Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Issue 2, 2012

Research Article

EFFECT OF *TERMINALIA CHEBULA* RETZ ON DEN INDUCED HEPATOCELLULAR CARCINOGENESIS IN EXPERIMENTAL RATS

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Received: 3 Dec 2011, Revised and Accepted: 11 Jan 2012

ABSTRACT

Hepatocellularcarcinoma (HCC), a highly aggressive form of solid tumor, has been increasing in South East Asia. The lack of effective therapy necessitates the introduction of novel chemo preventive strategies to counter the mortality associated with the disease. Towards this goal, the present study evaluates the chemo preventive potential of *T.chebula* aqueous extract (TCE) by estimating the levels of lipid peroxidation and assaying activities of various marker enzymes in diethylnitrosamine (DEN) induced liver cancer bearing rats. The daily oral treatment of TCE (50 mg/kg bwt) to liver cancer bearing rats demonstrated a significant ($p<0.05$) decline in lipid peroxidation, pathophysiological marker enzyme (AST, ALT, ALP, LDH, γ-GT and 5'NT) levels and increase in enzymic antioxidants (SOD, CAT, GPx, GR and GST) status. HE (Hematoxylin and eosin) staining suggesting that the maintenance of cell structure and integrity by TCE in experimental rats. Transmission electron microscope (TEM) results support the stimulation of apoptosis by TCE. These findings confirmed the chemo preventive potential of TCE in DEN induced experimental liver carcinoma.

Keywords: *T.chebula,* DEN, Transmission Electron Microscope, Antioxidants

INTRODUCTION

The plants of genus *Terminalia*, comprising of 250 species, are widely distributed in tropical areas of the world[1]. Fruits of *Terminalia chebula* Retzius (T.chebula Retz.) (Combretaceae), commonly known as black Myroblans in English Harad in Hindi and Kadukkai in Tamil, indigenous in India and Pakistan. Among many Asian and African countries, is a popular folk medicine and has been studied for its homeostatic, laxative, diuretic and cardiotonic activities[2,3]. Atleast seven *Terminalia* species were traditionally used for the treatment of cancers. The fruit powder of *Terminalia chebula* is used in India to treat several diseases ranging from digestive, coronary disorders to allergic and infectious diseases like cough and skin disorders[4].

Primary hepatocellular carcinoma (HCC) is one of the most frequently occurring forms of a solid tumor. It exhibits a high prevalence with 620,000 cases per year reported worldwide of which more than eighty percent of cases are reported from China, Africa and South East Asia[5]. It is highly aggressive, as shown by the mortality of 595,000 cases per year that nearly matches the incidence of this tumor type[6]. HCC presents with limited therapeutic options. Hence, a thorough understanding of the biological bases of this malignancy might suggest new strategies for effective treatment[7]. Hepatocarcinogenesis induced by DEN is an ideal animal model to investigate liver tumor formation because it proceeds in stages similar to that of human liver cancer i.e., formation of preneoplastic foci, neoplastic nodules and HCC nodules[8].

Oxygen free radicals generated by a number of processes *in vivo* are highly reactive and toxic[9]. However, biological systems have evolved an array of enzymic and non-enzymic antioxidant defense mechanisms to combat the deleterious effects of oxygen free radicals. It is a well known fact that oxidative stress arises when there is an imbalance between oxygen free radicals formation and scavenging by antioxidants. Excessive generation of oxygen free radicals can cause oxidative damage to biomolecules resulting in lipid peroxidation (LPO), mutagenesis and carcinogenesis. In this connection, oxygen free radicals induced LPO has been implicated in neoplastic transformation[10].

Experimental investigations as well as clinical and epidemiological findings have provided evidence supporting the role of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anions ($O_2^{\bullet-}$), hydrogenperoxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) in the etiology of cancer[11]. In addition, certain aldehyde such as malondialdehyde (MDA), the end product of LPO arising from the free radical generation leading to the degradation of polyunsaturated fatty acids (PUFAs) can cause cross-linking in lipids, proteins and nucleic acids. Human body is equipped with various antioxidants viz. superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione (GSH), ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), etc., which can counteract the deleterious action of ROS and protect from cellular and molecular damage[12]. Antioxidants act as radical scavengers inhibiting LPO and other free radical-mediated processes thereby protecting the human body from various diseases[13, 14].

Lipid peroxidation (LPO) may result in several scream, including structural and functional membrane modifications, protein oxidation and generation of oxidation products such as acrolein, crotonaldehyde, malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), which are considered strong carcinogens[15, 16]. For many years cancer chemotherapy has been dominated by potent drugs that either interrupt the synthesis of DNA or destroy its structure once it is formed. Unfortunately, their toxicity is not limited to cancer cells but also normal cells are also harmed[17]. Efforts to develop less toxic drugs that affect antioxidant system, malignant cells and mechanism-based approach are necessary in prevention and therapy of cancer. The present study deals with the status of serum marker enzymes, liver tumor markers and antioxidant enzymes for prevention of DEN-induced hepatocarcinogenesis by *T.chebula* aqueous extract (TCE).The results are presented here.

MATERIALS AND METHOD

Preparation of *T.Chebula* aqueous extract

The finely powdered fruits of *T.Chebula* were mixed with eight parts of distilled water at about 70-80˚ C for two hours. The liquid extract was filtered through sieve. The filtrate was concentrated up to two parts on a rotary vacuum evaporator. The concentrated liquid was dried to get the dry powder of the extract.

Animals

Male wistar albino rats, weighing 150 – 180g, procured from the Small Animal Breeding Centre, Agricultural University, Mannuthy, Kerala were used. Animals were acclimatized under standard laboratory conditions at 25°± 2°C and normal photoperiod (12 h light: dark cycle). The animals were fed with standard rat chow and water *ad libitum*. The food was withdrawn 18–24 h before the experiment. The care and use of laboratory animals were done according to the guidelines of the Council Directive CPCSEA, India (No: 659/02/a) about Good Laboratory Practice (GLP) on animal experimentation. All animal experiments were performed in the

laboratory according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (IAEC).

Hepatocarcinogenesis initiation using diethylnitrosamine (DEN)

The experimental hepatocarcinogenesis was induced by using DEN (Sigma, USA). DEN is the most important environmental carcinogen among nitrosamines and primarily induces tumors of liver[18, 19]. The presence of nitrosamines and their precursors in human environment, together with the possibility of their endogenous formation in human body from ingested secondary amines and nitrites, have led to the suggestions of their potential involvement in HCC[20]. It is now widely used as a standard experimental model for HCC[21].

Experimental design

The experimental animals were divided into four groups, each group containing six animals, analyzed for a total experimental period of 16 weeks as follows: Group1, normal control rats fed with standard rat chow and pure drinking water. In Group 2 (TCE alone), rats were orally given TCE (50 mg / kg body weight) in the form of aqueous suspension daily once a day for 16 weeks. This dose of TCE is set based on the effective dosage fixation studies. Group 3 rats were induced with DEN (0.01%) alone in drinking water for 16 weeks. Group 4 rats were administered DEN (0.01%) in drinking water for the first 10 weeks followed by post-treatment with TCE as in group 2 for the remaining 6 weeks. At the end of 16 weeks, experimental rats (n =6 per group) were sacrificed. Blood was collected and serum was separated for the assays.

Biochemicalstudies.

The macromolecular damage such as LPO[22], the activities of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH)[23-25], gamma glutamyl transpeptidase (γ GT)[26], 5'-nucleotidase (5'NT)[27] and the activities of the antioxidant enzymes superoxide dismutase (SOD)[28], catalase (CAT)[29], glutathione peroxidase (GPx)[30], glutathione transferase (GST)[31], glutathionereductase (GR)[32] were estimated in liver.

Enzyme linked immunosorbent assay (ELISA) of AFP and CEA

Quantitative estimation of tumor markers α-fetoprotein (AFP) and carcino embryonic antigen (CEA) were based on solid phase enzyme linked immune sorbent assay (ELISA) using the UBIMAGIWELL (USA) enzyme immune assay kit[33,34].

Histopathological studies

A portion of the liver was cut into two to three pieces of approximately 6mm^3 size and fixed in phosphate buffered 10% formaldehyde solution. After embedding in paraffin wax, thin sections of 5 µm thickness of liver tissues were cut and stained with haematoxylin–eosin. The thin sections of liver were made into permanent slides and examined under high resolution microscope with photographic facility and photomicrographs were taken.

Transmission electron microscopy (TEM)

The liver samples were fixed in Karnovsky's fixative immediately after euthanization of rats for 6–8h at 4°C. These were post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 2h at 4 °C, dehydrated in ascending grades of acetone, infiltrated and embedded in araldite CY212 and polymerized at 60°C for 72 h. Thin (60–70nm) sections were cut with an ultra-microtome.The sections were mounted on copper grids and stained with uranyl acetate and lead citrate and observed under a transmission electron microscope[35].

Statistical Analysis

Data were analyzed by Analysis of Variance (ANOVA) and groups were compared by least significant difference [LSD] test using SPSS/10 software. P <0.05 was considered as significant. All the results were expressed as mean±S.D.

RESULTS

LPO mediated by free radical is considered to be a primary mechanism in the cell membrane destruction and cell damage. The effect of *T.chebula* aqueous extract on LPO in serum of control and experimental rats are given Figure 1. The level of LPO significantly ($p<0.05$) increased in DEN induced group 3 animals when compared to control group of animals. On the contrary, the administration of TCE (group 4) significantly reduced the LPO level when compared to group 3 animals.

Effect of TCE on tumor markers (AFP and CEA) in serum of control and experimental animals are shown in Figures (2 and 3). The level of tumor markers significantly increased ($p<0.05$) in group 3 animals when compared to control group of animals. On the other hand, the administration of TCE significantly reduced the tumor markers in group 4 experimental animals and it was compared with group 3 animals.

The analysis of marker enzymes can be used as an indication of neoplastic condition and therapy. The effects of TCE on the levels of pathophysiological marker enzymes are given in table 1. The levels of key marker enzymes significantly increased ($p<0.05$) in DEN induced group 3 animals when compared to control group of animals. Contrarily, TCE administration significantly reduced the key marker enzyme level and it was compared with cancer bearing group 3 animals.

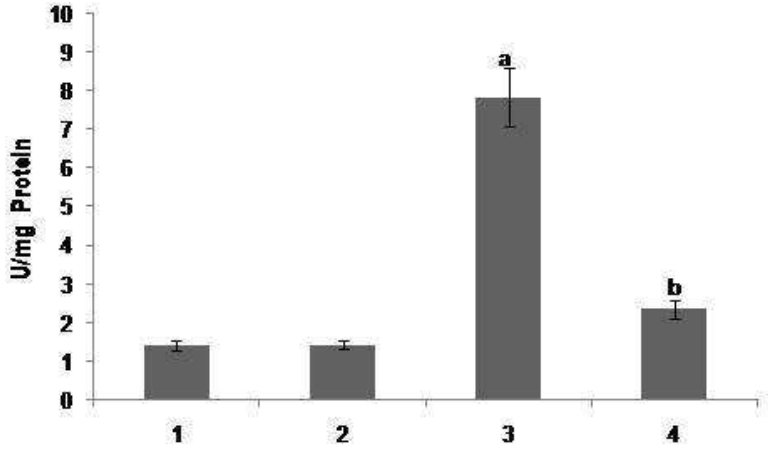


Fig. 1: Effect of TCE on the levels of lipid peroxide in the serum of control and experimental groups of rats.

Statistical significance P <0.05. LPO levels are expressed as nmol MDA formed/(minmgprotein). [a] Comparisons are made with group1 (control). [b] Comparisons are made with group3 (NDEA-induced).

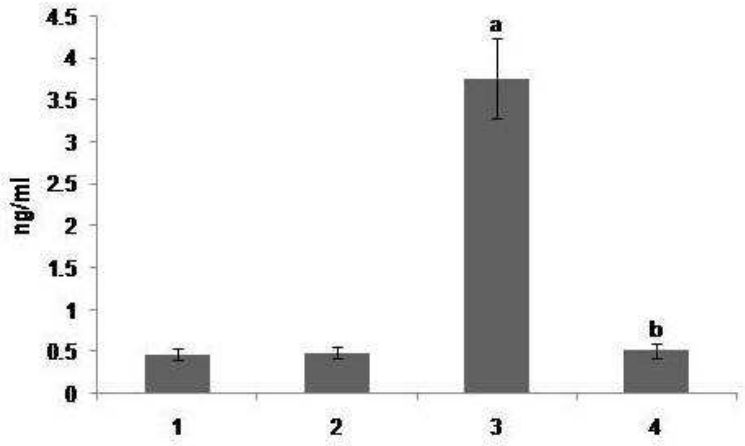


Fig. 2: Effect of TCE on the level of AFP in the serum of control and experimental rats.

Values are expressed as mean ± S.D. (n =6). Statistical significance p <0.05. Comparisons are made with [a] group1(control) and [b] group 3 (DEN-induced). AFP and CEA levels are expressed as ng/ml.

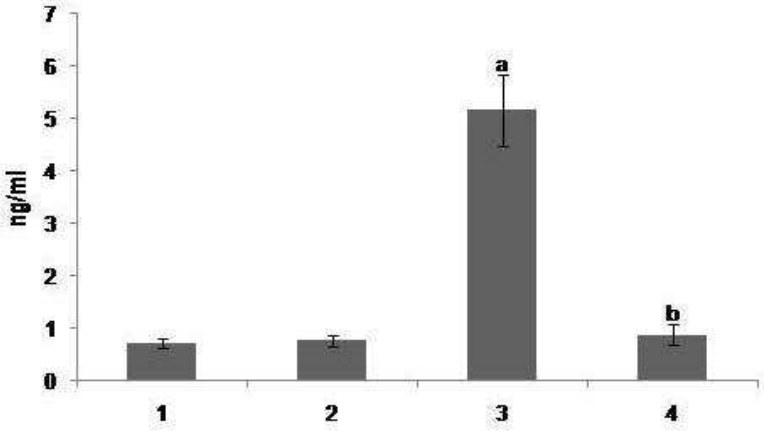
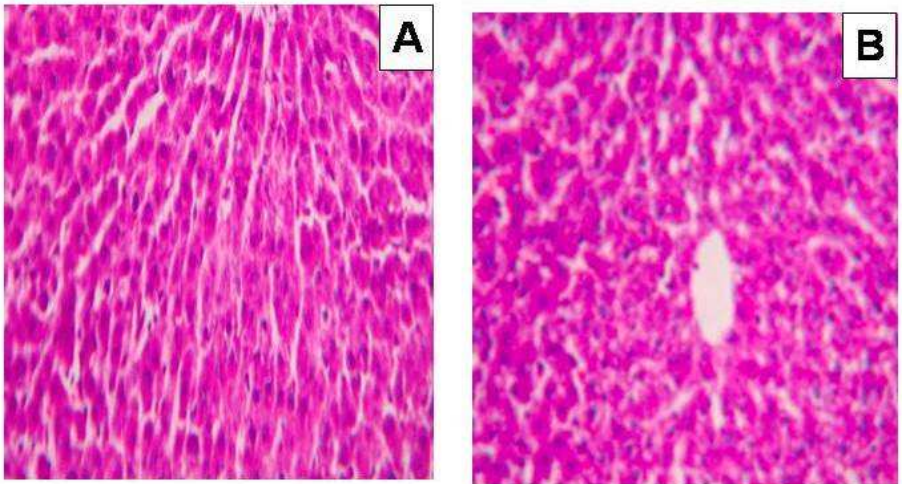


Fig. 3: Effect of TCE on the level of CEA in the serum of control and experimental rats.

Values are expressed as mean ± S.D. (n =6). Statistical significance p <0.05. Comparisons are made with [a] group1 (control) and [b] group 3 (DEN-induced). AFP and CEA levels are expressed as ng/ml.



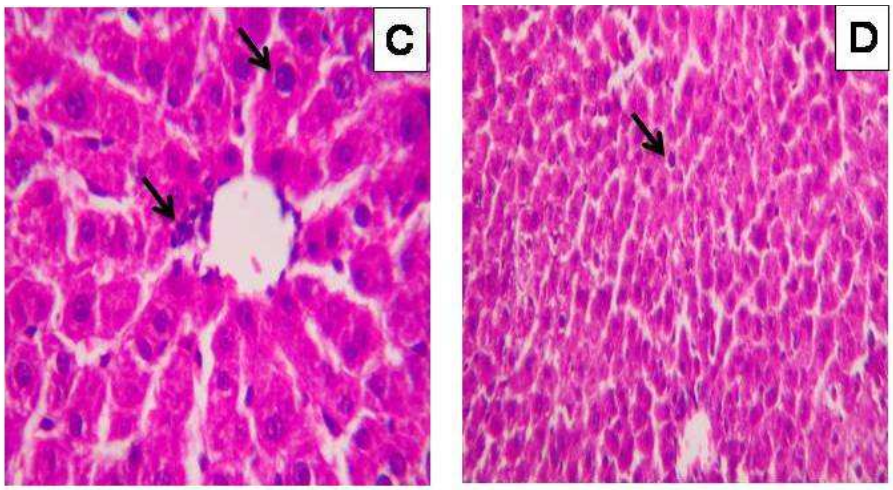


Fig. 4: H and E stained section of liver from a normal (group1) and TCE alone (group 2) rats showing normal hepatic cells with well preserved cytoplasm Prominent nucleus and nucleolus.

H and E stained section of liver from DEN treated group3 rat showing loss of architecture, granular cytoplasm and neoplastic cells. H and E stained liver section from TCE treated group4 rat showing normal architecture with few neoplastically transformed cells.

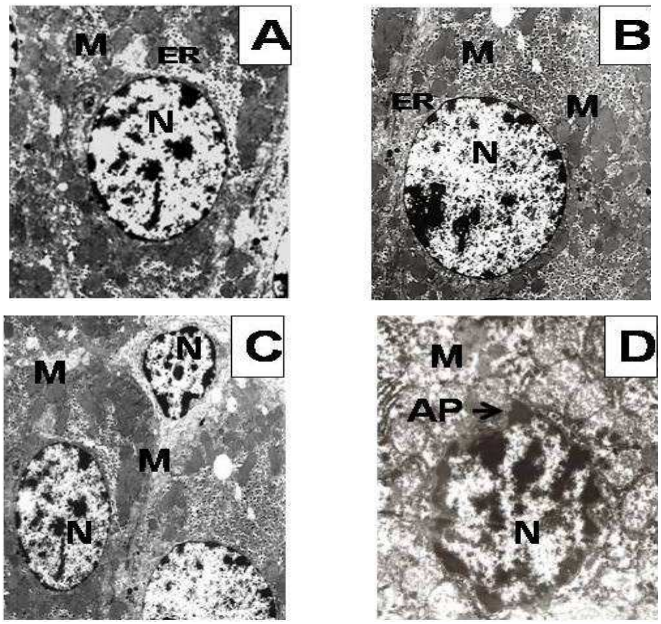


Fig. 5: Ultra-structural changes in liver cells viewed under transmission electron microscope in control and experimental animals.

A and B depict the normal structure of nucleus(N), mitochondria(M), endoplasmic reticulum(ER) and cytoplasm in the liver cells of control and EA alone treated animals (7,000×). C depicts the presence of multiple dysplastic nuclei close to each other with irregular cytoplasm in the liver cells of DEN administered rats (7000×). D depicts the liver cell nuclei with apoptotic bodies (AP), chromatin condensation, mitochondrial swelling and signs of apoptosis in DEN + EA post-treated rats (10,000×).

Table 1: Effects of TCE on the activities of marker enzymes in the serum of control and experimental group of rats

Groups	AST	ALT	ALP	LDH	γ GT	5'NT
Control	4.31±0.12	23.01±0.79	30.31±0.65	3.65±0.13	4.12±0.12	4.06±0.10
EA alone	4.62±0.18	23.43±0.81	30.26±0.60	3.78±0.13	4.01±0.10	4.03±0.10
DEN	9.83±0.85[a]	50.31±1.32[a]	55.08±1.06[a]	7.46±0.47[a]	11.63±0.68[a]	10.63±0.58[a]
EA+DEN	6.72±0.32[b]	33.36±0.97[b]	32.16±0.71[b]	4.16±0.23[b]	7.22±0.31[b]	6.12±0.27[b]

Values are expressed as mean ± S.D (n =6).Statistical significance p <0.05. Activity is expressed as IU/L for AST, ALT, ALP, LDH, γGT and 5'NT.

[a] Comparisons are made with group1 (control).

[b] Comparisons are made with group3 (DEN-induced).

Effect of TCE on the levels of antioxidants in serum of control and experimental rats are shown in table 2. In group 3 cancer bearing animals, the levels of antioxidants were found to be decreased significantly ($p<0.05$) when compared to control group of animals. Conversely, these decreased levels were significantly increased in TCE administered group 4 animals when compared to group 3 animals.

Histological examination of liver sections from control group1 (Figure 4A) and TCE alone (Figure 4B) group2 animals revealed normal architecture and cells with granulated cytoplasm and uniform nuclei. DEN induced group 3 (Figure 4C) animals showed loss of architecture with granular cytoplasm and larger hypochromatic nuclei, whereas group 4 (Figure 4D) animals treated with EA maintained near normal architecture with fewer neoplastically transformed cells.

The ultra-structural analysis of experimental animals is shown in figure 5. Control and TCE alone group 2 animals showed normal nuclei and cytoplasm with similar architecture. The presence of multiple nuclei with irregular cytoplasm were noted in DEN induced group 3 animals.In group 4 the occurrence of cell shrinkage, apoptotic bodies, chromatin condensation and mitochondrial swelling were observed.This clearly substantiated the initiation of apoptosis in group 4 animals.

Table 2: Effects of TCE on serum enzymic antioxidant status in control and experimental group of rats

Groups	SOD	CAT	GPx	GR	GST
Control	2.31±0.09	6.15±0.52	13.36±1.18	9.75±0.26	5.14±0.18
EA alone	2.27±0.08	6.32±0.58	13.09±1.16	9.70±0.24	5.19±0.19
NDEA	0.71±0.85[a]	1.58±1.06[a]	5.87±0.51[a]	3.38±0.75[a]	0.96±0.56[a]
EA+NDEA	1.95±0.43[b]	5.93±0.73[b]	11.84±0.91[b]	8.07±0.38[b]	4.03±0.36[b]

Values are expressed as mean ± S.D. (n =6). Statistical significance $p<0.05$.

Activity is expressed as µmol of GSH Oxidized per min per mg of protein for GPx; units per min per mg of protein for GST; 50% inhibition of epinephrine auto oxidation for SOD; µmole Of hydrogen peroxide decomposed per min per mg of protein for CAT and µmole of NADPH oxidized/(min mg protein) for GR**.**

[a] Comparisons are made with group1 (control).

[b] Comparisons are made with group3 (NDEA-induced).

DISCUSSION

LPO has long been known and has been suggested to be responsible for numerous deleterious effects observed in biological systems, especially after initiation, it concurrently proceeds by a free radicals reaction mechanism and it is regarded as one of the basic mechanism of cellular damage caused by free radical[36]. It is initiated by the obstruction of a hydrogen atom from the side chain of poly unsaturated fatty acids in membrane lipids. Increased LPO alters membrane fluidity and membrane potential and thereby leads to loss of cellular function and cell death[37]. However, the administration of TCE decreased the LPO levels in drug treated animals which may be due to the free radical scavenging activity of the TCE.

α-fetoprotein (AFP) an oncofetal serum protein, is progressively lost during development, such that it is virtually absent from the healthy adult[38]. It has long been recognized that exposure of rats to certain carcinogens like DEN causes an elevation of circulating AFP levels [39]. Carcino embryonic antigen (CEA), a member of the immunoglobulin supergene family, is a 180–200kDa heavily glycosylated protein used clinically as a tumor marker to detect recurrence of many types of tumors[40]. It functions as an adhesion molecule that can form both homotypic and heterotypic aggregates between cells. CEA is cleared from the circulation by the liver with significant traces taken up by the spleen and lungs. In this study, increase in serum AFP and CEA levels upon DEN induction associated with increase in tumor growth. The decrease in tumor markers after TCE administration might due to decrease in the production rate of tumors.

Biochemical marker enzymes are used to screen particularly cancer conditions for differential diagnosis, prognosis, monitoring the progress and for assessing the response to therapy[41]. These enzymes are more unique and changes in their activities reflect the effect of proliferation of cells with growth potential and its metabolic turnover. The rise in their activities is shown to be a good correlation with the number of transformed cells in cancer conditions[42]. In cancer conditions, there will be a disturbance in the transport function carried out by cell organelles including hepatocytes, resulting in the leakage of enzymes due to altered permeability of plasma membrane, and thereby causing a decreased level of these marker enzymes in the cells and increased level in serum. The structural integrity of the cells has been reported to be damaged in toxicity induced animals and this results in cytoplasmic leakage of enzyme into the blood stream[43]. In this study, increase in pathophysiological marker enzyme levels upon DEN induction might due to disturbance in the transport function and the leakage of the enzyme. TCE administration rectified the disturbance and enzyme leakage.

SOD is said to act as the first line of defense against sduperoxide radical generated as a by-product of oxidative phosphorylation[44]. Further, CAT or GPx converts H_2O_2 to H_2O. Depletion in the activities of these antioxidant enzymes can be owed to an enhanced radical production during DEN metabolism. Glutathione is required to maintain the normal reduced state of cells and to counter act all the deleterious effects of oxidative stress. GSH is said to be involved in many cellular processes including the detoxification of endogenous and exogenous compounds[45]. Decreases in the activities of SOD, CAT, GPx, GR, GSH and GST are seen in tumor bearing animals this might be due to increased tumor growth rate. Rate of increase in antioxidants after TCE administration suggesting that the scavenging of excessive free radicals in the body and hinder process of carcinogenesis by TCE.

Ultra-structural studies showed that the animals induced with DEN have multiple irregular shaped nuclei close to each other with irregular cytoplasm and with metastatic potential were seen which might be due to the excessive free radical generation during DEN administration[46]. Control and experimental groups showed similar kind of architecture whereas, DEN induced animals showed altered morphological structures such as dysplastic nuclei, membrane changes with irregular cytoplasm. The signs of stimulation of apoptosis were noted by the presence of liver cell with shrunken nucleus, condensed chromatin, membrane blebbing and formation of apoptotic bodies in TCE treated rats. Thus the results confirmed the stimulation of apoptosis by TCE. Therefore, in this study, TCE might render protection to macromolecules to avoid damage from xenobiotic such as DEN by maintaining the redox balance thereby exhibit anticancer activity during DEN induced liver cancer.

CONCLUSION

The results of the present study demonstrate that TCE attenuates LPO, normalizes pathophysiological marker enzymes and nucleic acid levels and increases antioxidant status. Further, TCE provides evidence for induction apoptosis. Thus the results of the present investigation have confirmed the efficacy of TCE as an effective chemotherapeutic agent.

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