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Enzymatic and Non-Enzymatic Antioxidant Activities of *Enicostemma littorale* in *p*-DAB Induced Hepatocarcinoma in Rats

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Abstract: The aim of this study was to investigate the effect of *Enicostemma littorale* (Gentianaceae) aerial part on antioxidant defense systems of plasma and liver in *p*-Dimethylaminoazobenzene (*p*-DAB)-induced hepatocarcinoma in rats. The levels of vitamin-E and vitamin-C were estimated in plasma of control and experimental groups of rats. The levels of reduced glutathione, glutathione-S-transferase and activities of superoxide dismutase, catalase and lipid peroxides were assayed in liver tissue of control and experimental groups of rats. Administration of *p*-DAB exhibited a significant increase in the levels of liver lipid peroxides, liver weight and a concomitant decrease in the levels of vitamin-E, vitamin-C in hepatocarcinoma rats. Thus, there was an alteration in the antioxidant enzyme system of hepatocarcinoma rats. These alterations were reverted back to near normal level after the treatment with *Enicostemma littorale* extract and vitamin-E. Histopathological studies also revealed that the protective effect of *Enicostemma littorale* on liver cells.

Key words: Antioxidants, *Enicostemma littorale*, *p*-dimethylaminoazobenzene (*p*-DAB), oxidative stress, hepatocarcinoma

INTRODUCTION

Liver is one of the most important organs for the metabolism of various chemicals and is responsible for the major detoxification. Liver damage/hepatocarcinoma, in general associated with the external agents comes under the classification as carcinogens. *p*-DAB is a coloring agent commonly used in foods and a powerful liver carcinogen appears to be metabolically activated to ultimate carcinogen probably being electrophile in the liver (Fabiana *et al*., 2001). The electrophilic nature of the metabolites of *p*-DAB has the capacity to covalently bind with RNA, DNA and tissue protein (Labuc and Blunck, 1979; Ohinishi *et al*., 2001) and has been restricted by the NADPH/NADH generating system and reduced glutathione system. The conjugation of reduced glutathione with electrophilic derivatives is catalyzed further by a soluble enzyme glutathione-S-transferase. Thus, the metabolism and the detoxification of carcinogens in liver were taken care of through enzymes of glutathione family. Study on quantification of glutathione families and their activity would clearly indicate the degree of damage or the metabolism of the chemical carcinogen.

In other words, reactive oxygen species such as superoxide, hydroxyl radicals and hydrogen peroxide (Halliwell, 1996) are released due to the induction of *p*-DAB, which further stimulate the damage of liver by the metabolized products of lipid peroxidation, consequently leading to DNA aberrations (Biswas and Bukhsh, 2002). Shamberger *et al*. (1974) reported that hydroxyl radicals are highly reactive and are the basis for the generation of lipid peroxide products such as malondialdehyde, which are considered to be a cause for carcinogenesis. Numerous plant products have been shown to have the anti cancer property (Davis and Kuttan, 2001; Ahmed and Khater, 2001) and the antioxidant vitamin, flavanoids and polyphenolic compound of the plant origin have been extensively reported as scavengers of free radicals and inhibitors of lipid peroxidation (Hanasaki *et al*., 1994; Formica and Regelson, 1995; Tapiero *et al*., 2002).

Enicostemma littorale, is traditionally used in rheumatism, abdominal ulcers hernia, swelling, itches and it was recently studied for hypoglycemic (Ravi *et al*., 2000) and anticancer activity (Kavimani and Manisenthkumar, 2000), our earlier studies had confirmed its hypolipidemic potential in *p*-DAB induced hepatotoxic animals (Gopal *et al*., 2004). Recently, aqueous extract of *Enicostemma littorale* was reported to show hypolipidaemic and antioxidant effect in cholesterol fed rats (Vasu *et al*., 2005). The complete characterization of the plant reveals that it has number of flavanoids, alkaloids, steroids, phenolic acids and aminoacids. The various components of the major group chemicals are summarized below.

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Major groups	Components
Ash (15%) (Dymock *et al*., 1893)	Iron, potassium, sodium, calcium, magnesium, silica, phosphate, chloride, sulphate and carbonate.
Alkaloids steroids and triterpenoids (Natarajan and Prasad, 1972; Ghosal *et al*., 1974)	Enicoflavin and gentiocrucine, catechins, saponins, betulin, triterpene sapogenin, swertiamarin
Flavonoids and xanthones (Ghosal and Jaiswal, 1980).	Apigenin, genkwanin, isovitexin, swertisin, saponarin, 5-O-glucosylswertisin and 5-O-glucosylisoswertisin
Phenolic acids (Daniel and Sabnis, 1978).	Vanillic acid, syringic acid, *p*-hydroxy benzoic acid, protocatechuic, acid, *p*-coumaric acid and ferulic acid
Amino acids	L-glutamic acid, tryptophane, alanine, serine, aspartic acid, L-proline, L-tyrosine, threonine, phenyl alanine, L-histidine monohydrochloride, methionine, iso-leucine, L-arginine monohydrochloride, DOPA, L-glycine, 2-amino butyric acid and valine

The present study aims to investigate the effect of *Enicostemma littorale* on enzymatic and non-enzymatic antioxidants in plasma and liver of *p*-DAB-induced hepatocarcinoma rats on par with a standard anticancer drug, vitamin-E.

MATERIALS AND METHODS

Chemicals: *p*-dimethylaminoazobenzene was procured from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals used were of analytical grade.

Plant material: Aerial part of *Enicostemma littorale* (Gentianaceae) was collected from Chennai, Tamil Nadu, India, in the month of December and the plant was identified and authenticated by Dr. T. Anandan, Research officer, Central Research Institute for Siddha Medicine, Chennai.

Preparation of plant material: The plant materials (aerial part alone) were dried under shade and ground well, sieved through a fine cloth and the resultant fine powder (100 μ size) was used as drug in the crude form along with physiological saline.

Animals: Male albino Wister rats, weighing about 150-200 g obtained from Tamil Nadu Veterinary and Animal Sciences University, Chennai, India were used for the present investigations. The animals were maintained on standard rat feet supplied by Pranav Agro Industries Ltd., Shangli, Maharashtra, India. The experiments were conducted according to the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines.

Experimental induction of hepatocarcinoma damage: The animals were fasted overnight and hepatocarcinoma was induced by a single intraperitoneal injection of a freshly prepared solution of *p*-dimethylaminoazobenzene (20 mg kg^{-1} body weight) in 3% DMSO injected intraperitoneally once in a week for a period of two months. Normal control rats were received only the vehicle of 3% DMSO alone once in a week for a period of two months.

Experimental design: The rats were divided into 4 groups consisting of six animals in each group as follows:

- **Group 1:** Normal control rats receiving vehicle 3% DMSO (5 mL kg^{-1} body weight) alone
- **Group 2:** Test control; *p*-DAB induced
- **Group 3:** Treatment with *Enicostemma littorale*. (Followed by the last dosage of *p*-DAB, the animals were orally administered by the method of gavage with *Enicostemma littorale* (1 g kg^{-1} body weight) in crude form along with physiological saline, once a day for a period of two months)
- **Group 4:** Positive control. Administration of vitamin E suspended in sunflower oil administered at regular interval for the two months at the concentration of 400 mg kg^{-1} body weight

Biochemical analysis: Biochemical analysis was carried out using 12 h fasting animals (after the last dosage of drug/carcinogen, the feeding was stopped). Animals were sacrificed by cervical decapitation and the blood was collected in two test tubes. Serum and plasma was separated as per the standard procedures. Liver samples were removed and the weight was measured followed by homogenization using 10% saline. The homogenate was centrifuged at 15,000-x g for 30 min at 4°C and the supernatant was used for the supernatant was used for the analysis of biochemical parameters. The tissue homogenate was placed at -20°C. Reduced glutathione, glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT) activity assays were determined as per the methods reported by Beutler and Kelley (1963), Habig *et al*. (1974) and Marklund and Marklund (1974). Vitamin-E and Vitamin-C content were determined in plasma as per the method of Desai (1984) and Omaye *et al*. (1979). Lipid peroxidative effect was evaluated by

measuring the hepatic content of thiobarbutric acid reacting substances (TBARS), expressed as malondialdehyde (MDA) equivalents (Ohkawa *et al*., 1979).

Statistical analysis: Results were statistically evaluated using one-way analysis of variance (ANOVA) for repeated measurements. p-values were below 0.05 were considered significant.

RESULTS

Throughout the experimental period, it has been noticed that all the experimental animals exhibited good survival and no lethal effect was observed.

Physiological effect: Table 1 showed the physiological effect of induction of *p*-DAB and the test plant. Regarding liver weight, about 12% increase in weight was observed in the *p*-DAB induced animals, whereas, administration of *Enicostemma littorale* plant powder decreases the liver weight from 3.16±0.21 g to 2.88±0.13 g/100 g body weight close to the normal liver weight of 2.79±0.32 g/100 g body weight and about 2.99±0.16 g/100 g was observed with the animals receiving vitamin E. Oral administration of the plant exhibited the significance at $p<0.05$.

Activity of glutathione family members: Regarding reduced glutathione activity assessed in the liver samples, as it has been explained in the Table 2, it was significantly reduced in the *p*-DAB induced animals compared to normal rats. Reduced glutathione concentration of about 11.01±0.75 (μg mg^{-1} protein) was observed with normal rats, whereas it was only 5.43±0.77 (μg mg^{-1} protein) in the *p*-DAB induced liver samples. Further treatment with the plant increases the glutathione level to about 39%, comparable to the experimental animals receiving vitamin E. Finally, the activity is restored back to that of normal groups in both standard and test drug treated animals.

When, considering GST level in the liver samples, GST activity was also reduced as in the case of glutathione activity, by the induction of the chemical carcinogen *p*-DAB at 20 mg kg^{-1} body weight. Oral administration of plant restores the GST activity almost equal to the normal experimental animals (Table 2). GST concentration of normal animals was estimated as 5.32±0.56 μmole of CDNB conjugate formed/min/mg protein and it has been reduced to 2.81±0.50 μmole of CDNB conjugate formed/min/mg protein by the induction of *p*-DAB, which is restored back to the level of 4.17±0.45 μmole of CDNB conjugate formed/min/mg protein after the oral administration.

SOD activity: The SOD content analyzed in the liver homogenate was drastically lowered by the induction of *p*-DAB and administration of plant *Enicostemma littorale* restores the activity back (Table 2). The *p*-DAB induced animals showed the percentage reduction of about 26% and about 12% restoration was with the test plant.

Catalase activity: Similar to SOD level, CAT level was also lowered by the administration of *p*-DAB and the level again reaches the normal value after the administration of *Enicostemma littorale* (Table 2). The CAT level for normal group animal was 21.26±1.82 μmole of H_2O_2 consumed/min/mg protein and it was about 11.93±1.33 μmole of H_2O_2 consumed/min/mg protein in the carcinogen induced animal group and was about 16.04±1.96 μmole of H_2O_2 consumed/min/mg protein in the test plant treated group animals.

Vitamin C: Analysis of vitamin C (non-enzymatic antioxidants) content (Table 2) in plasma was found to be decreased in the *p*-DAB induced animals (12.13±1.00 mg dL^{-1}) compared to normal group increase

Table 1: Liver weight measured in grams in normal, *p*-DAB induced and the plant treated animals

Experimental groups	Liver weight/100 g of body weight
Normal (n = 6)	2.79±0.32
p-DAB induced (n = 6)	3.16±0.21
Enicostemma littorale plant treated (n = 6)	2.88±0.13[c]
Vitamin E treated (n = 6)	2.99±0.16[c]

Values are expressed as mean±SD of six animals/group. Treated groups compared to Group 2 and Group 4 ([a]$p<0.001$, [b]$p<0.01$,[c]$p<0.05$)

Table 2: Various enzymatic antioxidant and non-enzymatic antioxidant level assessed in liver and plasma for normal, induced and for the *Enicostemma littorale* plant treated groups in the study

Experimental groups	GSH (μg mg^{-1} protein)	GST (μmole of CDNB conjugate formed/min/mg protein)	Superoxide dismutase (units/min/mg protein)	Catalase (μmole of H_2O_2 consumed/min/mg protein)	Vitamin C (mg dL^{-1})	Vitamin E (mg dL^{-1})	Lipid peroxidation (n moles of MDA released/mg protein)
Normal (n = 6)	11.01±0.75	5.32±0.56	44.28±1.79	21.26±1.82	18.95±1.80	21.26±0.51	79.01±2.43
p-DAB induced (n = 6)	5.43±0.77	2.81±0.50	32.77±2.38	11.93±1.33	12.13±1.00	18.21±0.55	168.80±4.76
Enicostemma littorale plant treated (n = 6)	9.02±0.79[a]	4.17±0.45[a]	37.43±1.55[b]	16.04±1.96[b]	17.94±1.13[a]	19.73±0.73	90.58±3.18[a]
Vitamin E treated (n = 6)	7.41±0.90	3.13±0.07	35.17±2.14	14. 42±1.69	15.45±0.93	22.21±1.4	99.88±1.42

Values are expressed as mean±SD. Treated groups compared to Group 2 and Group 4 ([a]$p<0.001$, [b]$p<0.01$,[c]$p<0.05$)

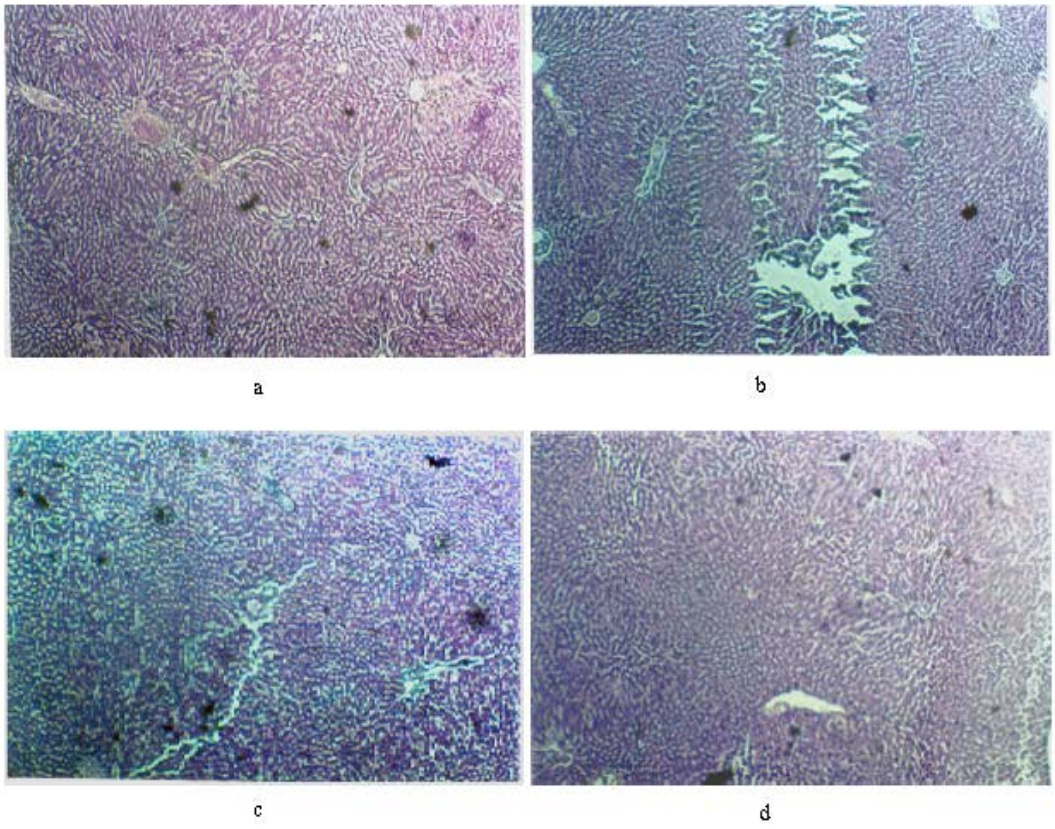

Fig. 1: Histopathological appearance of liver of rat, (a) normal (vehicle alone), (b) test control (*p*-DAB induced), (c) *Enicostemma littorale* treated and (d) positive control (Vitamin E administration)

in the concentration of vitamin E to the level of 22.21±1.4 (mg dL^{-1}). Significance between the groups was observed at the level of $p<0.01$ (Table 2).

Lipid peroxides: Malondialdehyde content of liver increases in the carcinogen induced group (168.80±4.76 nmoles of MDA released/mg protein). Administration of *Enicostemma littorale* plant significantly reduces the malondialdhyde content in the liver to the level of 90.58±3.18 nmoles of MDA released mg^{-1} protein. The normal group animals exhibited 79.01±2.43 nmoles of MDA released/mg protein.

Histological changes: Hyperplasia of hepatic parenchymal cells in *p*-DAB administered rats has been observed. Normal control rats show more compact and well distributed junctional complexes (Fig. 1a). In DAB administered rats, hepatic cells exhibit loss of contact inhibition (polarity) and damaged central vein of liver lobules (Fig. 1b). However, animals treated with *Enicostemma littorale* (Fig. 1c), show higher cell density with compact junctional complexes than DAB administered rats where the nuclear envelope is completely damaged. However, the protective effect of treatment with vitamin-E has been shown in reducing the cancer of the liver.

DISCUSSION

Liver is one of the most important components of our body system and the detoxifying enzymes generated in the liver system governed the entry or the exit of the unwanted/foreign material/toxic material. But still, some chemical carcinogens are also reported to damage the tissues and alter the DNA, which further complicate the physiological systems. Hence, studies have been focused on increasing the activity of detoxifying of enzyme system by the administration of various drugs formulated. *p*-DAB, a known carcinogen induces hepatocarcinoma. Much research has now been focused on detoxification of this carcinogen without affecting the other physiological and immunological systems. Now, more attention is being given to the exploitation of natural herbal plants to solve the problem associated with both the internal and the external chemical carcinogens. In the present study, restoration of liver functions, which was previously

affected by the induction of known carcinogen *p*-DAB, was effected through the oral administration of the plant *Enicostemma littorale* and comparing the efficiency of the plant powder with the standard drug vitamin E. Assessment of biochemical, physiological and antioxidants levels in liver homogenate and plasma further authenticate the restoration of liver activity.

In the present study, increase of liver weight as observed by the induction of *p*-DAB in the present study, may be due to combination of hypertrophy and hyperplasia, a pattern of liver growth usually observed during drug detoxification (Velanganni and Balasundaram, 2003). Similarly, Gross increase in liver enlargement occurs with frequent response to the administration of drugs, food additives, pesticides and other types of chemicals was observed by Pain *et al.* (1984). Administration of powdered plant orally reduces the liver weight considerably this might be due to the inactivation of the chemical by *Enicostemma littorale*.

GSH binds is excreted from the host system (Kweon *et al.*, 2003). Therefore, the increased GSH by *Enicostemma littorale* plant could partly protect the further activation of the carcinogen. GST plays a physiological role in initiating the detoxification of electrophilic ultimate carcinogens (Morse and Stoner, 1993). Chemicals like CCl_4 and 3'methyl-4-*p*-DAB alter the hepatic GST activity (Aniya and Anders, 1985; Karmakar *et al.*, 2002). GST level was significantly reduced in *p*-DAB treated animals and upward reversal was observed after the treatment with *Enicostemma littorale*.

In the present study, the diminution of SOD activities in *p*-DAB treated animals might be the result of their sensibility to at least one of the ROS generated under oxidative stress (Fabiana *et al.*, 2001). Inhibition of SOD could be the consequence of an irreversible autocatalytic process, in which the sustained increase of ROS, would finally lead to cellular death (Pigeolet *et al.*, 1990). In our experiments, we have shown a great increase in SOD activity that could be ascribed to the production of free radicals and the need to enhance the natural defense system of *Enicostemma littorale*. CAT is a hemoprotein which catalyses the reduction of hydrogen peroxides and protects the tissues from highly reactive hydroxyl radicals (Chance *et al.*, 1952). Decreased CAT activity is linked up to exhaustion of the enzyme as a result of oxidative stress caused by *p*-DAB (Gerez *et al.*, 1998). Thus, CAT activity was restored to normal level after treatment with the plant evidently supports the antioxidant defense mechanism of the plant chosen.

Ascorbic acid is an essential antioxidant that disappears faster than other antioxidants when plasma is exposed to reactive oxygen species (Frei *et al.*, 1989). *p*-DAB and its metabolites leads to the generation of ROS, further reduces the antioxidant level of the biological system of the carcinogen induced animals. Adminsitration of Vitamin A, Vitamin C and Vitamin E, in turn increases the antioxidant property against *p*-DAB induced hepatoma (Velanganni and Balasundaram, 2003). The treatment with the plant significantly increases the ascorbic acid and α-tocopherol level of the treated animals and thus exhibiting the protective nature of the plant. *p*-DAB and its metabolites induce lipid peroxidation products (Gerez *et al.*, 1998), such as malondialdehyde, were frequently regarded as a cancer promotive substance (Vaca *et al.*, 1988). Elevated levels of MDA in *p*-DAB induced group clearly reflect the over production of free radicals and/or the inability of antioxidant defense system. Level of lipid peroxidation in cells is controlled by various cellular defence mechanisms consisting of enzymatic and non-enzymatic scavengers system (Simmons, 1984). Several plant products have been reported to modulate the level of lipid peroxides (Stadler *et al.*, 1994; Imai *et al.*, 1994). Oral administration of the aerial part of plant *Enicostemma littorale* prevents lipid peroxidation and thus enhances the restoration activity of liver. When considering the components of the plant, it has been reported that several alkaloids, flavonoids and reducing sugars (Chopra *et al.*, 1998) are the major sources for the plant to perform its activity and the sustainability in reducing the liver damage. Though the rate of liver damage was found to be high by the induction of *p*-DAB, about 80-90% restoration of liver function was initiated after the administration of the plant. Thus, the present study evidently proves that the chosen plant enhances the activity of glutathione family members and also acts as a scavenger to prevent the damage caused by the free radicals released by induction of *p*-DAB and further prevents the lipid peroxidation activity. Exploitation of this plant for detoxification of other chemical carcinogens may provide the further understanding of the usage of the plant *Enicostemma littorale*.

Histopathological studies revealed that alterations occurred in the architecture of liver tissue in *p*-DAB-induced hepatocarcinoma rats. It is interesting to note that these alterations are corrected apparently near normal by *Enicostemma littorale* treatment.

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