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CITRAL ATTENUATES N-NITROSODIETHYLAMINE INDUCED LIVER CARCINOGENESIS IN EXPERIMENTAL WISTAR RATS

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Keywords:

Citral, Hepatocellular carcinoma, Diethyl nitrosamine, Antioxidants, Lipid peroxidation

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ABSTRACT: Antioxidants are one of the key troupes in tumorigenesis, several natural and artificial antioxidants were shown to have anticancer effects. The aim of the current study is to reveal the chemopreventive nature of citral during diethylnitrosamine (DEN) -induced liver cancer in male Wistar albino rats. Administration of DEN to rats caused in amplified relative liver weight and serum marker enzymes aspartate transaminase (AST), alkaline phosphatase (ALP), alanine transaminase (ALT), lactate dehydrogenase (LDH) and gamma glutamyltranspeptidase (γGT). The levels of lipid peroxides prominent (in both serum and tissue) with a succeeding reduction in the final body weight and tissue antioxidants like superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPx) and glutathione reductase (GR). Citral supplementation (100 mg/kg body weight) knowingly reduced these alterations, thereby showing powerful anticancer effect in liver cancer. Histological interpretations revisions were also conceded out, which supplementary supports to the chemopreventive action of the citral against DEN induction during liver cancer progression. These explanations suggest that citral inhibits lipid peroxidation, hepatic cell damage, and protects the antioxidant system in DEN-induced hepatocellular carcinogenesis.

INTRODUCTION: Hepatocellular carcinoma (HCC) is one of the most common types of malignant tumors and the third leading cause of cancer death Worldwide [1]. In spite of current advances in the use of synthetic drugs, several short comings such as ability and side effects still remain[2]. Thus, there is an imperative need for alternative and effective agents for the controlling of hepatocellular carcinoma, with better efficacy and less impairment [3].

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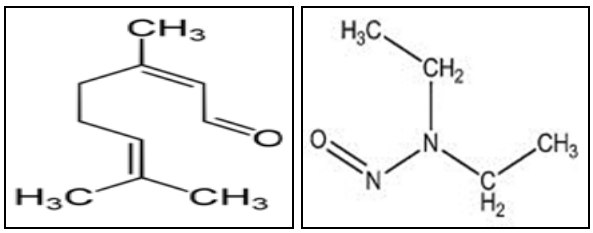
The major causes of HCC are known as hepatitis B or C virus infection, food additives, alcohol, fungal toxins (aflatoxins), toxic industrial chemicals, air and water pollutants [4, 5]. Recent studies have indicated that formation of reactive oxygen species (ROS) within hepatocytes would eventually result in the cytotoxic effect [6]. Furthermore, ROS-cause oxidation of target proteins or enzymes would negatively influence the normal functions, which might lead to hepatocarcinogenesis. Compelling evidences also showed that ROS could promote the invasive ability of hepatoma cells. Therefore simultaneous treatment with antioxidants, especially at the early stages, might be a breakthrough in HCC interventions. N-nitroso alkyl compounds, in particular DEN are an eloquent hepatotoxin, carcinogen and mutagen [7, 8].

N-nitroso compounds are considered to be a lethal, and they crop up in tobacco products, cheddar cheese, cured and browned meals, occupational surroundings, cosmetics, agricultural toxicants and pharmaceutical agents [9]. DEN has been widely used as a precursor in initiating carcinogenesis in experimental animal models. Activation of DEN, which occurs chiefly in liver microsomes, has been demonstrated to stimulate Kuepfer cells, leading to high levels of ROS, capable of damaging liver cells and inducing hepatocarcinogenesis [10]. The cellular damage caused by ROS is measured in terms of lipid peroxidation [11].

Citral (3,7-dimethyl-2,6-octadienal) is a monoterpene that occurs naturally in herbs, plants and citrus fruits. It is a nature mixture of isomeric acyclic aldehydes geranial (trans-citral, citral A) and neral (cis-citral, citral B). Due to its intense lemon aroma and flavour, citral is widely used as an additive in food, cosmetics and detergents. In addition to these uses, citral possesses antifungal activity against both plant and human pathogens and inhibits seed germination. It also has bactericidal, insecticidal, deodorant, expectorant, appetite stimulating and spasmolytic properties, and weak diuretic and anti-inflammatory effects.



(a) Citral **(b) Diethylnitrosamine**

FIG. 1: STRUCTURE OF CITRAL AND DIETHYLNITROS-AMINE (DEN)

Recently, it was demonstrated that citral produces a long-lasting inhibition of TRPV1-3 and TRPM8 channels, whereas it produces a transient block of both TRPV4 and TRPA1 channels. Similarly, it was demonstrated that the main constituent of the fruit essential oil of *C. insularimontanum* is citral, and that this compound exerted a significant inhibitory effect on the production of nitric oxide in lipopolysaccharide-stimulated RAW 264.7 cells [12]. Moreover, citral exhibited an anti-inflammatory effect in a test of croton oil-induced mice ear edema. Therefore, in light of its anti-inflammatory effects and inhibition of ion channels, we decided to evaluate its anti-inflammatory effects and assess the gastric damage resulting from the systemic administration of citral. Even though many reports revealed that citral plays an important role in the prevention of malignant transformation and cancer development its effect on hepatocarcinogenesis *in-vivo*, is not yet documented. In this investigation, we delineated the role of citral on DEN induced hepatocarcinogenesis in rats.

MATERIALS AND METHODS:

Animals: Male Wistar albino rats weighing about 150-180 g were obtained from Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Madhavaram, Chennai, India. The animals were housed in cages under proper environmental conditions and were fed with a commercial pelleted diet (M/s Hindustan Foods Ltd., Bangalare, India). The animals had free access to water. All the experiments were designed and conducted according to the ethical norms approved by Institutional animal ethics committee guidelines (IAEC NO: 01/12/2018).

Chemicals and Their Sources: DEN and Citral were procured from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals used were from SRL (Mumbai, India).

Experimental Design: The experimental animals were divided into four groups, each group comprising of six animals.

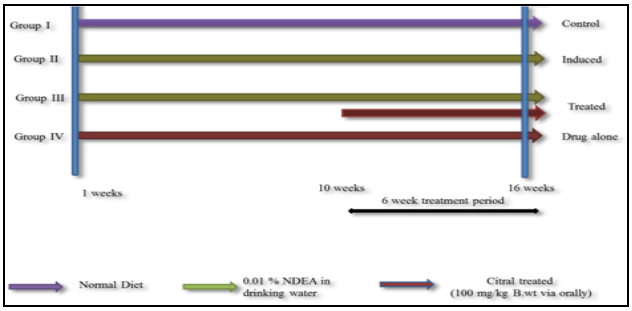


FIG. 2: SCHEMATIC REPRESENTATION OF THE EXPERIMENTAL PROTOCOL INVOLVING NDEA EXPOSED EXPERIMENTAL RAT HEPATOCELLULAR CARCINOGENESIS

Group 1: Normal control rats fed with standard diet and pure drinking water for 16 weeks.

Group 2: Rats were induced with HCC by providing 0.01% DEN through drinking water for 15 weeks [13].

Group 3: Rats treated with citral (100 mg/kg body weight) [14] administration of 0.01% DEN and

continued till the end of the experiment (*i.e.*, 16 weeks).

Group 4: Rats were treated with citral alone by oral gavage daily at a dose of 100 mg/kg body weight for 16 weeks.

After the experimental period, the rats were fasted overnight, anaesthetized with diethyl ether and then sacrificed by cervical decapitation.

Biochemical Parameters: For biochemical estimation, Blood samples were allowed to clot at room temperature and centrifuged at 1500 r/min for 10 min to separate the serum. The biochemical parameters such as AST, ALT, ALP, $_{\gamma}$GT, were estimated using Mindray-BS-200E Biochemical fully automated analyzer. For lipid peroxidation studies and enzymatic antioxidant 10% liver tissue homogenate (0.1 M Tris-HCl buffer, pH 7.4) was prepared, the homogenate was then centrifuged at 3500 r/min for 10 min at 4 °C in a refrigerated centrifuge. The resultant supernatants were maintained in an ice bath. The activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) were estimated by the methods of respectively. Levels of lipid peroxidation (LPO) products were estimated in serum and liver tissue homogenate as per the protocol [15].

Histological Examination: Animals were sacrificed by cervical dislocation. Histological examination portion of the liver tissue was fixed in 10% neutral buffered Formalin and embedded in paraffin wax for histological evaluation. Sections with thickness 5 µm were stained with hematoxylin and eosin (H and E), examined under high power light microscope.

Statistical Analysis: The data were analyzed with SPSS/10 Software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. P values of <0.05 were considered to indicate the statistically significance. All the results were expressed as mean ± standard error (SE) for six animals in each group.

RESULTS:

Effect of Citral on Body Weight, Liver Weight and Relative Liver Weight: The hepatoprotective effect of Citral against DEN-induced HCC was elucidated in male Wistar albino rats. **Fig. 3(a)** and **(b)** shows initial body weight, final body weight, liver weight and relative liver weight of control and experimental group of animals. In DEN-induced group II animals, there is a significant decrease in the absolute body weight and significant increase in liver weight when compared with group I control animals. The Citral treated groups III showed a significant increase in the absolute body weight when compared with group II DEN-induced animals during the course of the experiment, all rats showed greater tolerance to treatment with Citral. In group II animals, the relative liver weight is significantly improved when compared with group I animals and there is a significant reduced in the liver weight in citral treated groups III animals when compared with group II DEN-induced animals. No obvious changes were observed between the control and citral alone treated group which is an indicative of nontoxic nature of citral.

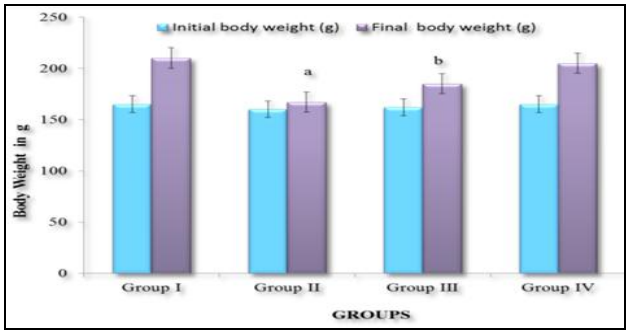


FIG. 3 (a): BODY WEIGHT CHANGES IN CONTROL AND EXPERIMENTAL GROUPS OF RATS. Results are expressed as mean ± S.D for six rats in each group. Statistical significance at P\0.05 compared with [a] group 1, [b] group 2. Body weight change is expressed in grams

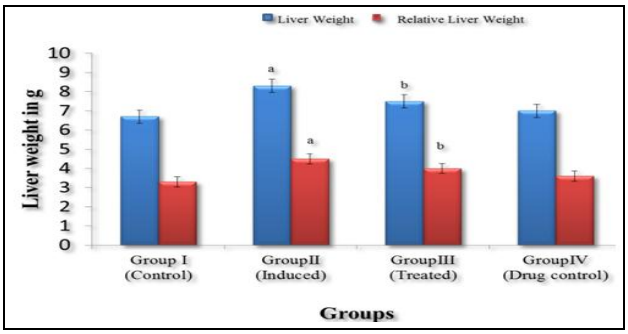


FIG. 3(b): LIVER WEIGHT AND RELATIVE LIVER WEIGHT OF CONTROL AND EXPERIMENTAL GROUPS OF RATS. Results are expressed as mean ± S.D for six rats in each group. Statistical significance at P\0.05 compared with [a] group 1, [b] group 2. Liver weight is expressed in grams. Relative liver weight is the average of liver weight at final body weight multiplied by 100.

Citral Lipid Peroxidation: Fig. 4 shows the level of LPO in the serum and liver of control and experimental groups of animals which was analyzed for oxidative stress.

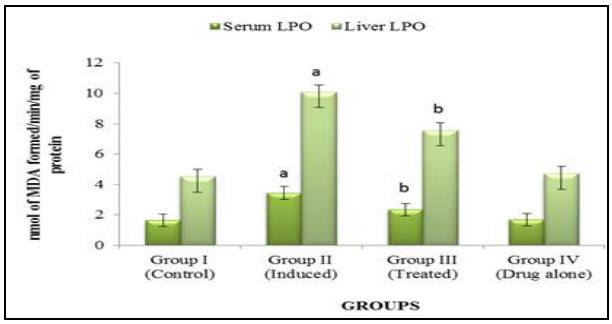


FIG. 4: EFFECT OF CITRAL ON THE LEVELS OF LIPID PEROXIDES IN THE SERUM AND LIVER OF CONTROL AND EXPERIMENTAL GROUPS OF RATS. Results are expressed as mean ± S.D for six rats in each group. Statistical significance at P\0.05 compared with [a] group I, [b] group II. LPO levels are expressed as nmol of MDA formed/ min/mg protein.

In DEN-induced group II animals, there is a significant increase in the levels of lipid peroxides when compared with group I control animals. Whereas in Citral treated groups III animals, there is a significant decrease in the levels of lipid peroxides when compared with group II induced animals. However, animals treated with Citral alone group IV did not show any significant changes when compared with group I animal.

Effect of Citral on the Activities of Marker Enzymes in the Liver Tissue of Control and Experimental Groups of Rats: Fig. 5 (a, b, c) shows the effect of Citral on the levels of markers enzymes AST, ALT , LDH, ALP and γ-GT in the serum of control and experimental group of rats. The marker levels were significantly increased in DEN-induced group II animals when compared with control animals. Whereas group III treated animals significantly decreased the levels of marker enzymes when compared with DEN-induced group II animals. No significant changes observed between control and citral alone treated animals.

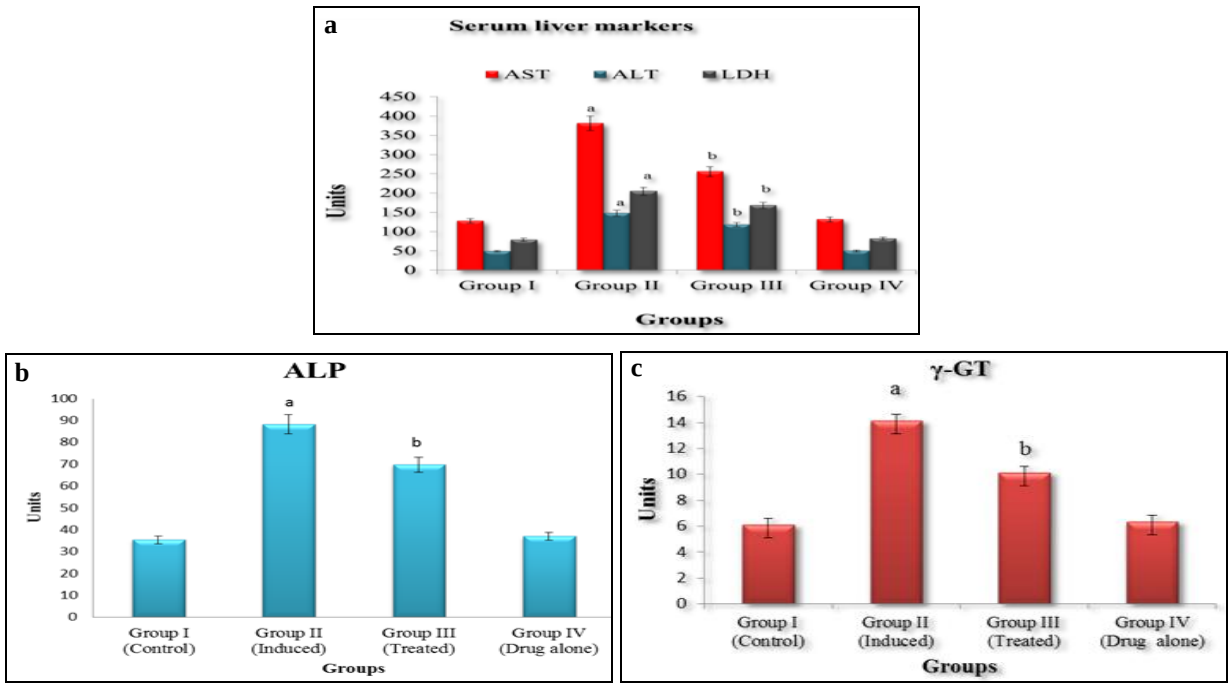


FIG. 5: EFFECT OF CITRAL ON THE ACTIVITIES OF MARKER ENZYMES IN THE LIVER TISSUE OF CONTROL AND EXPERIMENTAL GROUPS OF RATS. Units: µ moles of pyruvate liberated mg protein per min for AST, ALT and LDH; µ moles of phenol liberated mg protein per min ALP; n moles of p-nitroaniline formed mg protein per min for GGT.

Effect of Citralon the Enzymic and Non-Enzymic Antioxidant in the Liver of Control and Experimental Groups of Animals: Table 1 indicates the enzymatic and non-enzymatic antioxidant activities in the liver of the control and experimental groups. DEN induced group II animals showed significant decrease in the activities of enzymatic antioxidants such as SOD, CAT, GPx, GR and GST when compared with control Group I animals. Whereas treated group III animals showed significant increase in these enzymes when compared with DEN induced group II animals.

Non enzymatic antioxidants such as GSH, G6PD, Vitamin C, Vitamin E and Vitamin A also found significantly decreased activities during DEN induced Group II animals when compared with control animals. In treated group III animals, there is significant increase in the activities of GSH, G6PD, Vitamin C, Vitamin E and Vitamin A when compared with DEN induced animals. No significant change was observed in citral alone treated Group IV animals when compared with control animal.

TABLE 1: EFFECT OF CITRALON THE ENZYMIC AND NON-ENZYMIC ANTIOXIDANT IN THE LIVER OF CONTROL AND EXPERIMENTAL GROUPS OF ANIMALS

Particulars	Group 1 (Control)	Group 2 (Induced)	Group 3 (Treated)	Group 4 (Drug alone)
		Enzymatic		
SOD	9.45± 0.85	3.98 ± 0.47a	5.05 ± 0.50b	8.95 ± 0.76
CAT	69.75± 6.10	42.05 ± 4.60a	50.75 ± 3.32b	67.55 ± 6.05
GPx	99.53± 8.98	59.32 ± 5.89a	71.75 ± 5.40b	97.67 ± 8.75
GR	160.47± 7.50	103.65 ± 6.75a	125.22 ± 7.52b	160.03 ± 7.67
GST	1.12 ± 0.05	3.90± 0.17a	2.31 ± 0.13 b	1.14 ± 0.06
		Non-enzymatic		
GSH	41.79 ±3.79	14.09 ±1.35a	23.98± 1.96b	41.55 ± 3.60
G6PD	6.10 ± 0.55	3.23 ± 0.20 a	4.15 ±0.30b	6.67± 0.60
Vitamin C	3.56 ± 0.19	1.45 ±0.05a	2.07 ± 0.10	3.56 ± 0.19
Vitamin E	5.68 ± 0.30	1.89 ±0.08a	2.25 ± 0.10	5.59 ± 0.30
Vitamin A	3.57 ± 0.17	1.98 ±0.08a	2.48 ±0.11b	3.55 ± 0.18

Results are expressed as mean ± S.D for six rats in each group. Statistical significance P\0.05 compared with [a] group 1, [b] group 2. Units: SOD, (superoxide dismutase in units/mg protein, CAT (catalase in l mol of H_2O_2 decomposed/min/mg protein), GSH (glutathione in lg/mg protein), GPx (glutathione peroxidase in l mol of GSH utilized/min/mg protein), GR (glutathione reductase in l mol of NADPH oxidized/min/mg protein)

Histology Examination: Histopathological alterations of liver tissue sections, stained with hematoxylin and eosin (H and E) was assessed under a light microscope. Control animals revealed the normal architecture of the liver and citral alone supplemented animals also showed the normal histological appearance as compared to normal control animals. The DEN induced animals showed myocytes inflammation and hyalinization at focal, whereas DEN + Citral treated liver. Showing almost normal myocytes and the abnormal pathological findings are reduced. These restorations may be due to the protective effect of citral against oxidative stress and tissue damage induced by DEN.

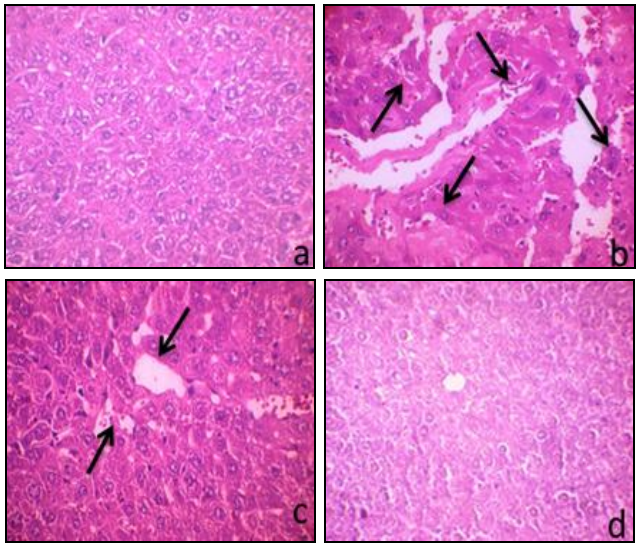


FIG. 6: HISTOPATHOLOGICAL EXAMINATION OF LIVER TISSUE IN CONTROL AND EXPERIMENTAL GROUPS OF RATS. (a) Control shows a normal architecture, (b) DEN alone slides showing loss of architecture, a marked tendency to spread by intrahepatic veins, both hepatic and portal with significant tumor thrombi within portal vessels. Cytological tumor cells are slightly larger, have more irregular nuclei and also numerous mitotic figures. (c) Citral treated showing few neoplastically transformed cells and hepatocytes maintaining near normal architecture. (d) Drug control slides showing normal liver architecture. (20X, HE).

DISCUSSION: Hepatic injury affected by DEN generally re-addresses, instability of liver metabolism, which leads to representative changes in the serum enzyme activities. Intracellular enzymes, such as transaminases, ALP, LDH and γGT are useful indicators for liver function and their increased levels are indicative of liver impairment. Aminotransferases (AST and ALT) are dependable marker enzymes of liver and they are the first enzymes to be used in diagnostic enzymology when liver damage has occurred [16].

Because of their intracellular location in the cytosol, toxicity affecting the liver with subsequent breakdown in membrane architecture of the cells leads to their spillage into serum, and their concentration rises in the latter. The liberation of LDH reflects a nonspecific modification in the plasma membrane integrity and/or permeability.

LDH is a conversant sensitive marker of compact neoplasm and many revisions discovered amplified LDH activity in various types of tumor. γGT is an enzyme embedded in the hepatocyte plasma membrane, again the emancipation of this enzyme into serum indicates damage to the cell and thus injury to the liver. It is a point excursion that serum γGT activity is measured to be one of the best indicators of liver impairment.

In the present study, citral cure sensitively reduced the increased activities of these enzymes. It is reported that citral helps with parenchymal cell regeneration in the liver, thus protecting membrane integrity and thereby minimizing enzyme leakage. Oxidative stress is supplementary with damage to a wide range of macromolecular species, including lipids, proteins, and nucleic acids, thereby producing major interrelated derangements of cellular metabolism including peroxidation of lipids.

Free radicals and non-radicals oxidizing species were produced in animals treated with carcinogens and also in human tissues. Reactive oxygen species (ROS) are formed from endogenous or exogenous sources are highly reactive, toxic, and mutagenic. DEN has been to fabricate free radicals a resolute free radical generation in the liver engulfs the antioxidant status and ultimately proceeds to oxidative stress pavement to the carcinogenesis. Lipid peroxidation shows an important role in carcinogenesis is the most studied biologically important free radical chain reaction.

Induction of DEN has been described to generate lipid peroxidation products like malondialdehyde and 4-hydroxy nonenal that may interact with several molecules leading cause oxidative stress and carcinogenicity. Increased level of LPO was recently testified through DEN-induced hepato-carcinogenesis.

This dynamic action may supplementary lead to the uncompromised fabrication of free radicals irresistible the cellular antioxidant resistance. It has been widely reported that free radicals participated in DEN-induced hepatocarcinogenesis. LPO generation at the initiation stage can be prohibited by free radical scavengers and antioxidant action of citral.

Animals treated with citral exhibited significantly lowered the levels of LPO, both in the liver and serum, when compared with animals induced by DEN. This shows the antilipidperoxidative role of citral may be mediated by its ability to scavenge free radical generation.

Antioxidants may protect the membrane from ROS toxicity by inhibition of ROS establishment by the intermission of ROS occurrence, by facilitating the repair caused by ROS and by providing cofactors for the effective of other antioxidants. Development of life threatening diseases like cancer is linked for the convenience of these antioxidants.

Natural antioxidants are accomplished preventing the ROS production and thereby reducing the complementary intracellular oxidative stress. SOD is the first line of defense in the antioxidant system against the oxidative damage mediated by superoxide radicals. Superoxide dismutases catalyze the mutation of superoxide radical to hydrogen peroxide and water.

Additionally, CAT or GPx catalyze the conversion of H_2O_2 to harmless byproducts. Glutathione, a cysteine-containing tripeptide, is required to maintain the normal reduced state of cells and to counteract all the deleterious effects of oxidative stress. GSH is said to be elaborate in many cellular progressions including the detoxification of endogenous and exogenous compounds. DEN, an electrophilic carcinogen may interact with the large nucleophilic pool of GSH thereby reducing the macromolecule and carcinogen interaction [17].

In citral preserved animals, there was an expressively higher level of GSH in the liver when compared to DEN-induced animals consistent with the idea of attenuation of DNA-carcinogen interaction and in that way prevention a promising environment for carcinogenesis. Decreases in the activities of SOD, CAT, GPx, GR, and GSH are seen in tumor cells.

The composites that can scavenge excessive free radicals in the body are suggested to deter the process of carcinogenesis. Such studies support our findings as we had seen a significant decrease in the activities of antioxidant enzyme in both serum

and liver of animals treated with carcinogenic in evaluation with normal animals.

On the other indicator, there is a significant proliferation in the activities of antioxidant enzymes in liver of the animals and it can administered both citral and carcinogen alone.

Histopathological studies were performed to further confirm the occurrence of apoptotic morphological changes at the cellular level. The control animals showed normal nuclei and cytoplasm. The animals induced with DEN showed the occurrence of several irregular shaped nuclei was very close to each other in that irregular cytoplasm were also seen and which might be due to the extreme free radical generation during DEN administration. Citral alone treated animals showed normal architecture, so did not induce any intracellular morphology of liver cell, which ultimately shows its nontoxic nature at a given dosage.

Thus, the results of the ultrastructural studies undoubtedly confirmed that control has the ability to cause apoptosis in cancer cells.

CONCLUSION: In conclusion, the current study establishes that the citral possesses effective free radical scavenging and antioxidant activities. From the results, it is evident that citral is proficient of reducing the levels of LPO and significantly increases the endogenous antioxidant protection mechanisms in DEN - induced hepatocellular carcinogenesis. Our results also show that the substantial increase in the levels of serum markers was prohibited by citral treatment. Then, we suggest that citral may be developed as an effective chemotherapeutic agent. Further studies are under way to elucidate the molecular mechanisms involved to prove citral efficacy as an anticancer agent.

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CONFLICTS OF INTEREST: No conflict of interest.

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