Diosmin anti-tumor efficacy against hepatocellular carcinoma.

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Abstract

Background: Cancer is a life threatening deadly disease considered as a major health concern globally. Hepatocellular Carcinoma (HCC) is a foremost health anxiety in developing and developed nations due to a variety of etiologies factors, including Hepatitis virus, superfluous storage of iron in the body, exposure to toxins and carcinogens which directly affect the cellular mechanism.

Objective: The present investigation involved in analyzing anti-cancer potential of diosmin through in vitro and in vivo examination.

Materials and methods: The present study absorbed the anti-cancer activity of diosmin at 50, 100, 150, 200 μM/ml for 24 hours of treatment on dose dependent manner. Group II animals were administrated 0.01% NDEA to induce primary liver carcinoma. Cancer bearing experimental animals were treated orally with the drug diosmin at the dosage level of 200 mg per kg/body weight for 28 successive days.

Results: Diosmin treatment on HepG2 cells constrains nearly half of the cell population and proliferation at the dose of 100 μM/ml and in vivo experimental studies showed remarkable repossession in biochemical and morphological characteristics.

Conclusion: These results summarizes that both in vivo and in vitro anti-carcinogenic activity of diosmin could be an outstanding flavonoid in the treatment of HCC because of its therapeutic and pharmacological properties.

Keywords: Diosmin, Flavonoids, N-nitrosodiethylamine, SEM, HCC, HepG2 cells.

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Introduction

Cancer is the life aggressive metabolic disease with high mortality rate and the rate of incidence are also endlessly intensifying year by year. Carcinogenesis is an active phenomenon induced by one or several agents, and it is proceeds in three steps such as initiation, promotion, and progression. Liver cancer arises in the hepatocytes and is the common and fatal amongst altogether other types of cancer [1]. The liver cancer epidemiology is similarly growing internationally. Males and progressive age are accompanying with a higher occurrence of liver cancer. The incidence rate of the liver cancer is twofold higher in developing countries compared to developed countries [2].

Flavonoids are natural materials in plants that are believed to have progressive effects on human wellness. They are the largest and most widespread classes of plant compounds and possess diverse pharmacological and biological properties. Flavonoids are also deliberated significant ingredients in the human diet, although their daily consumption differs with dietary practices [3]. Numerous therapeutic properties have been recognized to flavonoids, particularly for their anti-carcinogenic, anti-oxidant, and anti-inflammatory properties [4]. In contemporary days, flavonoids as strong free radical scavengers have concerned a remarkable attention as promising therapeutics against free radical facilitated ailments. They serve as a tremendous free radical trapper because they are extremely reactive as electron donors or hydrogen [5]. Citrus plants are great interest because of their leaves and fruits contains huge number of flavonoid glycosides. Citrus flavonoid has been reported to have a wide-ranging of biological action with anti-cancer activities. In addition, it has healthiness related possessions, which including medicinal effects against many viral pathogens, favourable effects against inflammatory, effects on capillary fragility and human platelet aggregation stoppage potential [6]. Based on this activity, citrus plant and its flavonoids can be considered as chemopreventive molecules. In this juncture, as a flavonoid diosmin derives to play a substantial part to extravagance numerous ailments. Diosmin is a significant flavonoid in citrus, it is a naturally derived from flavone glycoside. It could be collected from numerous plant sources or derived from the hesperidin, a flavonoid [7]. Diosmin is the chief constituent of Buchu leaf (Barosma betulina, Rutaceae) and is also occur in other
Materials and Methods

In vitro investigation

Cell culture: Liver cancer cell line (HepG2) was procured from NCCS, Pune, India. Human hepatoma cells were periodically grown at 37°C as monolayer cultures in a humidified condition of 5% CO₂ in 95% O₂ in Dulbecco Modified Eagles Medium comprising 50 IU/ml of antibiotic penicillin and 50 µg/ml of streptomycin. 10% (v/v) with FBS (Fetal Bovine Serum).

Chemicals: Diosmin, was purchased from Sigma chemicals, DMEM medium and sodium pyruvate were bought from Biochrome, Germany. Penicillin, streptomycin and fetal bovine serum were acquired from Gibco, Germany. Trypsin-EDTA was acquired from Hi-media, India. Culture plates were purchase from TTP, Switzerland.

Cytotoxicity assays: Cell sustainability was assessed by MTT method [9]. Lactate dehydrogenase (LDH) activity was assessed [10]; reduced glutathione was assayed [11].

In vivo studies

Chemicals: Carcinogen N-nitrosodiethylamine were purchased from Sigma Chemical Company, St Louis, MO, U.S.A.

Animals: The experimental protocol was approved by the Institutional Animal Ethics Committee for experimental clearance (IAEC No 07/018/08.). Adult healthy male Wistar albino strain rats weighing between 160 ± 20gm were used from the Central Animal House facility of Dr. ALM PGIBMS, University of Madras, Chennai 600 113, India. The animals were independently housed, retained in clean polypropylene cages and fed. The animals were given commercially available pelleted rat feed were provided with clean drinking water ad libitum. Throughout the experimentation period all the ethics guidelines were firmly followed.

Dose fixation: The dose regimen of diosmin was selected based on LD₅₀ was found to be 3000 mg/kg body weight [12]. Based on LD₅₀, the sub lethal dose i.e. one third of the LD₅₀ (150, 200, and 250 mg/kg body weight) were designated. During the administration of diosmin at the dose of 200 mg/kg b/w did not displayed any irregularities such as toxic, rotating, lacrimation, lowered breathing etc. till end of the study. Based on the acute toxicity studies 200 mg/kg b/w of diosmin was elected for the present investigation.

Experimental design: The rats were divided into four separate sets with six animals in each group. Group I administrated with DMSO as a vehicle at the dose of 1 ml/kg b.w and served as control group. Group II animals were induced cancer in hepatocytes by administrating 0.01% NDEA through consumption of water for the duration of 16 weeks. Group III animals HCC rats subsequently treated with diosmin at the dose of 200 mg/kg/b.w orally for 28 successive period. Group IV animals were given diosmin alone at the dose as same as the previous group for 28 days.

Collection of samples: Animals were anaesthetized at the end of the experimental period, and the blood was collected and serum was parted by centrifugation. The liver and kidney tissues were removed, washed in ice-cold saline. Using 0.1 M Tris–HCl buffer (pH 7.4), a 10% of the liver homogenate and kidney homogenate tissue were prepared for further biochemical investigation.

Biochemical parameters: The SOD was assayed [13], the catalase was estimated [14], Glutathione peroxidase was estimated [15], reduced glutathione was assayed [11], ascorbic acid was assessed [16], Vitamin E was assessed [17], Na+ K+-ATPase was estimated [18], Ca²⁺-ATPase was assayed [19], Mg²⁺-ATPase was estimated [20], The Isocitrate dehydrogenase ICDH was estimated [21], SDH was assayed [22], The enzyme MDH (Malate dehydrogenase) activity was assayed [23], the activity of α-ketoglutarate dehydrogenase was estimated [24], Hexokinase was assayed [25], Phosphogluco-isomerase was assayed [26], Aldolase was estimated [21], Glucose-6-phosphatase and Fructose 1,6-diphosphatase was assayed [27], Hexose was estimated [28], Hexosamine was estimated [29], Sialic acid was determined [30].

Scanning electron microscopic examination of liver tissues: Samples were fixed with modified Karnovsky’s fluid 19 and 0.1 M sodium phosphate buffer at pH 7.4 was added. Fixation was performed for 10-18 hours at 4°C; subsequently in fresh buffer the tissue was washed. With 1% osmium tetroxide with the 0.1 M sodium phosphate buffer at 4°C the post fixation was done. After several washes, the specimens were dehydrated. Samples were additionally dried, and then the tissue samples were fixed on the aluminium stumps, and gold coating about 35 nm thicknesses of the tissue samples was attained. Finally, the samples were examined under scanning electron microscope.

Results

The cytotoxic effects of natural substances and various chemicals on tumour cell lines have been widely considered as a foremost tool for identifying anti-tumour actions. MTT dye reduction has been used successfully to quantify the cytotoxicity of various compounds and to enumerate anti-proliferative efficacy of the drug. The results of the viability of control and diosmin treated (50, 100,150 and 200 µM/ml) HepG2 cells are presented in the Figure 1. In this present investigations, the diosmin remarkably inhibits the hepatoma cell line (HepG2) after 24 hours of treatment. From this result, it is inferred that diosmin treatment showed marked inhibition of HepG2 cells viability in a concentration basis method. Quantification of LDH Leakage has been shown to be more reliable method for measuring cellular damage or impairment.
in cytotoxicological studies. Cytosolic leakage of LDH enzymes, as a sign of cytotoxicity. The lactate dehydrogenase (LDH) levels released into the medium of control and diosmin treated (50, 100, 150 and 200 μM/ml) HepG2 cells are presented in Figure 2. From this study, it was observed that LDH activities establish to be considerably elevated after 24 hours of exposure in the medium incorporated with diosmin. Glutathione is a universal molecule that plays a significant role in cellular free radical metabolism and xenobiotic detoxification mechanism. It has been found that the measure of GSH are bigger in cancer cells with drug resistance when matched to drug sensitive cells. The GSH levels of normal and diosmin treated HepG2 cells were displayed in Figure 3. From this present investigations, it is concluded that a substantial reduction of GSH was observed in diosmin treated HepG2 cells in a dose dependant manner.

Figure 1. Effect of diosmin on HepG2 cells for 24hour-MTT assay. Each Bar represents mean ± SD of six observations, a-Control vs. DMSO, 50, 100, 150, and 200 μM 24 hours.

Figure 2. Level of LDH in control and diosmin treated HepG2 cells.

Living tissues are accomplished with antioxidant defence mechanisms encompassing enzymatic and non-enzymatic antioxidants. A reduction in the activities of these antioxidant enzymes relate to the deposit of enormously reactive free radicals and significant destructive toxic effects on cell membranes, macromolecules and their functions. In this association, the importance of diosmin on the antioxidant enzymes levels were estimated from the serum, liver and kidney were presented in Figure 4 and Tables 1 & 2 respectively.

The NDEA induced cancer bearing animals showed a noteworthy diminution of enzymic antioxidants such as CAT, SOD, GPX (p<0.001) and non-enzymic antioxidants such as Vit-C GSH, and Vit-E (p<0.001). These antioxidants enzymes activities were considerably amplified (p<0.01) in drug treated rats, when compared to animals with cancer. The activities of antioxidants (p<0.001) in liver and kidney of cancer induced animals is noticeably decreased. These antioxidant (p<0.001) were significantly increased (p<0.01) when compared to group II animals. No such distinguished deviations were noticed in drug control group IV animals when matched to DMSO alone administrated control rats.

ATPases have been referred to as conspicuous energy linked enzymes perceived in all living organisms which supplies metabolic energy. The ATPase enzymes activities specify an active passage system that may be liable for the active transport of ions such as K⁺, Na⁺, Mg⁺ and Ca⁺⁺ through cell membranes. It is also susceptible to oxyradical-linked impairment and lipid peroxidation. The membrane bound ATPase (Ca⁺⁺, Na⁺/K⁺, and Mg⁺⁺) in erythrocyte membrane and in liver tissues were presented in Figure 5 and Table 3. A statistically substantial decrease (p<0.001) of Na⁺/K⁺ and Mg⁺⁺ ATPase levels were perceived in group II cancer bearing animals, when compared to group I rats. The level of serum and liver Ca⁺⁺ ATPase was significantly augmented (p<0.001) in group II cancer bearing rats. In contrast the level of Ca⁺⁺ ATPase was considerably decreased (p<0.001) in diosmin treated group rats. Interestingly, these abnormal levels were brought back to near normal (p<0.001) in diosmin treated animals. Nevertheless, there was no substantial modification were observed in group IV diosmin alone treated rats when equated to the group I control.
The dysfunction of TCA cycle has been concerned to play an imperative role in the cancer formation. Reduction in the activities of TCA cycle enzymes might be owing to the modification in cell structure, morphology and role of mitochondria to endure metabolic alterations and mitochondrial quantity is considerably abridged in malignancy cells. The levels of TCA cycle enzymes in the liver of experimental and control animals were illustrated in Figure 6. Substantial decline (p<0.001) in the activities of SDH, ICDH, α-KGDH and MDH enzymes were detected in group II cancer animals, when compared to control group I. On the contrary, the levels of SDH, ICDH, α-KGDH and MDH were expressively increased (p<0.001) in diosmin treated animals. No evident alteration was detected in diosmin alone treated animals.

In Table 4, the activities of diosmin on carbohydrate metabolizing enzymes in liver of control and experimental animals were presented. The activities of these enzymes such as aldolase hexokinase and phosphoglucoisomerase were significantly (p<0.001) increased. On the other hand, enzymes like glucose-6-phosphatase and fructose-1-6-diphosphatase were reduced (p<0.001) in malignance bearing rats when compared with normal group I rats. Instead, all the above-mentioned enzymes were considerably (p<0.001) reverted to near normal in diosmin treated rats. No remarkable variations were spotted in diosmin treated group IV rats.

Glycoproteins are found in highest concentration in animal fluids such as plasma and urine as well as in connective tissues. Elevation of glycoprotein substances are significant indicator of cancerous conditions and these variations modify the rigidity of cell membranes. Tumour cell plasma membrane alteration and their outflow may be the reason for the detected raises of these glycoproteins during uncontrolled cell proliferation condition. The level of the sialic acid hexose and hexosamine in liver of experimental and control groups were depicted in Figure 7. The level of sialic acid hexose and hexosamine were found to be augmented considerably in cancer bearing animals (p<0.001). In contrast, the level of glycoproteins was reverted to normal level in diosmin treated animals (p<0.05). There was no noteworthy variation in diosmin alone treated animals.

Scanning electron microscopic results were depicted in Figure 8. Under microscopic observation, group I control and drug control group IV liver tissues presented normal architecture of hepatocytes with regular nuclei whereas in cancer induced group III exhibited disturbed and abnormal hepatocytes with binucleated and enlargement in the size of the cells. Carcinogen treated liver tissue section presented asymmetrical sinusoids, nuclei were injured cracked hepatocytes and tumoral vacuoles. These structural abnormalities were recovered in drug treated liver sections under microscopic examination. SEM analysis displayed morphological restoration of liver tissues in drug treated animals.
Figure 6. The level of mitochondrial TCA cycle enzymes in the liver of control and experimental animals. (Note: Each value represents means ± SD of six animals). Units: ICDH is n moles of α-ketoglutarate formed/mg protein/min; SDH is μ moles of succinate oxidised/mg protein/min; MDH is n moles of NADH oxidised/mg protein/min; α-KGDH is μ moles of potassium ferrocyanide liberated/mg protein/min ; a-Group II, III & IV compared with Group I; b-Group III compared with Group II; *p<0.001, #p<0.01, @p<0.05, NS-Not Significant.

Figure 7. The levels of glycoproteins in the liver of control and experimental animals. (Note: Each value represents means ± SD of six animals). a-Group II, III & IV compared with Group I; b-Group III compared with Group II; *p<0.001, #p<0.01, @p<0.05, NS-Not Significant.

Table 1. The level of enzymic and non-enzymic antioxidants in liver of control and experimental animals.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I (Control)</th>
<th>Group II (DEN)</th>
<th>Group III (DEN+Diosmin)</th>
<th>Group IV (Diosmin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD IU/mg protein/min</td>
<td>14.2 ± 0.35</td>
<td>7.61 ± 0.79*</td>
<td>8.92 ± 0.56</td>
<td>14.28 ± 0.57NS</td>
</tr>
<tr>
<td>Catalase (µ mol of H₂O₂ consumed/mg protein/min)</td>
<td>64.4 ± 1.87</td>
<td>44.91 ± 4.87*</td>
<td>51.72 ± 3.8*</td>
<td>63.97 ± 2.6NS</td>
</tr>
<tr>
<td>GPx (µg of GSH utilized/mg protein/min)</td>
<td>10.22 ± 0.2</td>
<td>7.78 ± 0.75*</td>
<td>8.95 ± 0.61</td>
<td>10.14 ± 0.42NS</td>
</tr>
<tr>
<td>Vitamin – C (mg/dl)</td>
<td>3.33 ± 0.08</td>
<td>2 ± 0.21*</td>
<td>3.03 ± 0.22</td>
<td>3.25 ± 0.11NS</td>
</tr>
<tr>
<td>Vitamin – E (mg/dl)</td>
<td>5.09 ± 0.09</td>
<td>3.51 ± 0.31*</td>
<td>4.1 ± 0.26</td>
<td>5.22 ± 0.19NS</td>
</tr>
<tr>
<td>GSH (µg of GSH/mg protein/min)</td>
<td>12.55 ± 0.08</td>
<td>9.2 ± 0.21*</td>
<td>11.11 ± 0.22</td>
<td>12.28 ± 0.11NS</td>
</tr>
</tbody>
</table>

Each value represents means ± SD of six animals
a-Group II, III & IV compared with Group I
b-Group III compared with Group II
Table 2. The level of enzymic and non-enzymic antioxidants in kidney of control and experimental animals.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I (Control)</th>
<th>Group II (DEN)</th>
<th>Group III (DEN+Diosmin)</th>
<th>Group IV (Diosmin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (IU/mg protein/min)</td>
<td>5.34 ± 0.14</td>
<td>4.41 ± 0.48</td>
<td>4.92 ± 0.37@B</td>
<td>5.38 ± 0.27NS</td>
</tr>
<tr>
<td>CAT (µ mol of H₂O₂ consumed/mg protein/min)</td>
<td>46.14 ± 1.80</td>
<td>30.24 ± 3.34</td>
<td>39.61 ± 2.85@B</td>
<td>46.01 ± 2.37NS</td>
</tr>
<tr>
<td>GPx (µg of GSH utilized/mg protein/min)</td>
<td>3.23 ± 0.06</td>
<td>2.69 ± 0.26</td>
<td>2.94 ± 0.22@B</td>
<td>3.27 ± 0.14NS</td>
</tr>
<tr>
<td>Vitamin – C (mg/dL)</td>
<td>2.41 ± 0.08</td>
<td>1.52 ± 0.15</td>
<td>2.18 ± 0.16@B</td>
<td>2.45 ± 0.11NS</td>
</tr>
<tr>
<td>Vitamin – E (mg/dL)</td>
<td>3.37 ± 0.08</td>
<td>2.21 ± 0.18</td>
<td>2.62 ± 0.16@B</td>
<td>3.40 ± 0.11NS</td>
</tr>
<tr>
<td>GSH (µg of GSH/mg protein/min)</td>
<td>2.72 ± 0.08</td>
<td>1.56 ± 0.14</td>
<td>2.24 ± 0.15@B</td>
<td>2.81 ± 0.12NS</td>
</tr>
</tbody>
</table>

Each value represents means ± SD of six animals
a-Group II, III & IV compared with Group I
b-Group III compared with Group II
*p<0.001, #p<0.01, @p<0.05, NS-Not Significant

Table 3. The level of ATPase in the liver of control and experimental animals.

<table>
<thead>
<tr>
<th>Parameters (µ mol of inorganic phosphate liberated/mg protein/ min)</th>
<th>Group I (Control)</th>
<th>Group II (DEN)</th>
<th>Group III (DEN+Diosmin)</th>
<th>Group IV (Diosmin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺/K⁺ ATPase</td>
<td>0.32 ± 0.007</td>
<td>0.19 ± 0.01</td>
<td>0.25 ± 0.01@B</td>
<td>0.30 ± 0.01NS</td>
</tr>
<tr>
<td>Ca²⁺ ATPase</td>
<td>0.29 ± 0.007</td>
<td>0.54 ± 0.05</td>
<td>0.39 ± 0.03@B</td>
<td>0.31 ± 0.01NS</td>
</tr>
<tr>
<td>Mg²⁺ ATPase</td>
<td>0.23 ± 0.05</td>
<td>0.12 ± 0.01</td>
<td>0.17 ± 0.01@B</td>
<td>0.25 ± 0.01NS</td>
</tr>
</tbody>
</table>

Each value represents means ± SD of six animals
a-Group II, III & IV compared with Group I
b-Group III compared with Group II
*p<0.001, #p<0.01, @p<0.05, NS-Not Significant

Table 4. The level of carbohydrate metabolizing enzyme in liver of control and experimental animals.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I (Control)</th>
<th>Group II (DEN)</th>
<th>Group III (DEN+Diosmin)</th>
<th>Group IV (Diosmin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase (n mol of glucose-6-phosphate liberated/mg protein/min)</td>
<td>13.79 ± 0.30</td>
<td>27.19 ± 2.96@</td>
<td>19.2 ± 1.46@B</td>
<td>13.39 ± 0.35NS</td>
</tr>
<tr>
<td>Phospho-glucosomerase (n mol of fructose liberated/mg protein/min)</td>
<td>26.40 ± 0.72</td>
<td>42.4 ± 3.56@</td>
<td>32.06 ± 2.39@B</td>
<td>27.38 ± 1.03NS</td>
</tr>
<tr>
<td>Aldolase (n mol of glyceraldehyde liberated/mg protein/min)</td>
<td>21.5 ± 0.42</td>
<td>36.44 ± 3.51</td>
<td>27.62 ± 1.73@B</td>
<td>22.8 ± 0.84NS</td>
</tr>
<tr>
<td>Glucose-6-phosphatase (n mol of inorganic phosphate liberated/mg protein/ min)</td>
<td>22.37 ± 0.61</td>
<td>14.63 ± 1.57@</td>
<td>18.69 ± 1.28@B</td>
<td>21.17 ± 0.84NS</td>
</tr>
<tr>
<td>Fructose-1,6-diphosphatase (n mol of fructose diphosphate liberated/mg protein/min)</td>
<td>31.35 ± 0.74</td>
<td>24.36 ± 2.23@</td>
<td>28.3 ± 2.05@B</td>
<td>31.84 ± 0.98NS</td>
</tr>
</tbody>
</table>

Each value represents means ± SD of six animals
a-Group II, III & IV compared with Group I
b-Group III compared with Group II
*p<0.001, #p<0.01, @p<0.05, NS-Not Significant
human hepatoma cells at the dosage of 100 and 200 μM/ml. Thus, the diosmin tempted cellular oxidative stress in upstream the signalling events that which modify HepG2 cells pro and anti-apoptotic equilibrium. Therefore, it is conceivable HepG2 cells with diosmin treatment decline the GSH level and promotes oxidation induction which regulate through programmed cell death or apoptosis.

Antioxidants are potent free radical scavengers and restrict reactive oxygen species induced cellular damage. Superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidases (GPx) antioxidant enzymes has been considered as primary defence mechanism against cell damage. It has been reported that the levels of SOD were significantly lower in malignant cells and excess formation of radicals [34]. Oxidative stress in cells can be estimated through the levels of these antioxidant enzymes which safeguard the cells and tissues from reactive oxygen species induced cellular damage [35]. In the present investigation, NDEA administration decreased the antioxidant enzymes levels could be credited to the extreme application of these antioxidants in inactivating the free radicals generated during the breakdown of NDEA. Activities of the enzymic antioxidants are reverted to ordinary status in diosmin treated animals which clearly designates the antioxidant potency of the drug. The non-enzymic scavenger’s namely glutathione, ascorbic acid, and α-tocopherol, which scavenge residual free radicals escaping from decomposition [36]. The dropped glutathione in NDEA induced rats signifies the increased consumption of glutathione because of oxidative stress. It is reported that vitamins like E, C has several biological activities such as immunomodulation, and modification of metabolic stimulation of carcinogens. They can prevent genetic damages and protect lipid and lipoprotein against oxidative damage [37,38]. In this study, Vitamins C and E were found to be suggestively declined in malignance condition when compared to control. In contrast, upon treatment with diosmin the levels tend to become usual. From the results of our investigation, diosmin have remarkable characteristic of anti-radical and antioxidant activities.

Adenosine triphosphatase (ATPase) plays a predominant role in ions transportation, which regulates the cellular volume, membrane permeability and osmotic pressure. Ca$^{2+}$-ATPases Mg$^{2+}$-ATPase and Na$^+$/K$^+$-ATPase responsible for the maintenance of cell structure during diseased condition particularly cancerous stage cell membrane has been disturbed due to irregularity in the levels of membrane bound ATPase. During diethylnitrosamine administration these enzymes showed decreased activities due to the sensitivity to hydroperoxides and superoxide radicals [39,40]. In group II animals, because of membrane damage caused by NDEA there was an abnormality in the levels of ATPase. The amount of membrane bound ATPase was found to be pointedly normalised in erythrocyte membrane and hepatocytes of diosmin treated animals could be due to the membrane stabilizing activity of diosmin by preventing peroxidation of membrane lipids.

**Discussion**

Scientific community powerfully practicing that, in vitro cytotoxic screening parameters are the predominant and significant in recognizing potent anti-cancer agents. In this present investigation, cytotoxic assays like MTT, LDH leakage and GSH was performed to screen diosmin anti-tumour activity on HepG2 cell line. The results of these cytotoxic assays such as MTT, LDH and GSH diosmin showed a remarkable reduction in the cell viability and levels of marker enzymes in dose and time dependent manner [31]. The reduced cell viability and proliferation might be due to anti-cancer and anti-oxidant potential of diosmin. Since it has been reported that pharmacological potential of medicinal plants and their bioactive principles have cytotoxic effect on various cancer cells [32]. Estimation of lactate dehydrogenase (LDH) leakage, an example of intracellular enzyme is a noteworthy parameter in assessing the cytotoxic nature of the drug. During prolonged incubation and release of LDH in cancer cell line treatment, the damaged cells were fragment completely which is a consistent pointer to test cytotoxicity of the drug. The HepG2 cells were treated with diosmin for 24 hours of time, which display enhanced LDH levels in the medium might be due to medicinal characteristics of the drug. Since LDH is recognised as a notable marker for diseased condition.

Reduced glutathione is considered as a non-enzymatic antioxidant involving in plummeting various toxic metabolic products such as peroxides and radicals. The abridged intracellular GSH quantities leads to the development of ROS in cells treated with anti-tumour drugs which in turn leads to the damage of DNA and macromolecules [33]. In this analysis, GSH levels were expressively diminished in diosmin treated human hepatoma cells at the dosage of 100 and 200 μM/ml.

**Figure 8.** Scanning electron microscopic investigation of in the liver of control and experimental animals. (Note: Group I control and IV Drug control display normal morphological feature of liver cells. Group II exhibit cellular rearrangements and morphology of the hepatic cells due to carcinogen pathological effect. Group III demonstrate the anti-cancer activity of diosmin through recovering the degree of differentiation, structural aberrations and reconstruction of sinusoid).
Through the tricarboxylic acid (TCA) cycle, the mitochondrial enzymes (α-KGDH ICDH, SDH and MDH) catalyse the oxidation of numerous substrates yielding reducing counterparts and these counterparts were channelled through the respiratory chain for the formation of adenosine triphosphate (ATP) by oxidative phosphorylation. These mitochondrial enzymes were disturbed by ROS which ends in uncharacteristic mitochondrial substrate oxidation and cellular energy depletion. There is a massive morphological and functional difference between normal cell and tumour cell mitochondria [41]. In this present study, it was noted that the condensed actions of TCA cycle key enzymes in the liver of tumour bearing animals due to modifications in malignancy cell morphology and mitochondria undergo metabolic variations and the quantity of mitochondria tremendously condensed in cancer cells. The cancer cell possesses uncharacteristic design of energy metabolism comparatively with normal cells. Upon treatment with diosmin the mitochondrial abnormalities were reverted near normal due to anti-tumour property of the bioactive compound diosmin.

During hepatic cancerous condition, it has been observed that glucose metabolizing enzymes levels were nonstandard in the transformation of normal liver to high glucose utilization. The activities of glycolytic and gluconeogenic enzymes and tumour progression have strong association and this glucose metabolizing enzymes levels can be used as effective pointers of diagnosis and prognosis [42]. In the present investigation, the cancer animals illustrated uncharacteristic level of carbohydrate metabolising enzymes in liver may be due to the higher rate of glycolysis in the liver and subsequent leakage of this enzyme into the blood. These metabolising enzymes were brought back moreover to normal level because of medicinal treatment. Due to ameliorative potential of diosmin the damaged cells were recovered in the means of cell shape, nuclei shaped and regular cytoplasm. This proves the anti-cancer properties of the diosmin.

Conclusion

Founded on the outcomes of the entire investigations, it is suggested that diosmin has shown promising anti-cancer effects against NDEA induced experimental HCC. Our findings strongly reveal the anti-carcinogenic potential of diosmin which may be related directly or indirectly to its antioxidant properties. This novel approach will throw more light on the cognitive mechanism of diosmin against chemically induced hepatoma. To establish a broader implication, studies are needed in other cancer models. We expect that diosmin may be established as a hopeful anticancer agent in clinical practice in future.

Conflicts of Interest

The authors have declared that there is no conflict of interest.

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