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Anticancer Potency of *Terminalia arjuna* Bark on N-nitrosodiethylamine-Induced Hepatocellular Carcinoma in Rats

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Abstract – The anticancer potency of the ethanolic extract of *Terminalia arjuna* on N-nitrosodiethylamine (DEN) induced hepatocellular carcinoma in Wistar albino rats was studied. Single intraperitoneal injection of DEN was administrated to induce liver cancer. After two weeks, phenobarbital (PB) was given orally for fourteen weeks to promote the cancer. The cancer bearing animals treated with ethanolic extract of *T.arjuna* (400 mg/kg body weight) for 28 days. Nucleic acids such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were estimated in liver and kidney of control and experimental animals. Certain marker enzymes viz, alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), acid phosphatase (ACP), alkaline phosphatase (ALP), 5'-nucleotidase (5'ND) and lactate dehydrogenase (LDH) were assayed in serum, liver and kidney of control and experimental animals. The levels of DNA and RNA were significantly increased in cancer bearing animals. The activities of ALT, AST, ACP, ALP, 5'ND, and LDH were significantly ($P < 0.001$) increased in serum of cancer bearing animals. On the other hand, the levels of ALT, AST were decreased ($P < 0.001$) and ACP, ALP, 5'ND, and LDH were significantly increased ($P < 0.001$) in liver and kidney. These changes were reversed to near normal in drug treated animals. These observations suggest that the ethanolic extract of *T.arjuna* possess anticancer activity.

Key words – *Terminalia arjuna*, N-nitrosodiethylamine, Hepatocellular carcinoma, Phosphatases, Aminotransaminases, Lactate dehydrogenase

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequently occurring malignancy in developing countries. According to Tea (2001) is of the opinion that in worldwide one million deaths occur per year due to the HCC. In this context, Rocken (2001) suggested that the occurrence of HCC may be due to multifactorial environmental conditions such as infectious, nutritional, metabolic, endocrine factors, aflatoxin exposure and alcohol consumption. The chemotherapeutic agents such as mitomycin, adriamycin and cisplatinum are effective against liver cancer. Most of the chemotherapeutic agents are produce cytotoxic and side effects. Therefore, there is a need of alternatives for this. Many research work have been carried out from the natural sources for cure of human diseases, particularly to cancer.

Tropical and subtropical countries have a number of medicinal plants, which have some potential effects to cure liver cancer. Numerous medicinal plants and their formulations are used for liver disorder in ethanomedical practices as well as traditional system of medicine in India (Gupta, 2003). *Terminalia arjuna* Roxburgh. (Family: Combretaceae) known as "Arjuna" in Indian medicine is a large tree with huge trunk and horizontally spreading branches. All the parts of the plant are used in Indian medicine for cure of number of diseases such as cardiotonic, diuretic, antidysentric, fractures, ulcers, blood diseases, intoxications, urinarydischarges, biliousness, aneamia, asthma, tumours, leucoderma, wounds, aphordisial and hypertension (Chopra, 1958; Chopra, 1956; The Wealth of India, 1976; Chopra, 1929; Caius, 1930).

Hartwell (1982) reported that atleast seven *Terminalia arjuna* species have been used in cancer Treatment. The various parts of the *T. arjuna* have yielded a variety of compounds such as flavonoids, pentacyclic triterpenoids and ellagic acid (Huang, 1983; Kapoor, 1990; Bone, 1996). Therefore, it is of interest to investigate the effect of ethanolic extract of *T. arjuna* bark on nucleic acids and certain marker enzymes of DEN induced HCC in rats.

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Material and Methods

Plant material – The fresh bark of *Terminalia arjuna* was collected during September 2002 in Chennai, India. The plant was authenticated by botanist, Captain Srinivasa Murti Drug Research Centre for Ayurveda, Chennai, Tamil Nadu, India. 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 suspended 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 weights were 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, phenol, flavonoids, tannin and saponins.

Animals – Male Wistar albino rats (60 – 80 g) were used in the present study and were obtained from Tamil Nadu University of Veterinary and Animal Sciences (TANUVAS), Chennai, India. The animals were maintained at 25° ± 2°C and they were given with standard commercially available pellet diet, marketed by M/S Hindustan Lever Limited, Mumbai, India and water *ad libitum*.

Experimental design – The rats were divided into four different groups, each consists of six animals. Group I animals were given normal saline, Group 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 chaw for upto 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 served as plant extract control.

After the experimental period all the rats were weighed and killed by cervical decapitation. The weight of the liver and kidney were noted. The grayish white hyperplastic nodules were obtained from all the groups treated with DEN and were easily identified from the surrounding reddish brown liver tissues. In carcinogen treated group the total number of nodules were 192 where as in drug treated group, it were decreased to 58. The carcinoma was proved by pathological examination. Blood was collected and allowed to clot for 45 mins and serum was separated by centrifugation at 3000 × g at 30°C for 15 mins.

The levels of nucleic acids were estimated in liver and kidney of control and experimental animals. The extractions of nucleic acids were followed by the method of Schneider (1957). The DNA and RNA were estimated by the method of Burton (1956) and Rawal *et al.* (1977) respectively. Serum, liver and kidney samples were used for the estimation of alanine and aminotransaminase, aspertate aminotransaminase, acid and alkaline phosphatase phosphastase, 5'-nucleotidase and lactate dehydrogenase according to the method of King (1965a,b,c) and Luly (1972) respectively.

Statistical analysis – Statistical differences between the groups were calculated by independent sample-'*t*' test by using SPSS 7.5 student version. The Mean ± S.D was compared in each group. Comparisons were made between group II and I, group II and III, group I and IV.

Results

The effect of ethanolic extract of *Terminalia arjuna* on body, liver and kidney weight are presented in Table 1. Body weight were significantly decreased ($P < 0.001$) in group II DEN induced HCC rats. On the other hand, liver weight was slightly increased ($P < 0.01$) in cancer bearing animals. However, no significant differences were observed in liver and kidney weight during treatment. Table 2. represents the levels of nucleic acids (DNA and RNA) in liver and kidney of control and experimental animals. In group II animals the levels of nucleic acids were significantly elevated. These were slightly decreased in drug treated animals. However, no significant changes were observed.

The effect of ethanolic extract of *Terminalia arjuna* on

Table 1. Effects of ethanolic extract of *Terminalia arjuna* on body, liver and kidney weight of control and experimental animals

Parameters	Group I	Group II	Group III	Group IV
Body weight (gm)	293.33 ± 10.80	236.66 ± 10.80 a*	273.33 ± 10.80 b*	296.66 ± 12.90 a^{NS}
Liver weight (gm)	7.416 ± 0.43	8.4 ± 0.46 $a^{\#}$	8.0 ± 0.34 b^{NS}	7.543 ± 0.174 a^{NS}
Kidney weight (gm)	5.698 ± 0.18	5.876 ± 0.10 a^{NS}	5.746 ± 0.14 b^{NS}	5.738 ± 0.201 a^{NS}

Values are mean ± S.D.
a-as compared with group I; b-as compared with group II.
*$P < 0.001$; $^{\#}P < 0.01$; $^{@}P < 0.05$; NS-not statistically significant

Table 2. The levels of nucleic acids in liver and kidney of control and experimental animals

Parameters mg/g of wet tissue		Group I	Group II	Group III	Group IV
Liver	DNA	7.598 ± 0.19	8.630 ± 0.234 a*	8.361 ± 0.22 b^{NS}	7.646 ± 0.213 a^{NS}
	RNA	5.436 ± 0.205	6.560 ± 0.209a*	5.798 ± 0.180 b*	5.320 ± 0.201a^{NS}
Kidney	DNA	4.331 ± 0.22	4.816 ± 0.226$a^{\#}$	4.616 ± 0.210b^{NS}	4.320 ± 0.218 a^{NS}
	RNA	6.211 ± 0.210	8.156 ± 0.242 a*	7.936 ± 0.230b^{NS}	6.160 ± 0.238 a^{NS}

Values are mean ± S.D.
a-as compared with group I; b-as compared with group II.
*$P < 0.001$; $^{\#}P < 0.01$; $^{@}P < 0.05$; NS-not statistically significant.

Table 3. Activities of tumor marker enzymes in serum of control and experimental animals

Parameters	Group I	Group II	Group III	Group IV
ALT (μ mol of pyruvate liberated/mg protein/min)	28.701 ± 1.712	39.531 ± 2.023 a*	31.274 ± 1.25 b*	27.381 ± 1.292 c^{NS}
AST (μ mol of pyruvate liberated/mg protein/min)	4.593 ± 0.444	6.224 ± 0.465 a*	5.001 ± 0.183 $b^{\#}$	4.753 ± 0.424 c^{NS}
ACP (μ mol of phenol liberated/mg protein/min)	25.029 ± 1.198	37.568 ± 1.079 a*	26.181 ± 1.038 b*	25.187 ± 0.988 c^{NS}
ALP (μ mol of phenol liberated/mg protein/min)	180.53 ± 15.446	272.226 ± 14.177 a*	191.608 ± 9.79 b*	173.885 ± 15.086 c^{NS}
5'-ND (n mol of phosphate liberated/mg protein/min)	2.889± 0.240	5.720 ± 0.401 a*	3.106 ± 0.215 b*	2.709 ± 0.160 c^{NS}
LDH (μ mol of pyruvate liberated/mg protein/min)	0.90 ± 0.092	1.52 ± 0.083 a*	1.082 ± 0.186 $b^{\#}$	0.911 ± 0.075 c^{NS}

Values are mean ± S.D.
a-as compared with group I; b-as compared with group II.
*$P < 0.001$; $^{\#}P < 0.01$; $^{@}P < 0.05$; NS-not statistically significant.

aminotransaminases, phosphatases, 5'-nucleotidase and lactate dehydrogenase levels in serum of control and DEN induced liver cancer animals are presented in Table 3. There was a significant ($P < 0.001$) increase in serum ALT, AST, ACP, ALP, 5'-ND, and LDH in cancer bearing animals. In *T. arjuna* extract administrated animals, the levels of ALT, ACP, ALP, and 5'-ND were reduced and are statistically highly significant ($P < 0.001$) where as AST and LDH were reduced significantly ($P < 0.01$).

In liver of group II cancer bearing animals, the activities of ALT and AST were decreased significantly ($P < 0.001$) where as other marker enzymes such as ACP, ALP, 5'ND, and LDH were increased significantly ($P < 0.001$) (Fig. 1). In *T. arjuna* treated group III animals, the levels of AST, ALT, ACP, ALP, 5'ND, and LDH were reversed to near normal. Similarly in kidney of cancer bearing animals there was a significant decrease in the activities of ALT and AST enzyme levels ($P < 0.001$). On the contrary significant increase in the levels of ACP, ALP, 5'ND, and LDH ($P < 0.001$) were observed (Fig. 2). When the administration of ethanolic extract of *T. arjuna* the levels of AST, ALT, ACP, ALP, 5'-ND, and LDH were return back to near normal. However, there was no significant changes in these parameters were observed in group IV drug control animals when compared with group I control animals.

Discussion

The mortality due to liver carcinoma is higher than other malignant tumors, because of its difficulty in early diagnosis. Most of the clinically diagnosed are large ones when the patients lose the opportunity of operation. Therefore, non-operation therapy becomes more and more important (Laconi *et al.*, 2001; Hidvegi *et al.*, 1998). In this connection, medicinal plants play an important role in curing liver carcinogenesis.

The body weight of DEN induced cancer bearing animals were declined when compare to the control animals. Tessitore *et al.* (1987) have shown that, tumor growth elicited marked loss of body weight in growing ascetic hepatoma bearing rats. This may be due to decreased food intake and or absorption (Pain *et al.*, 1984). The steadily increase body weight were noted in group III drug treated animals may be due to the therapeutic efficacy of ethanolic extract of *T. arjuna.* In

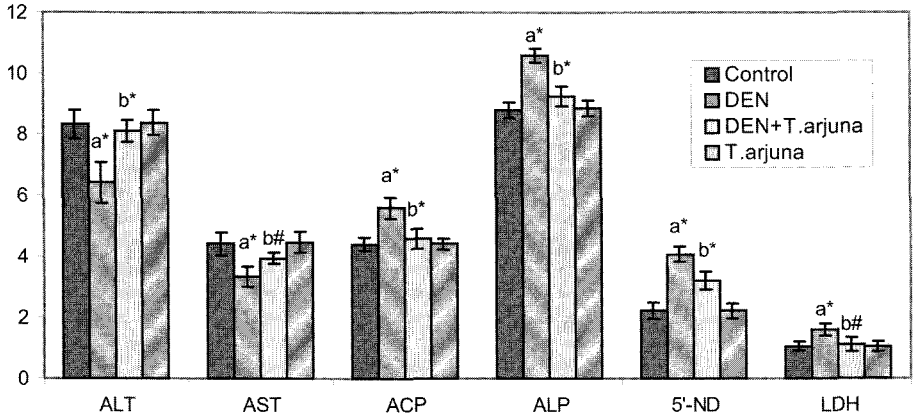

Fig. 1. Activities of marker enzymes in liver of control and experimental animals. Values are expressed as follows: AST and ALT-μmol of pyruvate liberated/mg protein/min; ACP and ALP-μmol of phenol liberated/mg protein/min; 5'ND-nmol of phosphate liberated/mg protein/min; LDH-μmol of pyruvate liberated/mg protein/min.
Values are mean ± S.D., a-as compared with group I; b-as compared with group II.
$P < 0.001$; $^{\#}P < 0.01$; $^{@}P < 0.05$.

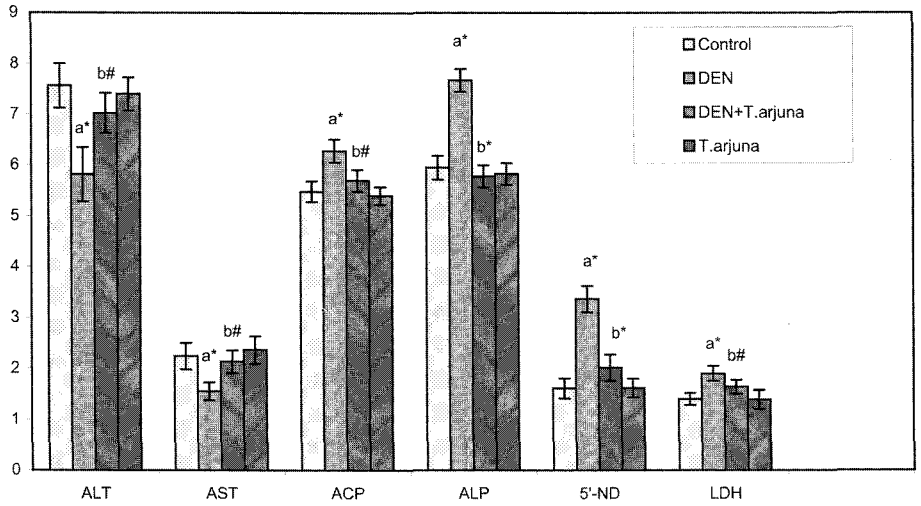

Fig. 2. Activities of marker enzymes in kidney of control and experimental animals. Values are expressed as follows: AST and ALT-μmol of pyruvate liberated/mg protein/min; ACP and ALP-μmol of phenol liberated/mg protein/min; 5'ND-nmol of phosphate liberated/mg protein/min; LDH-μmol of pyruvate liberated/mg protein/min.
Values are mean ± S.D., a-as compared with group I; b-as compared with group II.
$^{*}P < 0.001$; $^{\#}P < 0.01$; $^{@}P < 0.05$.

drug treated animals, nodules were smaller than those observed in DEN induced animals indicate the cytotoxic nature of plant extract and suggested that the *T. arjuna* can restrain the proliferation of rat hepatocellular carcinoma. The level of DNA and RNA of liver and kidney were found to be progressively increased in cancer bearing animals. It has been observed that the tumor growth corresponds to the elevated levels of DNA and RNA synthesis. The administration of *T. arjuna* decrease the synthesis of DNA and RNA. The studies of Dixit and Gold (1986) have shown that polyphenolic (ellagic acid) can inhibit the activity of the direct acting mutagen methylnitrosourea (MNU) and reported that the antimutagenic activity of ellagic acid against the MNU is due to inhibition of methylation of the O^6 position of guanine in double stranded DNA. Hence, the regression of tumor and decrease of nodule size and number may be due to the interaction of this polyphenolic of *T. arjuna* with DNA and inhibit the nucleic acid synthesis.

More sensitive and specific liver cancer marker enzymes viz, lactate dehydrogenase and aminotransaminases were used as a diagnostic tool for liver injury (Kadem *et al.*, 1982). Alteration in serum enzyme levels have been reported for many years as possible indicators of malignancy and to be help in following up the progression and or regression of malignant tumors during therapy.

Damaged cells lose their internal mileu including enzymes, distributed all over the extra cellular space. The rise in their activity in serum has been shown to be in good correlation with the member of transformed cells in cancer conditions (Kadem *et al.*, 1982).

In the present investigation, the serum of hepatocellular carcinoma induced rats, the levels of aspartate and alanine aminotransaminases were increased compare to the normal rats, where as in liver and kidney these enzymes levels were decreased. The elevated levels of serum enzymes are indicative of cellular leakage and lose of functional integrity of the cell membrane in liver as suggested by Drotman and Lowhorn (1978). In view of this, the extract mediated reduction in levels of ALT and AST towards the normal values may be the stabilization of plasma membrane as well as repair of hepatic tissue damage induced by DEN. This is in accordance with the finding of Thabrew (1987).

Phophatases are related to the function of hepatic cell. Increase in serum level of ALP and ACP is due to increased synthesis because of the billary pressure (Moss and Butterworth, 1974). DEN induced elevation of this enzymatic activity in serum is correlated with high level of serum bilirubin content. In this study, the administration of *T. arjuna* extract showed these enzymes were revert back to normal level in liver. This may be possibility of the extract being able to stabilize the billary dysfunction in rat liver during DEN exposure.

LDH have been successfully used as indicators for prognosis during chemotherapy. Stark weather *et al.* (1966) and and Massey *et al.* (1971) reported that serum isoenzyme changes provided another means of evaluating the effects of antitumor agents in patients during therapy. A gradual decrease in serum LDH to normal values following treatment was found to correlate with a favorable patient response to therapy. LDH is a tetrameric enzyme and is recognized as a potential tumor marker in assessing the progression of the proliferating malignant cells. It is a fairly sensitive marker for solid neoplasma (Lippert *et al.*, 1981). The elevated activity of LDH may be due to the over production of tumor cells (Helmes *et al.*, 1998) or it may be due to the release of enzyme from destroyed tissues (Mirmomeni *et al.*, 1979). The elevated LDH activity in cancer bearing rats may also be due to the enhanced glycolysis during the growth of tumor (Nakashimhan, 1988). Drug treated animals reverted the activity to near normal, which may be due to the inhibition of glycolysis and render the protection to membrane integrity. This proved the antineoplastic property of the drug.

5'-ND enzyme hydrolyses nucleotidase with a phosphate group on carbon atom 5γ of the ribose. It is widely found to be distributed in tumor tissue. 5'-ND activity was found to be elevated in cancerous animals. Dao (1980) have reported that the increased activity of 5'-ND seems to have originated from the proliferating liver tumor cells. This elevation of the marker enzyme may be correlated with the progression of the malignancy. Walia *et al.* (1995) have reported higher activities of 5'-ND activity in cancerous breast tissue patients. In our study, the drug treated animals brought back this enzyme activity to near normal level indicating their antitumor property.

Terminalia arjuna is widely used in the Indian system of medicine; its bark particularly is extensively used in Ayurvedic medicine. Sumitra (2001) reported that flavonoids and oligomeric proanthocyanidins from *T. arjuna* provides free radical antioxidant activity and vascular strengthening against myocardial necrosis in rats. Hence, it was assumed that the removal of free radicals induced by DEN which cause the membrane damage of tissues, might be due to the antioxidant properties of active principles such as flavonoids present in the extract. Moreover, large number of studies have been reported that *T. arjuna* possess antimutagenic activity. For instance, Tannin fraction from the *T. arjuna* on the genotoxicity of mutagens in both TA98 and TA 100 tester strains of *Salmonella typhimurium* using Ames assay (Kaur, 2000). From this point view, it was suggested that inhibition of DEN induced liver cancer may also be due to the antimutagenic properties of *T. arjuna.*

From this investigations it can be concluded that the administration of *T. arjuna* bark in at dose of 400 mg/kg body weight showed antineoplastic action through normalize the marker enzymes by preventing the leakage of enzymes from the cells in to the blood and increases the stability of membrane leads to regression of tumor. Thus, the present data provide a rationale for the use of *T. arjuna* as a suitable herbal treatment for the chemotherapy of liver cancer.

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