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Antioxidant activity of *Terminalia arjuna* bark extract on *N*-nitrosodiethylamine induced hepatocellular carcinoma in rats

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Abstract

The present investigation was carried out to evaluate the antioxidant nature of ethanolic extract of *Terminalia arjuna* bark (EETA) on *N*-nitrosodiethylamine (DEN) induced liver cancer in male Wistar albino rats. Liver cancer was induced by single intraperitonial injection of DEN (200 mg/kg). After 2 weeks of DEN administration, Phenobarbital (PB) was given to promote the cancer for up to 14 successive weeks. EETA extract (400 mg/kg) was given post-orally for 28 days to hepatocellular carcinoma-bearing rats. After the experimental period, all the animals were sacrificed and serum, liver and kidney samples were collected for further biochemical analysis. The levels of lipid peroxides (LPO) under basal and also in the presence of inducers (H_2O_2, ascorbate and $FeSO_4$) were estimated in serum, liver and kidney of control and experimental animals. Enzymic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and non-enzymic antioxidants like Vitamin C (Vit-C) and Vitamin E (Vit-E) levels were determined in all the groups of animals. A significant increase in LPO levels were observed while the levels of enzymic and non-enzymic antioxidants were decreased, when subjected to DEN induction. These altered enzyme levels were ameliorated significantly by administration of EETA at the concentration of 400 mg/kg in drug-treated animals. This protective effect of EETA was associated with inhibition of LPO induced by DEN and to maintain the antioxidant enzyme levels. Our results show an antioxidant activity of *T. arjuna* bark against DEN-induced liver cancer. (Mol Cell Biochem **281:** 87–93, 2006)

Key words: antioxidants, free radicals, liver cancer, *N*-nitrosodiethylamine, *Terminalia arjuna*

Introduction

Hepatocellular carcinoma (HCC) constitutes about 85% of primary liver cancer. Globally around 440,000 new cases of HCC occur annually, accounting for around 5.5% of all human cancer incidence. Almost the same number die of this cancer annually [1]. In general, hepatic chemical carcinogenesis is a multistep process in experimental animals [2]. Carcinogens initiate the process, which is followed by regeneration, growth and clonal proliferation, eventually leading to cancer [3]. *N*-Nitrosodiethylamine (DEN) is a representative chemical of a family of carcinogenic *N*-nitroso compounds. Administration of DEN to animals has been shown to cause cancer in liver and at low incidence in other organs also. The involvement of free radicals in DEN-induced liver cancer has been extensively studied [4]. Initiation during or

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after DEN exposure is thought to be a rapid metabolism of DEN to reactive metabolites that interact with DNA, forming various DNA adducts that can lead to mutations. Hence, O4-ethyldeoxythymidine adduct (O4-Etdt) accumulates in hepatocyte DNA following DEN administration which is thought to be important in tumour initiation [5].

There is extensive evidence that the free radicals participate in DEN-induced hepatocarcinogenesis, which was confirmed by overexpression of 8-hydroxyguanine in DEN-administered rat liver [6]. Generally, oxygen free radicals are natural physiological products, but also extremely reactive oxygen species (ROS). They have been proved to cause numerous cellular anomalies, including but not limited to protein damage, deactivation of enzymatic activity, alteration of DNA and lipid peroxidation of membranes [7]. When the excess amount of ROS accumulates, numerous pathological effects may manifest in the cells including carcinogenesis [8]. Continuous interaction of the animal with these free radicals causes damage of proteins, lipid, DNA, carbohydrates and membrane, resulting in oxidative stress. In order to maintain cellular health, it is essential to have a specific and effective chemical scavenger to target multiple types of radicals. Most of the commercially based antioxidant supplements are single oxidant [9]. Therefore, it is important to find a specific scavenger to efficiently and effectively reduce multiple ROS. It was also observed that majority of the antioxidants originate from natural sources. These accelerated the research to find out a novel antioxidant from natural resources and further modification and refinement of active antioxidant molecules. It has been noticed that many of the plants, rich in phenolic compounds, are widely used as antioxidant and antimutagenic.

T. arjuna (Combretaceae) Roxenberg is an important medicinal plant rich in tannins and triterpenes and used extensively as a cardiotonic in the Ayurveda [10]. A few randomised clinical trials of unstandardised concoction of the tree bark have been performed in coronary heart disease (CHD) patients in India [11]. Interestingly, it was reported that several species of *Terminalia* have been used in traditional treatment of cancer [12]. In the present investigation, we studied the antioxidant activity of ethanolic extract of *T. arjuna* bark on DEN-induced HCC in rats.

Materials and methods

Plant material

The fresh bark of *T. arjuna* was collected during September 2002 in Chennai, Tamil Nadu, India. The plant was authenticated by Botanist, Captain Srinivasa Murti Drug Research Centre for Ayurveda, Chennai. A voucher specimen (No. 064) has been deposited in the herbarium of the same department.

Preparation of plant extract

The shade dried *T. arjuna* bark was coarsely powdered (1 kg) and soaked in 1000 ml of ethanol for 10 days at room temperature. The extract was filtered and concentrated to obtain the solid residue and the final weight was noted and stored. The yield of the total ethanolic extract was 8.5%. Primary phytochemical screening of the ethanolic extract of *T. arjuna* bark revealed the presence of triterpenoids, phenols, flavonoids, tannins and saponins.

Animals

Healthy male Wistar albino rats aged 6 weeks were procured from Tamil Nadu Veterinary and Animal Science University, Chennai. The animals were randomised and housed in polypropylene cages (four per cage) with rice husks for bedding and maintained in an airconditioned room at $25 \pm 2\,^{\circ}C$, a relative humidity of $36 \pm 6\%$ with 12 h dark cycle; they were fed with normal rat chow, marketed by M/s Hindustan Lever Limited, Mumbai, India and water *ad libitum*. The protocol was approved by the Institutional Animal Ethics Committee (IAEC no. 07/023/03).

Experimental design

The rats were divided into four groups, each consisting of six animals. Group I – control animals were given normal saline (0.9%), Groups II and III animals were administered with single intraperitoneal injection of *N*-nitrosodiethylamine (DEN, Sigma Chemical Company, USA) at a dose of 200 mg/kg body weight in saline to induce liver cancer. Two weeks after administration of DEN, Phenobarbital (PB, Sigma) at a concentration of 0.05% was incorporated into rat chow for up to 14 successive weeks to promote the cancer. After the induction period, Group III animals were treated with ethanolic extract of *T. arjuna* tree bark orally at a concentration of 400 mg/kg body weight for 28 days. Group IV animals were served as plant extract control.

At the end of the experimental period, all the animals were sacrificed by cervical decapitation. The liver cancer nodules were observed, counted and hepatocellular carcinoma was proved by pathological examination [13]. Blood samples were allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2500 rpm at $30\,^{\circ}C$ for 15 min. The liver and kidney were immediately excised, weighed and homogenised in 0.1 M ice-cold Tris-HCl buffer (pH 7.4) to give 10% homogenate.

Biochemical estimation

All the samples were used for following biochemical investigations: Total protein [14], lipid peroxidation (LPO)-thiobarbituric acid reactive substances (TBARS) [15] and *in vitro* induction of peroxidation with inducers (H_2O_2, ascorbate and $FeSO_4$) [16], enzymic antioxidants, such as superoxide dismutase (SOD) [17], catalase (CAT) [18], glutathione peroxidase (GPx) [19] and non-enzymic antioxidants like vitamin C (Vit-C) [20] and vitamin E (Vit-E) [21].

Statistical analysis

Statistical differences were calculated by one-way ANOVA followed by LSD multiple comparisons using SPSS 7.5 student version. The values were expressed as mean ± S.E.M. in each group. Comparisons were made between Groups II, IV and I, and between Groups III and II. $p < 0.05$ was considered statistically significant in all the cases.

Results

The effect of EETA on LPO in serum and liver of control and experimental animals are presented in Figs. 1 and 2. The levels of LPO were found to be significantly increased in Group II DEN-induced cancer-bearing animals compared with control animals under basal conditions and also in the presence of inducers ($p < 0.05$). Conversely, the administration of EETA reduced the peroxidation reaction in Group III drug-treated animals when compared with Group II cancer-bearing animals ($p < 0.05$). Table 1 shows the levels of LPO in kidney of control and experimental animals. Under basal and in the presence of inducers (H_2O_2 and ascorbate), the levels of LPO were significantly increased ($p < 0.05$) in Group II cancer-bearing animals. However, the levels of LPO were decreased in Group III drug-treated animals when compared with DEN-induced liver cancer animals.

Table 2 shows the activities of SOD, CAT, GPx, Vit-C and Vit-E in serum of control and experimental animals. Serum of Group II DEN-induced cancer-bearing animals shows a significant decrease in enzymic and non-enzymic antioxidants levels ($p < 0.05$). However, the levels of SOD, CAT, GPx and Vit-E were increased significantly in DEN + EETA group ($p < 0.05$) when compared with Group II animals. Similarly, liver of Group II cancer-bearing animals shows a significant decrease in enzymic and non-enzymic antioxidants levels ($p < 0.05$) (Table 3). However, a significant increase of these enzymes was observed in DEN + EETA treated group when compared with Group II animals. Table 4 shows the levels of enzymic and non-antioxidants in kidney of control and experimental animals. The levels of enzymic and non-enzymic antioxidants were significantly decreased in Group II animals ($p < 0.05$) when compared with Group I

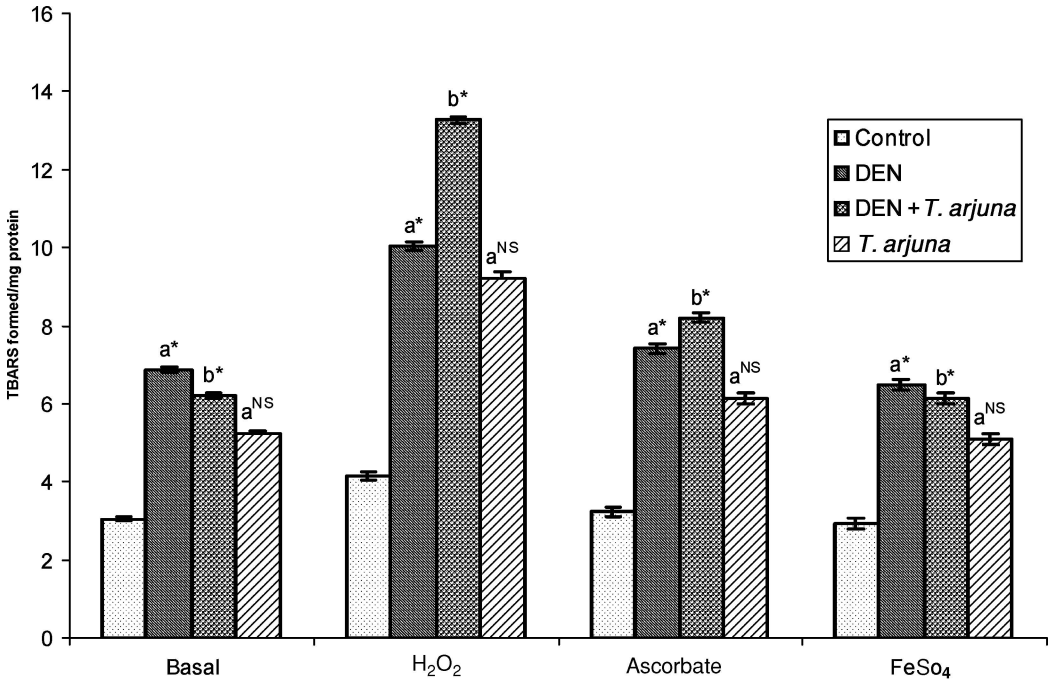

Fig. 1. Levels of lipid peroxidation in serum of control and experimental animals. Values are mean ± S.E.M.: (a) Groups II and IV compared with Group I; (b) Group III compared Group II; $^*p < 0.05$; NS: not significant; d.f. = 3, 20, F ratio: basal, 87.19; H_2O_2, 646.95; ascorbate, 225.66; $FeSO_4$, 188.90.

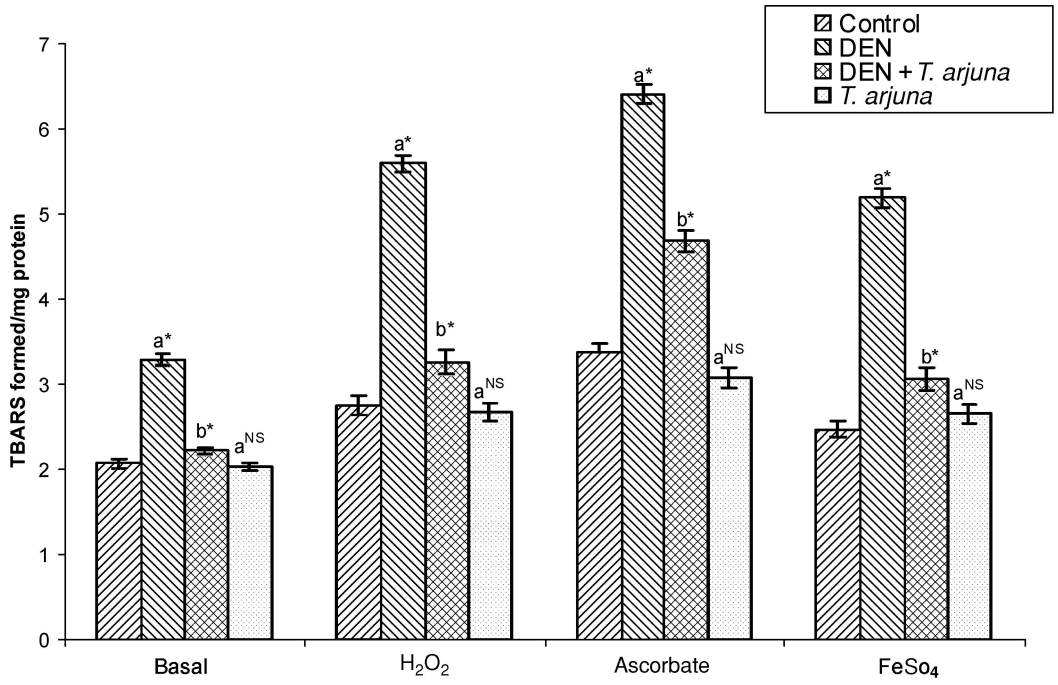

Fig. 2. Levels of lipid peroxidation in liver of control and experimental animals. Values are mean ± S.E.M.: (a) Groups II and IV compared with Group I; (b) Group III compared Group II; $^{*}p < 0.05$; NS: not significant; d.f. = 3, 20; *F* ratio: basal, 109.21; H_2O_2, 151.98; ascorbate, 115.96; $FeSO_4$, 132.21.

Table 1. Levels of lipid peroxidation in kidney of control and experimental animals

Parameters	Group I (Control)	Group II (DEN)	Group III (DEN + *T. arjuna*)	Group IV (*T. arjuna*)	*F* ratio
Basal	1.85 ± 0.05	2.35 ± 0.06[a*]	2.05 ± 0.05[b*]	1.75 ± 0.05[a] (NS)	21.54
H_2O_2 induced	5.46 ± 0.11	8.92 ± 0.13[a*]	6.96 ± 0.12[b*]	5.19 ± 0.08[a] (NS)	214.70
Ascorbate induced	3.46 ± 0.10	4.04 ± 0.12[a*]	3.58 ± 0.12[b*]	3.24 ± 0.14[a] (NS)	7.21
$FeSO_4$ induced	5.86 ± 0.11	6.00 ± 0.14[a] (NS)	5.95 ± 0.20[b] (NS)	5.54 ± 0.13[a] (NS)	1.79

Note. Each value represents mean ± S.E.M.; values are expressed as TBARS formed/min/mg protein. NS: not significant; d.f. = 3, 20.
[a]Groups II and IV compared with Group I.
[b]Group III compared with Group II.
$^{*}p < 0.05$.

animals. A significant increase of these enzymes was observed in Group III drug-treated animals when compared with Group II cancer-bearing animals.

However, the Group IV drug control animals do not show noticeable changes in these parameters when compared with the control animals indicating no adverse side effects due to the administration of *T. arjuna* in Group IV animals.

Discussion

Lipid peroxidation refers to the reaction of oxidative deterioration of polyunsaturated lipids. Peroxidation involves the direct reaction of oxygen and lipid to form radical intermediates and to produce semistable peroxides, which in turn damage the enzymes, nucleic acids, membranes and proteins. The increased levels of LPO under basal and also in the presence of inducers (H_2O_2, ascorbate and $FeSO_4$) in Group II cancer-bearing animals may be due to free radicals produced by DEN administration. Malondialdehyde (MDA), which is a major end product and an index of LPO, cross-links protein and nucleotides on the same and opposite strands [22]. Thirunavukkarasu and Sakthisekaran [4] reported that there was a significant increase in MDA level after DEN administration. Furthermore, it was documented that MDA is mutagenic in mammalian systems, which readily reacts with deoxynucleodies to produce adducts and cause DNA damage. Hence, the present study shows the increase in MDA levels.

Table 2. The activities of enzymic and non-enzymic antioxidants in serum of control and experimental animals

Parameters	Group I (Control)	Group II (DEN)	Group III (DEN + *T. arjuna*)	Group IV (*T. arjuna*)	*F* ratio
SOD	4.92 ± 0.09	$3.27 \pm 0.09^{a*}$	$3.83 \pm 0.09^{b*}$	5.15 ± 0.09^{a} (NS)	91.99
CAT	29.45 ± 0.54	$18.37 \pm 0.56^{a*}$	$23.40 \pm 0.58^{b*}$	30.51 ± 0.57^{a} (NS)	99.37
GPx	3.68 ± 0.09	$2.49 \pm 0.08^{a*}$	$3.32 \pm 0.08^{b*}$	3.81 ± 0.09^{a} (NS)	42.32
Vit-C	1.74 ± 0.05	$1.38 \pm 0.04^{a*}$	1.45 ± 0.05^{b} (NS)	1.77 ± 0.04^{a} (NS)	15.11
Vit-E	1.54 ± 0.05	$1.16 \pm 0.05^{a*}$	$1.45 \pm 0.06^{b*}$	1.56 ± 0.05^{a} (NS)	9.58

Note. Values are mean ± S.E.M.; NS: not significant; d.f. = 3, 20; enzyme units are expressed as SOD: units/mg protein, CAT: μmol of H_2O_2 utilised/min/mg protein, GPx: μg of glutathione utilised/min/mg protein, Vit-C and E: mg/dl.
[a]Groups II and IV compared with Group I.
[b]Group III compared with Group II.
$^{*}p < 0.05$.

Table 3. Changes on enzymic and non-enzymic antioxidant activities in liver of control and experimental animals

Parameters	Group I (Control)	Group II (DEN)	Group III (DEN + *T. arjuna*)	Group IV (*T. arjuna*)	*F* ratio
SOD	8.88 ± 0.15	$4.41 \pm 0.11^{a*}$	$8.19 \pm 0.10^{b*}$	$9.00 \pm 0.18^{a*}$	197.02
CAT	62.53 ± 1.43	$39.62 \pm 1.46^{a*}$	$59.40 \pm 1.62^{b*}$	63.03 ± 1.43^{a} (NS)	55.66
GPx	4.08 ± 0.09	$2.29 \pm 0.07^{a*}$	$3.41 \pm 0.09^{b*}$	4.14 ± 0.15^{a} (NS)	62.52
Vit-C	0.88 ± 0.03	$0.55 \pm 0.03^{a*}$	$0.81 \pm 0.03^{b*}$	0.94 ± 0.03^{a} (NS)	26.36
Vit-E	6.30 ± 0.10	$3.04 \pm 0.14^{a*}$	$3.79 \pm 0.15^{b*}$	6.40 ± 0.12^{a} (NS)	163.74

Note. Values are mean ± S.E.M.; NS: not significant; d.f. = 3, 20; enzyme units are expressed as SOD: units/mg protein, CAT: μmol of H_2O_2 utilised/min/mg protein, GPx: μg of glutathione utilised/min/mg protein, Vit-C and E: mg/g wet tissue.
[a]Groups II and IV compared with Group I.
[b]Group III compared with Group II.
$^{*}p < 0.05$.

Table 4. The activities of enzymic and non-enzymic antioxidants in kidney of control and experimental animals

Parameters	Group I (Control)	Group II (DEN)	Group III (DEN + *T. arjuna*)	Group IV (*T. arjuna*)	*F* ratio
SOD	5.24 ± 0.12	$4.39 \pm 0.12^{a*}$	$4.87 \pm 0.13^{b*}$	5.49 ± 0.11^{a} (NS)	91.99
CAT	45.50 ± 0.51	$18.37 \pm 0.63^{a*}$	$38.53 \pm 0.57^{b*}$	46.63 ± 0.53^{a} (NS)	99.37
GPx	3.18 ± 0.09	$2.77 \pm 0.10^{a*}$	2.85 ± 0.09^{b} (NS)	3.29 ± 0.07^{a} (NS)	42.32
Vit-C	2.36 ± 0.05	$1.54 \pm 0.05^{a*}$	$2.15 \pm 0.06^{b*}$	2.43 ± 0.06^{a} (NS)	15.11
Vit-E	3.34 ± 0.05	$2.24 \pm 0.05^{a*}$	$2.75 \pm 0.06^{b*}$	3.44 ± 0.06^{a} (NS)	9.58

Note. Values are mean ± S.E.M.; NS: not significant; d.f. = 3, 20; enzyme units are expressed as SOD: units/mg protein, CAT: μmol of H_2O_2 utilised/min/mg protein, GPx: μg of glutathione utilised/min/mg protein, Vit-C and E: mg/g wet tissue.
[a]Groups II and IV compared with Group I.
[b]Group III compared with Group II.
$^{*}p < 0.05$.

It also suggests that enhanced LPO and failure of antioxidant defence mechanism lead to tissue damage in cancer-bearing animals.

SOD has been reported as one of the most important enzymes in the enzymic antioxidant defence system. It scavenges the superoxide anion to form hydrogen peroxide, hence diminishing the toxic effect caused by this radical [23]. The increased superoxide radical levels in tumour cells [24] as compared with normal cells may explain the decrease of the enzymic activity in malignant than normal tissues. In the present study, the decreased level of SOD as observed in cancer-bearing animals may be due to the utilisation of the enzyme to scavenge H_2O_2 radicals. Catalase decomposes hydrogen peroxide and protects the tissue from highly reactive

hydroxyl radicals [25] and it is thought to be the first line of defence against oxidative damage caused by hydrogen peroxide and other radicals induced by carcinogen. The decreased level of CAT activities in Group II cancer-bearing animals may be due to the utilisation of this enzyme in the removal of hydrogen peroxide radicals caused by DEN administration. Glutathione peroxidase (GPx) is also considered to be an important H_2O_2 removing enzyme in mammalian cells and is more important than catalase for removing H_2O_2 [26]. GPx is involved in the defence mechanism against oxidative damage, it reduces the H_2O_2 and hydroperoxide levels. The present study reveals that the activity of GPx in liver and kidney was significantly decreased in HCC-bearing animals. The decreased activity of GPx in cancer condition may be due to excessive production of lipid hydroperoxides. GPx levels are also relatively low in hepatoma [27]. Reduction in SOD and GPx in hepatoma conditions would be expected to have dire consequences. But reduction in GPx is found to be more deleterious than SOD. The greater relative importance of GPx over SOD can be attributed to the ability of GPx to detoxify H_2O_2 formed by SOD [28].

Enzymic antioxidants are inactivated by hydroxyl radicals, and hence the presence of non-enzymic antioxidant is presumably essential for the removal of these radicals. Vit-C is a water soluble antioxidant that removes free radicals from cytosol by reacting directly with them [29]. Thus, the decreased level of Vit-C found in Group II cancer-bearing animals may be due to the utilisation of antioxidant to scavenge the free radicals. The availability of Vit-C is a determined factor in controlling and potentiating many aspects of host resistance against cancer. The ascorbate molecule must be involved in the feedback inhibition of lysosomal glycosides responsible for malignant invasiveness [30]. The Vit-C can protect cell membrane and lipoprotein particles from oxidative damage by regenerating the antioxidant from Vit-E [31, 32]. Thus, Vit-C and Vit-E act synergistically in scavenging wide variety of ROS. Vit-E is the major lipid soluble radical scavenger that prevents the LPO by terminating the chain reactions initiated in the membrane lipids [33]. Vit-E is a chain breaking antioxidant by donating its labile hydrogen atom from phenolic hydroxyl groups to propagating lipid peroxyl and alkoxyl radical intermediates of LPO [34]. Decreased Vit-E content in Group II cancer-bearing animals might be due to the excessive utilisation of this antioxidant for quenching enormous free radicals produced in these conditions. Besides, Vit-E has been found to have potent antioxidant activity due to its ability to penetrate to a precise site into the membrane, which may be the important feature of protection against highly reactive radicals [35].

The above biochemical alterations observed in Group II HCC animals may be due to the induction of LPO and the decrease of antioxidant enzymes following DEN administration. These alterations were significantly reversed towards normal level in Group III EETA-treated animals at a concentration of 400 mg/kg body weight.

It was noticed that many of the plants, rich in polyphenolic compounds, harbour antimutagenic substances [36]. Polyphenols are known to form a complex with proteins. The association of polyphenols with proteins is principally a surface phenomenon [37] and this association depends on the oligomeric nature of the polyphenol. The more the oligomeric nature, the more is the association which results in the increased antimutagenic activity. In this context, Wang *et al.* [38] reported that hydrolysable tannins are natural polyphenolic antioxidants capable of inhibiting H_2O_2 production and tumour promotion. Teel has shown that the anticarcinogenic and antimutagenic activity of plant phenols is due to an interaction of the compound with target tissue DNA which in turn blocks the sites of DNA to electrophilic attack by reactive carcinogenic moieties [39]. Hence, the regression of HCC in drug-treated animals may be due to the anticancer activity of *T. arjuna* bark extract.

Sumitra *et al.* [40] reported that arjunolic acid from *T. arjuna* prevents the decrease of enzymic antioxidants, such as SOD, CAT, GPx and Ceruloplasmin and non-enzymic antioxidants like ascorbic acid, reduced glutathione in isoproterenol-induced myocardial necrosis in rats. This is in accordance with the findings of Karthikeyan *et al.* [41], according to which alcoholic extract of *T. arjuna* bark augments endogenous antioxidant enzymes. Moreover, Kaur *et al.* [36] reported that tannin fraction of *T. arjuna* possesses antimutagenic activity against 4-nitro-phenylenediamine (NPD) in TA 98, tester strain of *Salmonella typhimurium* using the Ames assay and suggested that the activity of this compound is due to the interaction with DNA. Previous studies carried out in our laboratory proved the anticancer activity of *T. arjuna* ameliorates the marker enzyme levels [13] and regulates the carbohydrate metabolism [42] in DEN-induced liver cancer animals. Thus, the anticancer effect of ethanolic extract of *T. arjuna* in Group III animals might be due to synergistic mechanism of the plant extract, i.e., scavenging the free radicals induced by the DEN administration via antioxidant enzyme systems and binding of phenolic groups with target DNA to block the sites of DNA to electrophilic attack from pre-existing free radicals of carcinogenic moieties.

Thus, EETA significantly ameliorates the changes on both enzymic and non-enzymic antioxidants in drug-treated carcinogenic animals at the concentration of 400 mg/kg body weight. Therefore, it can be concluded that the *T. arjuna* possesses the anticancer activity through quenching free radicals induced by the DEN. Hence, it was suggested that the regression of liver cancer may be due to the antioxidant activity of *T. arjuna*. The potential ability of EETA against liver cancer in different constituents of *T. arjuna* remains to be studied.

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