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## RESEARCH COMMUNICATION

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# Ethanollic Neem Leaf Extract Protects Against N-methyl -N'-nitro-N-nitrosoguanidine-induced Gastric Carcinogenesis in Wistar Rats

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### Abstract

We evaluated the effects of ethanollic neem leaf extract on N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced gastric carcinogenesis in Wistar rats. The extent of lipid peroxidation and the status of the antioxidants superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPx), and glutathione-S-transferase (GST) in the stomach, liver and erythrocytes were used as biomarkers of chemoprevention. Animals were divided into four groups of six animals each. Rats in group 1 were given MNNG (150 mg/kg bw) by intragastric intubation three times with a gap of 2 weeks in between the treatments. Rats in group 2 administered MNNG as in group 1, in addition received intragastric intubation of ethanollic neem leaf extract (200 mg/kg bw) three times per week starting on the day following the first exposure to MNNG and continued until the end of the experimental period. Group 3 animals were given ethanollic neem leaf extract alone, while group 4 served as controls. All the animals were killed after an experimental period of 26 weeks. Diminished lipid peroxidation in the stomach tumour tissue was associated with enhanced antioxidant levels. In contrast to tumour tissue, enhanced lipid peroxidation with compromised antioxidant defences was found in the liver and erythrocytes of tumour bearing animals. Administration of ethanollic neem leaf extract significantly reduced the incidence of stomach tumours, modulated lipid peroxidation and enhanced antioxidant status in the stomach, liver and blood. From the results of our study, we suggest that ethanollic neem leaf extract may exert its chemopreventive effects by modulating lipid peroxidation and enhancing the antioxidant status in the stomach, liver and erythrocytes.

**Key Words:** neem leaf - gastric carcinogenesis - lipid peroxidation - antioxidants - chemoprevention - stomach cancer - MNNG

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### Introduction

Stomach cancer, the second most common cancer in the world, represents a very important health problem with about 900,000 new cases diagnosed every year, and is a major cause of mortality in Chennai, India. Despite advances in diagnosis and treatment, the 5-year survival rate of stomach cancer is only 25 per cent (Terry et al, 2002). The aetiology of stomach cancer is multifactorial and predominantly dietary. Humans are exposed to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-like N-nitroso compounds (NNC) through luminal nitrosylation of naturally occurring guanidine compounds by dietary nitrates, nitrites and secondary amines. NNC have been implicated as major risk

factors for stomach cancer (Bartsch et al, 1992).

Chemoprevention has evolved as an effective strategy to control the incidence of stomach cancer. Gastric cancer induced by MNNG in Wistar rats, which shows similarities to human gastric tumours, is an ideal model for investigating the development of stomach cancer and the effects of intervention by chemopreventive agents. Accumulating evidence supports the hypothesis that several medicinal plants and phytochemicals offer chemoprotection against toxic mutagenic and carcinogenic chemicals such as MNNG (Arivazhagan et al 1999; Kim et al, 1997; Uedo et al, 1999). Studies in this laboratory have documented the protective effects of neem leaf, garlic and lycopene in the MNNG model (Arivazhagan et al, 1999; 2001; Velmurugan et al, 2002).

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Medicinal plants are known to exert their anticarcinogenic effects by scavenging reactive oxygen species (ROS) and modulating antioxidant defence mechanisms. The enzymes glutathione peroxidase (GPx) and glutathione-S-transferase (GST) that use reduced glutathione (GSH) as substrate as well as superoxide dismutase (SOD) and catalase (CAT) have therefore assumed significance as biomarkers of chemoprevention owing to their antioxidant and detoxification properties (Weisburger, 2001). Previously, we have demonstrated that evaluation of ROS-induced lipid peroxidation and the status of antioxidants in the liver and blood, in addition to the target organ is a reliable method for screening putative chemopreventive agents (Velmurugan et al, 2003).

*Azadirachta indica* A Juss, commonly known as neem, has attracted the focus of attention owing to its insecticidal and medicinal properties. Neem elaborates a vast array of biologically active compounds that are chemically diverse and structurally complex. All parts of the neem tree- leaves, flowers, seeds, fruits, roots and bark are recognized to possess a wide range of pharmacological effects. The medicinal utilities have been described especially for neem leaf. Neem leaf and its constituents have been demonstrated to exhibit immunomodulatory, anti-inflammatory, antiulcer, antioxidant, antimutagenic and anticarcinogenic properties (Biswas et al, 2002; Puri, 1999). In previous reports from this laboratory, we have demonstrated the chemopreventive effects of aqueous neem leaf extract against experimental oral and gastric carcinogenesis (Nagini and Manoharan, 1997; Balasenthil et al, 1999; Arivazhagan et al, 2001).

Several experimental studies have demonstrated that alcoholic extracts of neem leaf are more effective than aqueous extracts and exhibit antihyperglycaemic, antifertility, and anti-inflammatory effects (Chattopadhyay et al, 1993; Chattopadhyay, 1998; Parshad et al, 1997). Most notably, alcoholic extracts of neem leaf have been reported to protect against gastric ulcer, a premalignant lesion and risk factor for gastric carcinogenesis (Koley et al, 1994). However, there are no reports on the effect of ethanolic neem leaf extract on MNNG-induced genotoxicity and stomach carcinogenesis. Recently, we documented the chemoprotective effects of ethanolic neem leaf extract against MNNG-induced genotoxicity and oxidative stress (Subapriya et al, 2003a; b). The present study was undertaken to investigate the chemopreventive potential of ethanolic neem leaf extract against MNNG-induced gastric carcinogenesis. We used lipid peroxidation and the status of the antioxidants SOD, CAT, GSH, GPx, GST as well as the GSH/GSSG ratio in the stomach, liver and erythrocytes as biomarkers of chemoprevention.

## Materials and Methods

### Chemicals

Bovine serum albumin, 2-thiobarbituric acid, trichloroacetic acid, 2,4-dinitro phenylhydrazine, reduced glutathione (GSH), 5,5'-dithiobis(2-nitrobenzoic acid)

(DTNB), and 1-chloro-2,4-dinitrobenzene (CDNB), phenazine methosulfate (PMS), nitroblue tetrazolium (NBT) nicotinamide adenine dinucleotide reduced (NADH), nicotinamide adenine dinucleotide phosphate (NADP) and glutathione reductase were purchased from Sigma Chemical Company, St. Louis, USA. MNNG of purity  $\geq 97\%$  was obtained from Fluka-Chemika-Biochemika, Buchs, Switzerland.

### Animals

The experiment was carried out with male Wistar rats aged 8-10 weeks weighing 120-130g obtained from the Central Animal House, Annamalai University, India. The animals housed six to a polypropylene cage were provided food and water ad libitum and maintained under controlled conditions of temperature and humidity with an alternating light/dark cycle. All animals were fed standard pellet diet (Mysore Snack Feed, Mysore, India). They were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India and approved by the ethical committee, Annamalai University.

### Collection of plant material

Fresh matured leaves of *A.indica* were collected locally during May-June and were identified by a pharmacognosy expert. These leaves were dried in shade, powdered and the powders were used for the extraction. Voucher specimens were deposited at the herbarium of the Botany Department, Annamalai University.

### Preparation of neem leaf extract

The ethanolic extract of neem leaf was prepared according to the procedure described by Chattopadhyay (1998). Air-dried powder (1 kg) of *A. indica* leaves was mixed with 3L of 70% ethyl alcohol and kept at room temperature for 36 h. The slurry was stirred intermittently for 2h and left overnight. The mixture was then filtered and the filtrate was concentrated under reduced pressure (bath temperature 50°C) and finally dried in a vacuum dessicator. The residue collected (yield 48g/kg of neem leaf powder) was a thick paste, green in colour and gummaceous in nature. The extract was dissolved in normal saline to obtain a final concentration of 20 mg/mL and used for the experiment.

### Treatment schedule

Animals were randomized into experimental and control groups and divided into four groups of six. Rats in group 1 were given MNNG (150 mg/kg bw) by intragastric intubation three times with a gap of 2 weeks in between the treatments (Saravanan and Nagarajan, 1993). Rats in group 2 administered MNNG as in group 1, in addition received intragastric intubation of ethanolic neem leaf extract (200 mg/kg bw) three times per week starting on the day following the first exposure to MNNG and continued until the end of the experimental period (Chattopadhyay, 1998). Group 3 animals were given ethanolic neem leaf extract alone as in

group 2 but without MNNG. Group 4 received basal diet and tap water throughout the experiment and served as the untreated control. All animals had free access to food and water.

The experiment was terminated at 26 weeks and all animals were killed by cervical dislocation after an overnight fast. Fresh tissues were used for estimations. Biochemical estimations were carried out in stomach tissue, liver and blood samples of experimental and control animals.

*Preparation of tissue homogenate and erythrocyte lysate*

The tissue samples after weighing were homogenized using appropriate buffer in an all glass homogeniser with Teflon pestle. Blood samples were collected in heparinised tubes and the plasma was separated by centrifugation at 1000 g for 15 minutes. After centrifugation, the buffy coat was removed and the packed cells washed three times with physiological saline. The erythrocyte samples (0.5 mL) were lysed with 4.5 mL of hypotonic phosphate buffer, pH 7.4. The hemolysate was separated by centrifuging at 2500 g for 15 min at 2°C.

*Biochemical Estimations*

Lipid peroxidation as evidenced by the formation of thiobarbituric acid reactive substances (TBARS) was assayed in tissues by the method described by Ohkawa et al (1979) and in erythrocytes by the method of Buege and Aust (1987). The pink coloured chromogen formed by the reaction of 2-thiobarbituric acid with the breakdown products of lipid peroxidation was read at 535 nm. Lipid hydroperoxides (LOOH) were estimated by the method of Jiang et al (1992). Hydroperoxides are detected by their ability to oxidize ferrous iron leading to the formation of a chromophore with an absorbance maximum at 560 nm. Conjugated dienes (CD) were assayed by the method of Rao and Recknagel (1968). SOD was assayed by the method of Kakkar et al (1984) based on 50% inhibition of the formation of NADH-phenazine methosulfate nitrobluetetrazolium (NBT) formazan at 520 nm. The activity of catalase was assayed by the method of Sinha (1972) based on the utilization of

hydrogen peroxide by the enzyme. GSH was determined by the method of Anderson (1985) based on the development of a yellow colour when DTNB is added to compounds containing sulfhydryl groups. Oxidised glutathione (GSSG) was estimated by following the oxidation of NADPH using glutathione reductase at 340nm based on the method of Srivastava and Beutler (1968).

GPx activity was assayed by the method of Rotruck et al (1973) with modifications. A known amount of enzyme preparation was incubated with hydrogen peroxide in the presence of GSH for 10 minutes. The amount of hydrogen peroxide utilized was determined by estimating GSH content by the method of Anderson (1985). The activity of glutathione-S-transferase (GST) was determined as described by Habig et al (1974) by following the increase in absorbance at 340 nm using CDNB as substrate. The protein content was estimated by the method of Lowry et al (1951).

*Statistical analysis*

Statistical analysis on the data for lipid peroxidation, antioxidants and detoxifying enzymes were analysed using analysis of variance (ANOVA) and the group means were compared by the least significant difference test (LSD). The results were considered statistically significant if the P<0.05.

**Results**

Table 1 shows the mean body weight, incidence of gastric tumours and the mean tumour burden in control and experimental animals. The mean body weights of MNNG treated animals (group 1) were significantly lower than that of control (group 4). No significant differences in body weights were observed in groups 2 through 4. The incidence of gastric tumours in group 1 was 83.3 per cent (5/6 animals) with a mean tumour burden of 113.12 mm<sup>3</sup>. Although no tumours were observed in groups 2 and 3, two of six animals in group 2 had small multiple nodules.

Figures 1 illustrate the effect of treatment with ethanolic neem leaf extract on MNNG-induced lipid peroxidation as evidenced by the formation of TBARS, lipid hydroperoxides

**Table 1. Body Weight, Tumour Incidence and Mean Tumour Burden in Different Groups. (Mean ± SD; n=6)**

Group	Treatment	Initial weight g	Final weight g	Tumour incidence	Tumour Burden <sup>b</sup> (mm <sup>3</sup> )
1.	MNNG	123.8 ± 4.5	130.1 ± 7.0 <sup>a</sup>	5/6 (83.3)	113.12
2.	MNNG + Ethanolic neem leaf extract	127.1 ± 7.0	148.1 ± 7.1	0	0
3.	Ethanolic neem leaf extract	124.0 ± 4.1	159.2 ± 7.7	0	0
4.	Control	126.43 ± 6.6	152.6 ± 6.5	0	0

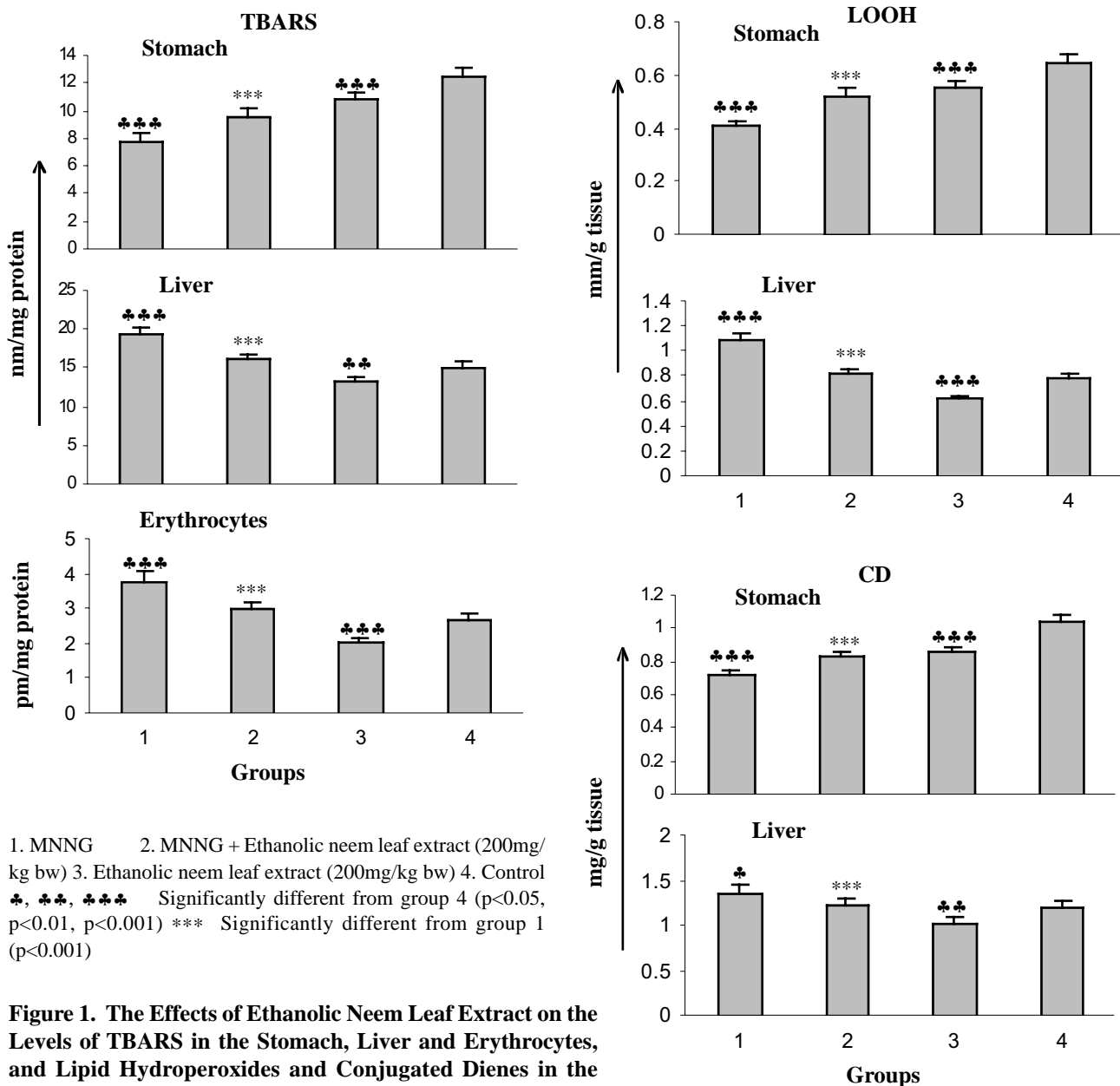
a - Significant at P<0.05

b - Mean tumour burden was calculated by multiplying the mean tumour volume (4/3πr<sup>3</sup>) with the mean number of tumors. (r = \_ tumour diameter in mm).

and conjugated dienes in the stomach, liver and erythrocytes of experimental and control animals. Administration of MNNG significantly lowered the extent of lipid peroxidation in the stomach of group 1 animals compared to control (group 4). Treatment with 200 mg kg<sup>-1</sup> bw ethanolic neem leaf extract significantly increased lipid peroxidation levels in group 2 animals as compared to group 1. In contrast to diminished lipid peroxidation in the stomach, the extent of lipid peroxidation in the liver and erythrocytes was significantly increased by MNNG (group 1) compared to group 4. Treatment with ethanolic neem leaf extract significantly reduced MNNG-induced lipid peroxidation in group 2 animals compared to group 1. Administration of ethanolic neem leaf extract alone (group 3) significantly reduced the extent of lipid peroxidation in the stomach, liver

and erythrocytes compared to control.

The influence of treatment with ethanolic neem leaf extract on the antioxidant profile in the stomach, liver and erythrocytes are shown in Figures 2-4. The concentrations of GSH and GSSG, GSH/GSSG ratio and the activities of SOD, CAT, GPx and GST were significantly increased in the stomach, whereas in the liver and erythrocytes, all the antioxidants were significantly decreased in MNNG treated animals compared to control (group 4). Treatment with ethanolic neem leaf extract significantly increased all the antioxidants in group 2 animals compared to group 1. Administration of ethanolic neem leaf extract alone (group 3) significantly enhanced the antioxidant status compared to group 1.



**Figure 1. The Effects of Ethanolic Neem Leaf Extract on the Levels of TBARS in the Stomach, Liver and Erythrocytes, and Lipid Hydroperoxides and Conjugated Dienes in the Stomach and Liver. (Mean ± SD; n=6)**

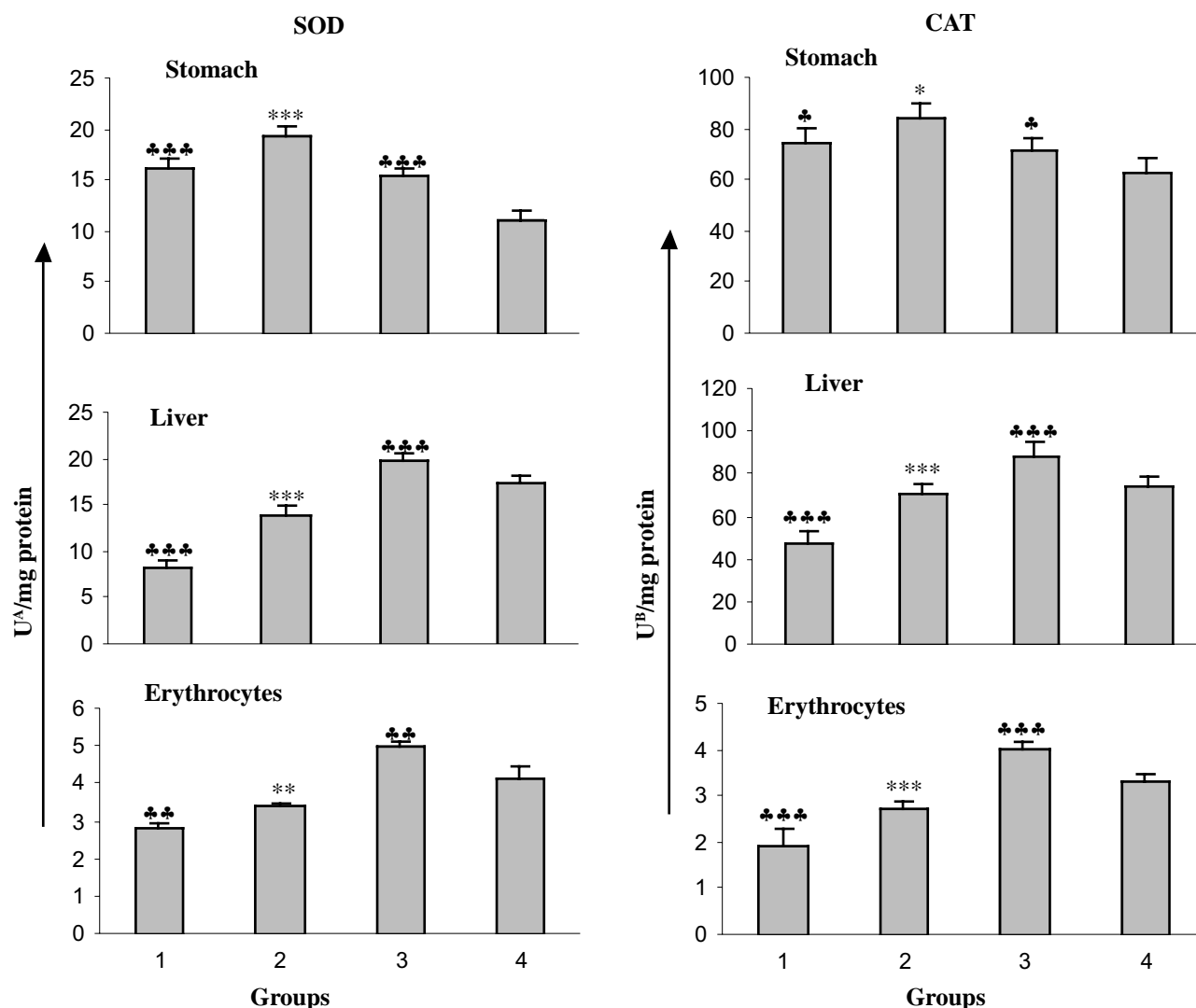
## Discussion

MNNG, an alkylating carcinogen used in the present study is known to methylate all the oxygen and most nitrogen atoms of DNA (Bignami et al, 2000). MNNG has been found to induce DNA strand breaks, point mutations and sister chromatid exchanges which if not adequately repaired, may result in activation of oncogenes leading to intestinal metaplasia, dysplasia and finally carcinoma (Salmenova et al, 1997).

In MNNG- induced stomach tumours, the extent of lipid peroxidation was low. Lipid peroxides have been reported to play an important role in the control of cell division. Low concentrations of ROS have been reported to stimulate cell

proliferation, whereas high levels induce cytotoxicity and cell death. Several studies have demonstrated that tumour cells are more resistant to lipid peroxidation than normal cells (Das, 2002).

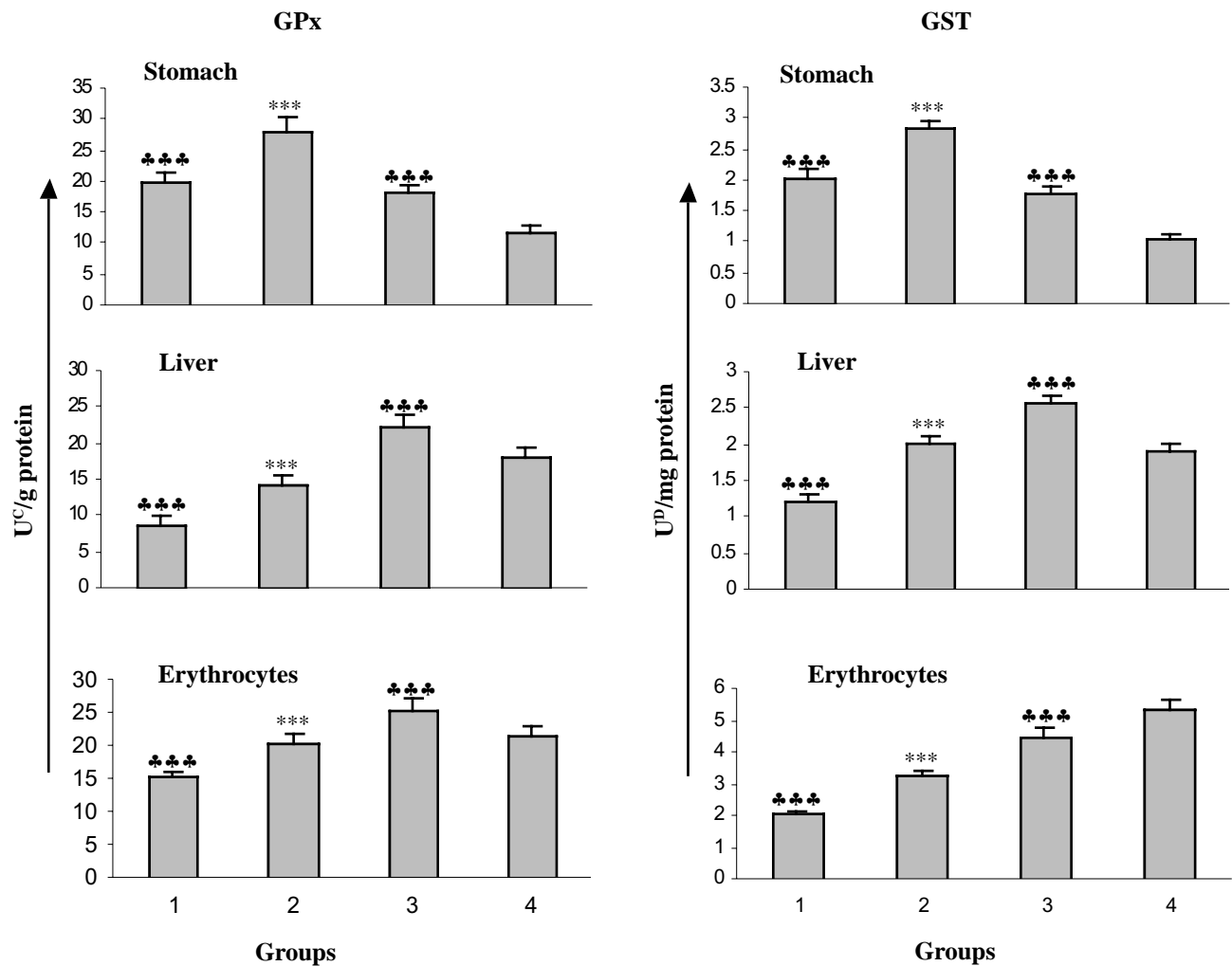
The decreased susceptibility of stomach tumours to lipid peroxidation seen in the present study may be attributed to enhanced antioxidant capacities. Increased generation of ROS such as  $O_2^{\bullet-}$  and  $H_2O_2$  is recognized to induce SOD, CAT and GPx. Higher activities of antioxidant enzymes have been observed in malignant tumours compared to controls (Kumaraguruparan et al, 2002; Izutani et al, 1996). In particular, synthesis of GSH, which has a central role in the maintenance of the cellular redox status was found to be increased in rapidly proliferating tumours. GSH in



1. MNNG    2. MNNG + Ethanol extract (200mg/kg bw)    3. Ethanol extract (200mg/kg bw)    4. Control  
 \*\*, \*\*\*, \*\*\*\* Significantly different from group 4 (p<0.05, p<0.01, p<0.001)    \*, \*\*, \*\*\* Significantly different from group 1 (p<0.05, p<0.01, p<0.001)

A - Amount of enzyme required to give 50% inhibition of NBT reduction    B -  $\mu$ moles of  $H_2O_2$  utilised/sec

**Figure 2. The Influence of Treatment with Ethanol Neem Leaf Extract on SOD and CAT Activities in the Stomach, Liver and Erythrocytes.** (Mean  $\pm$  SD; n=6)



1. MNNG 2. MNNG + Ethanollic neem leaf extract (200mg/kg bw) 3. Ethanollic neem leaf extract (200mg/kg bw) 4. Control  
 \*\*\* Significantly different from group 1 (p<0.001) \*\*\*C Significantly different from group 4 (p<0.001)  
 C - μmoles of GSH utilised/min D - μmoles of CDNB-GSH conjugate formed/min

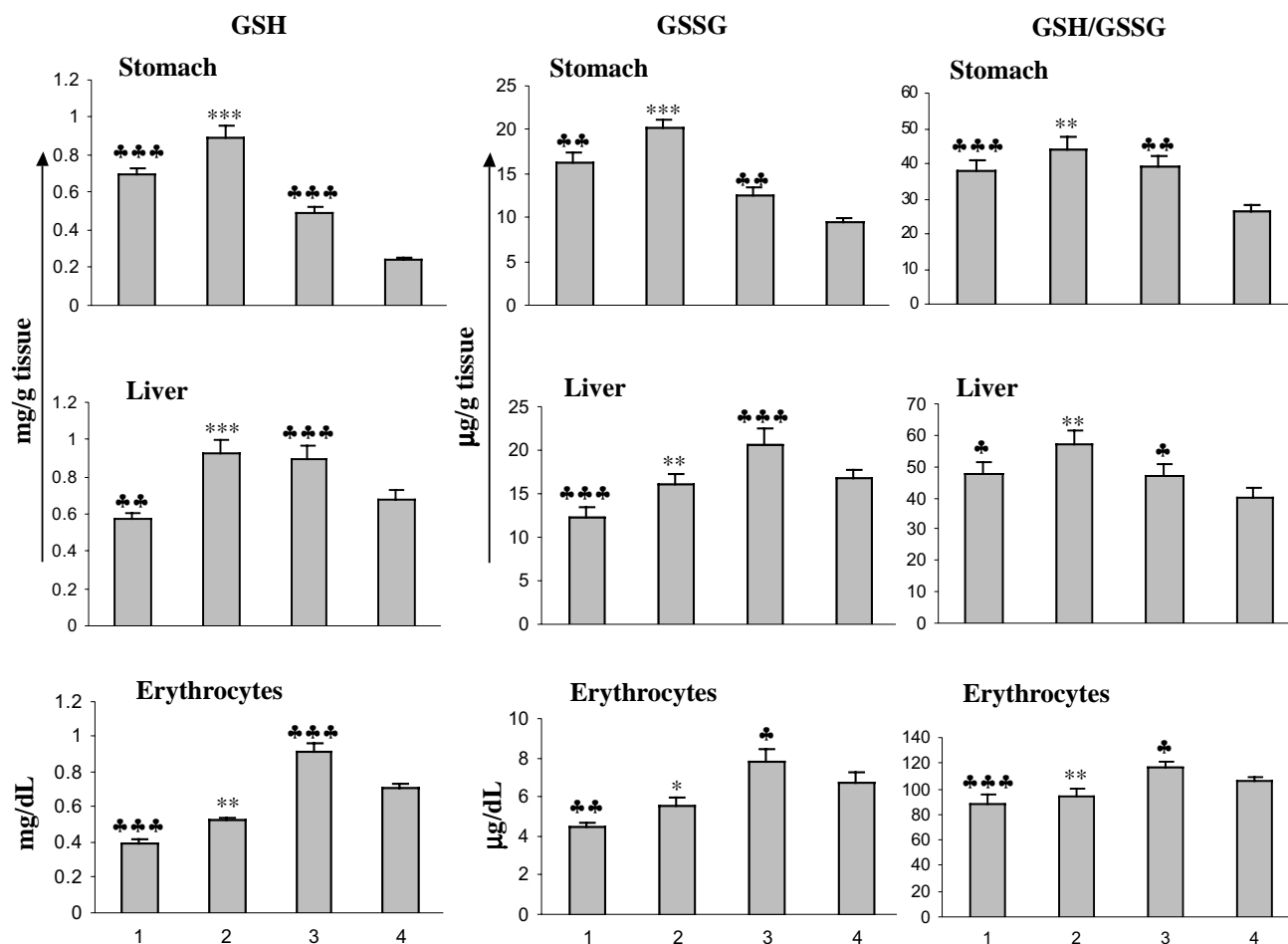
**Figure 3. The Influence of Treatment with Ethanollic Neem Leaf Extract on GPx and GST Activities in the Stomach, Liver and Erythrocytes.** (Mean ± SD; n=6)

conjunction with GPx and GST, regulates cell proliferation (Obrador et al, 1997). Overexpression of GSH and GSH-dependent enzymes has been documented in a wide range of tumours (Kumaraguruparan et al, 2002; Ghalia and Fouad, 2000). Thus, diminished lipid peroxidation combined with enhanced antioxidant capacity of MNNG-induced gastric tumours may serve to maintain a reduced environment which facilitates cell proliferation offering a selective growth advantage to tumour cells.

The tumour and host tissue appear to comprise two separate metabolic compartments with respect to the cellular redox state. In contrast to MNNG-induced stomach tumours, the liver and erythrocytes of tumour bearing animals showed enhanced lipid peroxidation associated with antioxidant depletion. Hepatic metabolism of MNNG is reported to produce deleterious effects in the liver and erythrocytes (Mikuni and Tatsuta, 2002). An increase in lipid peroxidation was observed when hepatocytes were exposed to MNNG

(Reitnan et al, 1988). The erythrocytes are major targets for lipid peroxidation because of their high content of polyunsaturated fatty acids and iron and their role as oxygen transporters (Hebbel, 1986). Compromised antioxidant defences in the host liver and erythrocytes may be due to increased utilization to scavenge lipid peroxides in these tissues as well as sequestration by the tumour cells.

Administration of ethanollic neem leaf extract significantly decreased stomach tumour incidence and tumour burden. This may be related to the modulatory effects of ethanollic neem leaf extract on lipid peroxidation and antioxidant enzymes in the target organ as well as in the liver and blood. These findings potentiate reports by us as well as other workers that chemopreventive agents exert their tumour inhibitory effects by an electrophilic ‘counterattack response’ characterized by the elevation of phase II enzymes that utilize GSH as substrate (Arivazhagan et al, 2001; Velmurugan et al, 2002; Prestera et al, 1993). Furthermore,



1. MNNG 2. MNNG + Ethanolic neem leaf extract (200mg/kg bw) 3. Ethanolic neem leaf extract (200mg/kg bw) 4. Control  
 \*, \*\*, \*\*\* Significantly different from group 1 (p<0.05, p<0.01, p<0.001)  
 †, ††, ††† Significantly different from group 4 (p<0.05, p<0.01, p<0.001)

**Figure 4. The Influence of Treatment with Ethanolic Neem Leaf Extract on Concentrations of GSH and GSSG and GSH/GSSG Ratio in the Stomach, Liver and Erythrocytes. (Mean ± SD; n=6)**

plant based chemopreventives are reported to upregulate the ability of the liver to metabolise carcinogens and induce antioxidant enzymes thereby blocking tumour development at extrahepatic sites (Johnson, 1997).

The results of the present study substantiate the anticarcinogenic and antioxidant activities of neem preparations reported by us as well as other workers. (Balasenthil et al, 1999; Chattopadhyay, 1998; Kusamran et al, 1998; Labadie et al, 1989). Neem leaf is reported to decrease the extent of lipid peroxidation (Chattopadhyay, 1998). Yadav and Rathore (1976) have documented the mitotic inhibition activity of neem leaf extract. Neem flowers are known to induce phase II enzymes such as GST in rats (Kusamran et al, 1998). Recently, Tepsuwan et al (2002) demonstrated the chemopreventive effects of neem flowers on DMBA-induced mammary carcinogenesis and aflatoxin B-induced liver carcinogenesis in rats. The inhibitory action of neem stem bark on superoxide anion production has been reported (Labadie et al, 1989).

Alcoholic neem leaf extract contains a number of potent antioxidants and anticarcinogens including β-carotene, nimbin, azadirachtin, nimbidiol, quercetin, nimbidin and nimbatiktam (Govindachari, 1992). β-Carotene is known to inhibit lipid peroxidation by trapping peroxy radicals (Miller et al, 1996). Azadirachtin has inhibitory effects on cell proliferation (Gogate, 1996). Quercetin, a highly ethanol soluble neem bioflavonoid and potent antioxidant, has been reported to decrease MNNG-induced DNA damage and induce phase II enzymes (Blasiak et al, 2002). Nimbidin and nimbatiktam have been known to exert protective effects against gastric ulcer (Pillai, 1978, 1995).

Although quantitation and characterization of individual components was not carried out, the results of the present study demonstrate that ethanolic neem leaf extract may mediate its chemopreventive effects by modulation of lipid peroxidation and enhancing antioxidant enzymes. These findings validate the hypothesis that medicinal plants rich in antioxidants are potential chemopreventive agents.

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