and downregulation of various oxidative stress markers in alloxan-induced diabetic nephropathy. Inflammation 39:1783–1797. https://doi.org/10.1007/s10753-016-0413-4
44. Shanmugam KR, Mallikarjuna K, Nishanth K, Kuo CH, Sathyavelu Reddy K (2011) Protective effect of dietary ginger on antioxidant enzymes and oxidative damage in experimental diabetic rat tissues. Food Chem 124:1436–1442
45. Zaanan A, Williet N, Hebbar M, Dabakuyo TS, Fartoux L (2013) Gemcitabine plus oxaliplatin in advanced hepatocellular carcinoma: a large multicenter AGEO study. J Hepatol 58:81–88. https://doi.org/10.1016/j.jhep.2012.09.006
46. Lobo V, Patil A, Phatak A, Chandra N (2010) Free radicals, antioxidants and functional foods: impact on human health. Pharmacogn Rev 4:118–126. https://doi.org/10.4103/0973-7847.70902
47. Devaki T, Binuclara J, Raghunandakumar S, Asokkumar S, Naveenkumar C, Premkumar T (2013) Anti-fibrotic effect of diosmin against dmn-induced liver fibrosisIn rats: a biochemical analysis. IJRPNS 2:283–292
48. Gönüllü U, Kato H (1995) Nuclear DNA and nuclear protein content of tumor cell in adenocarcinoma of the lung. Lung Cancer 13:13–19. https://doi.org/10.1016/0169-5002(95)00475G
49. Abdel-Daim MM, Khalifa HA, Abushouk AI, Dkhil MA, Al-Quraishy SA (2017) Diosmin attenuates methotrexate-induced hepatic, renal, and cardiac injury: a biochemical and histopathological study in mice. Oxid Med Cell Longev. https://doi.org/10.1155/2017/3281670
50. Kang MH, Reynolds CP (2009) Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. Clin Cancer Res 15:1126–1132. https://doi.org/10.1158/1078-0432.CCR-08-0144