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Effect of Diosmin on mTOR/Akt Signaling Molecules in NDEA-induced Hepatocellular Carcinoma in Experimental Rats

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Abstract

Background: Diosmin is a natural compound with a wide range of biological activity, e.g., it improves lymphatic drainage, supports microcirculation, and increases venous tone, and venous elasticity, hence, it is applied in the pharmacotherapy of chronic venous disorders (CVD). This work aims to analyze the effect of diosmin on mTOR and Akt-signaling molecules in NDEA-induced hepatocellular carcinoma (HCC) in experimental rats. Materials and Methods: Healthy adult rats were split into four groups randomly. Each group consisted of 6 animals. Group I: Control, Group II: NDEA-induced hepatocellular carcinogenic rats, Group III: Cancer-bearing animals treated with diosmin (200 mg) orally for 28 days. Group IV: Control animals treated with diosmin (200 mg) alone for 28 days. Liver function markers (ALP and AST) were measured by biochemical analysis while mTOR and Akt mRNA expression were analyzed by q-PCR analysis. Results: ALP and AST concentration in the serum and mRNA expression of the transcription factor, mTOR were found to be upregulated in HCC bearing rats but Akt mRNA expression was reduced. However, a 200 mg dose of diosmin controls the altered levels of liver function markers and signalling molecules. Conclusion: Results of this study suggest that diosmin may be one of the pharmacological and therapeutic natural compounds against hepatocellular carcinoma.

Keywords: *Akt, Diosmin, Liver Cancer, mTOR, Phytotherapeutics.*

Introduction

The most common primary liver cancer, hepatocellular carcinoma (HCC), causes up to one million fatalities annually worldwide. The fourth most common cause of cancer-related fatalities is HCC, which is a burden for world health [1]. Hepatocellular carcinoma is primarily caused by chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection. It is also linked to exposure to aflatoxins, alcohol misuse, and non-alcoholic fatty liver [2]. Chemoprevention is one method for reducing the risk of liver cancer. The injection of one or more non-toxic naturally occurring or manufactured medications significantly delays, prevents, or slows the disease. In this regard, naturally occurring polyphenols have recently attracted more interest due to their encouraging effectiveness in several cancer model studies [3].

For over a billion years, flavonoids, which are polyphenolic bioactive chemicals, have been known to exist naturally in practically all dietary plants, including fruits and vegetables. Flavonoids are found in many medicinal plants and are used in traditional medicine, thus herbal treatments containing them are used all over the world [4]. Because fruits contain polyphenolic chemicals, intake has been linked to lower cancer risk in numerous epidemiological studies. dietary polyphenols obtained from plants, such as Flavonoids are viewed as

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prospective anti-cancer medicines because they have chemopreventive potential and cancer cell-specific pro-apoptotic action [5]. A well-known flavonoid with a wide range of biological functions, such as an antioxidant, frequently utilized in medicine as a modulator of capillary permeability and anti-carcinogenic.

Treatments for chronic venous insufficiency, hemorrhoids, venous ulcers (particularly in the lower extremities), and the prevention of postoperative thromboembolism involve diosmin formulations [6]. In Europe, it is frequently prescribed as medicine due to its phlebotropic qualities, however, in the US, it is utilized as a nutritional supplement. Diosmin is a flavone glycoside which is found in a natural structure in the pericarps of different citrus fruits and is called 3′,5,7-trihydroxy-40-methoxyflavone-7-rhamnoglucoside [7]. This flavone glycoside is obtained by dehydrogenation of hesperidin. Diosmin was first isolated in 1925 from *Scrophularia nodosa* and was first introduced as a therapeutic agent in 1969. Clinical evidence suggests that diosmin is a safe, nontoxic drug, and well-tolerated drug. Our team has extensive knowledge and research experience that has translated into high-quality publications [8-13].

Diosmin is a natural compound with a wide range of biological activity, e.g., it improves lymphatic drainage, supports microcirculation, and increases venous tone, and venous elasticity, hence, it is applied in the pharmacotherapy of chronic venous disorders (CVD). To determine the effect of diosmin on angiogenic signalling molecules in NDEA, to experimentally analyze the NDEA-induced hepatocellular carcinoma in adult male Wistar rats [14-19].

This study aimed to analyse diosmin's effect on mTOR/Akt signalling molecules in NDEA-induced hepatocellular carcinoma through *in vivo* evidence of biochemical and gene expression analysis.

Materials and Methods

Animals

Animals were maintained as per the National Guidelines and Protocols approved by the Institutional Animal Ethics Committee (BRULAC/SDCH/SIMATS/IAEC/02-2019/016). 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 a constant 12 h light and 12 h dark schedule at the Biomedical Research Unit and Lab Animal Center (BRULAC), Saveetha Dental College & Hospitals, Saveetha Institute of Medical & Technical Sciences, Chennai – 600 077. They were fed with a 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, the diosmin dose (200 mg/kg body weight) was selected based on the study from our laboratory. 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 (200mg/kg/ body weight/day) orally for 30 days. Group IV-control rats were treated with diosmin (200 mg/kg/body weight/day) alone for 45 days. At the end of the experimental period, animals were subjected to ether anaesthesia; 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.

Assessment of Liver Function Markers

Liver function markers such as aspartate transaminase (AST) and alkaline phosphatase (ALP) kits procured from Spinreact, Spain. Results for the same were expressed as U/L [20].

Real-Time RT-PCR Analysis of mRNA Expression

The real-time RT-PCR analysis was performed using gene-specific primers. cDNA was synthesized from total RNA isolated from tissues using the first strand synthesis kit (Qiagen, Germany) [21]. The protocol to be used for real-time PCR is as follows: The PCR mixture consists of 5µl of cDNA sample (1:10 dilution), 300nM of each primer and 10µl of master mix for SYBR green I (Eurogentec, Belgium) in a final volume of 20µl. Amplification was carried out in the MX3000P Multiplex quantitative PCR system with initial denaturation at 95°C for 10 min, followed by denaturation at 95°C for 15 seconds annealing at 60°C for 1 min and extension at 72ºC for 30 seconds. Amplification of the internal control (β-actin) was performed simultaneously in separate tubes. All reactions were performed in triplicate along with no template control (NTC) and results were analyzed using MX3000P Multiplex quantitative PCR system software (Stratagene). Dissociation curve analysis was performed after each reaction to ensure the amplification of a single product. The relative number of mRNAs was calculated using the comparative CT method [22].

Statistical Analysis

Data were presented as the mean ± SEM of 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

Assessment of Liver Function Markers

Liver function markers ALT, ALT and ALP were significantly raised in NDEA-induced animals compared to control (Figure 1 & 2). Oral administration of diosmin reduced the same significantly ($p < 0.05$). The x-axis represents experimental groups, and the y-axis represents the activity. Each bar represents the Mean ± SEM of 3 independent observations. P value ≤ 0.05 was statistically significant. a-compared with control; b-compared with NDEA-Induced rats; c-compared with diosmin.

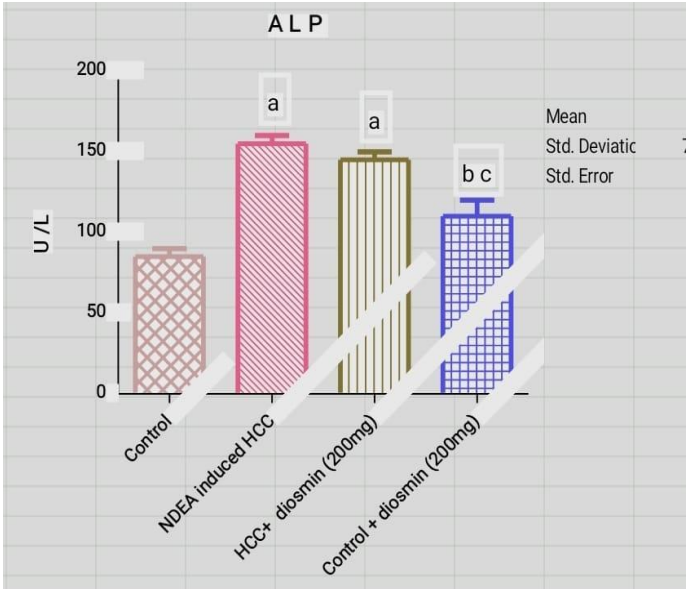

Figure 1. Effect of Diosmin on the Levels of ALP in Control and Treated Animals

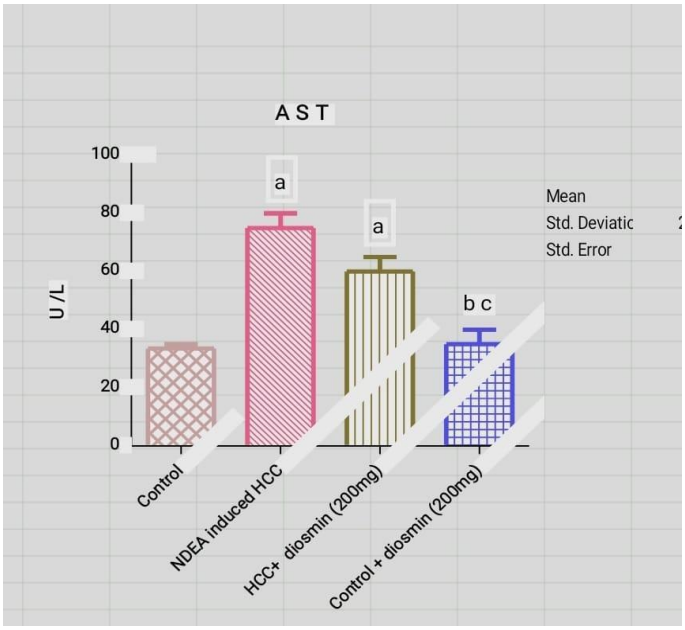

Figure 2. Effect of Diosmin on the Levels of AST in Control and Treated Animals.

From the study, we can able to notice the values of AST and ALP. Normally, AST and ALP are present in small quantities in the body, after injecting the NDEA-induced HCC their values will become elevated. Then after the addition of HCC with diosmin (200mg) their value seems to lower; when the control is added with diosmin (200mg) their value is further lowered. The goal of cancer chemoprevention is to slow, block or reverse the process of carcinogenesis through the use of natural or synthetic compounds. Dietary compounds are reported to have the innate ability to modify the deregulated intracellular pathways thereby delaying the process of carcinogenesis.

Effect of Diosmin on mTOR mRNA Expression on NDEA-Induced Animals

mTOR, also known as the mechanistic target of rapamycin, and occasionally referred to as FK506-binding protein 12-rapamycin-associated protein 1 (FRAP1), is a kinase encoded by the MTOR gene in humans. Belonging to the phosphatidylinositol 3-kinase-related kinase family, mTOR plays crucial roles in cellular signalling pathways. In the current study, mTOR mRNA expression level was elevated in NDEA-induced animals and their expression were significantly reduced ($p<0.05$) upon diosmin treatment compared to the control group (Figure 3).

Effect of Diosmin on Akt mRNA Expression on HCC Experimental Animals

Akt also called protein kinase B (PKB), is a 57kD serine/threonine protein kinase that is composed of an N-terminal pleckstrin homology (PH) domain, a central catalytic domain, and a C-terminal regulatory domain. Akt plays a major role in cell proliferation and metabolism via various signalling cascade events. Hence in this study, we measured the mRNA expression level of Akt in the experimental animals. NDEA-induced animals showed a lower expression level of Akt which is significantly raised upon diosmin treatment. This signifies the therapeutic potential of diosmin on HCC animals (Figure 4).

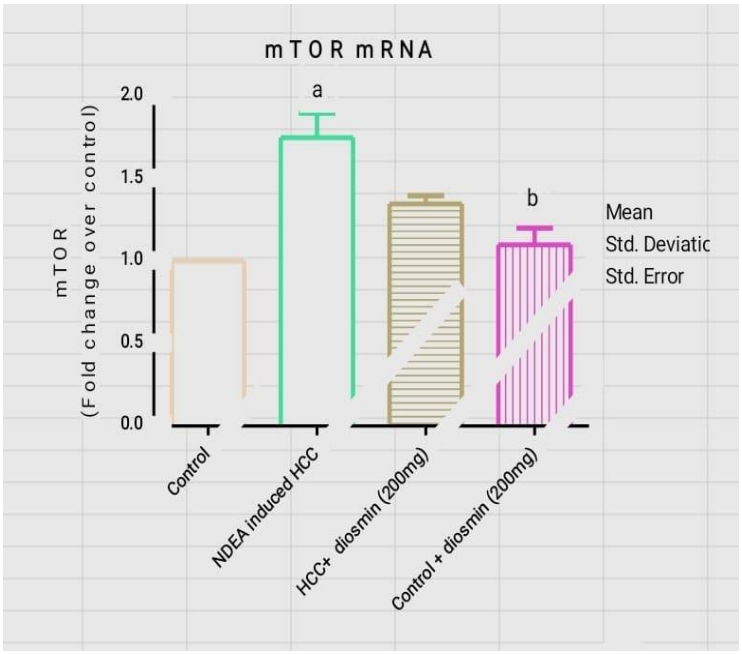

Figure 3. Effect of Diosmin on mTOR mRNA Expression in NDEA-induced Rats

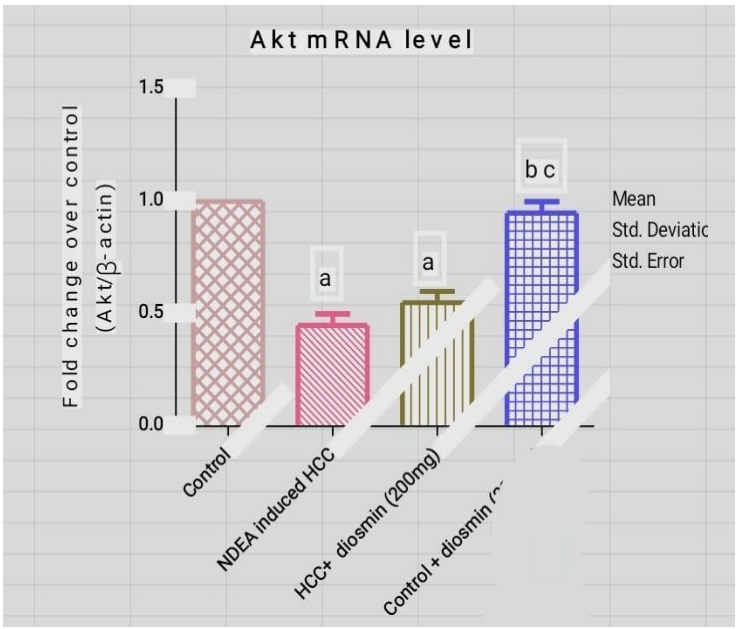

Figure 4. Effect of Diosmin on Akt mRNA Expression in NDEA-induced Rats

Discussion

Hepatocellular carcinoma (HCC) comprises a significant majority (85-90%) of liver malignancies, with its etiology linked to nitrosamines a group of dietary carcinogens present in various food items like smoked pickled fish, cheese, nitrite-cured meats, dried milk, and alcoholic beverages, as well as tobacco smoke. These substances contribute to chronic inflammation, oxidative stress, and cellular proliferation in response to tissue damage, ultimately leading to the development of hepatic neoplasia. The induction of hepatocarcinogenesis by N-nitrosodiethylamine (NDEA) serves as a common experimental model in anticancer studies due to the histological and biochemical similarities between rodent and human hepatic lesions. Despite significant progress in understanding the association between cell signaling, oxidative stress, and tumor

development in HCC, there is still a need for a more comprehensive understanding of the molecular and regulatory mechanisms involved [23-25].

Numerous epidemiological studies have highlighted that a diet rich in fruits and vegetables is linked to a reduced risk of cancer in humans, suggesting the potential effectiveness of certain dietary components in cancer prevention. These natural agents typically down-regulate activated signalling pathways in malignant cells and hinder the proliferation of initiated cells while causing minimal damage to normal cells [26-28]. Most cancer-preventive agents are natural phytochemicals that act by inhibiting enzymes involved in carcinogen activation and proliferation. Diosmin is a well-known flavonoid with a broad spectrum of biological activities, including antioxidant, anti-inflammatory and anti-apoptotic activities. Several studies reported that diosmin has a beneficial effect on many pathological conditions such as hyperlipidaemia, diabetes mellitus and peptic ulcer [29-32]. The molecular mechanism of diosmin against the HCC was still unclear. Hence this study is carried out to investigate the molecular mechanism of Diosmin administration in N-nitrosodiethylamine-induced liver cancer.

Real-time quantitative PCR was employed to examine the gene expression levels of mTOR and Akt. In rats induced with N-nitrosodiethylamine (NDEA), there was a significant ($p<0.05$) decrease observed in the mRNA level of Akt which gets restored to the normal level upon disomin treatment. The mRNA expression level of mTOR is elevated in NDEA-induced animals which is reduced significantly to normal level upon diosmin treatment ($p<0.05$). The level of liver marker enzymes such as ALP and AST were elevated in NDEA-induced animals compared to the control group ($p<0.05$). Upon diosmin treatment, these enzyme levels were restored to their normal levels which strongly signifies the therapeutic potential of diosmin on hepatocellular carcinoma. These findings suggest diosmin's potential to exert control over hepatocellular carcinoma (HCC).

Conclusion

Studies revealed that the treatment of diosmin has a prospective anti-cancer activity by rearranging hepatic cell structure and its integrity. Results of this study suggest that diosmin may be one of the pharmacological and therapeutic representatives against hepatocellular carcinoma. To conclude, the present study clearly shows that diosmin could be used as one of the therapeutic phytomedicines for the treatment of hepatocellular carcinoma.

Conflict of Interest

The authors declare that they have no conflict of interest.

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