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Chemoprotective effects of ethanolic extract of neem leaf against MNNG-induced oxidative stress

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We evaluated the modifying effects of ethanolic extract of neem leaves (*Azadirachta indica* A. Juss) on oxidative stress induced by the potent gastric carcinogen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) in male 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) were used as intermediate endpoints of chemoprevention. Three different concentrations of ethanolic neem leaf extract (100, 200 and 400 mg kg^{-1} body weight) were administered by intragastric intubation (i.g) for five consecutive days followed by MNNG (i.g) 1.5 h after the final administration. Enhanced lipid peroxidation was accompanied by compromised antioxidant defences in the stomach, liver and erythrocytes of MNNG-treated rats. Pretreatment with ethanolic neem leaf extract at a dose of 200 mg/kg body weight (bw) significantly lowered the concentration of lipid peroxides and increased antioxidant levels. Our results demonstrate that neem leaf exerts its chemoprotective effects on MNNG- induced oxidative stress by decreasing lipid peroxidation and enhancing the antioxidant status.

1. Introduction

Chemoprevention has evolved as a promising approach to control the incidence of cancer, a disease that accounts for 7 million deaths per year worldwide [1]. The current focus of chemoprevention is on intermediate biomarkers capable of detecting early changes that can be correlated with inhibition of carcinogenic progression. Many chemopreventive agents are recognized to exert their anticarcinogenic effects by scavenging oxygen free radicals (OFR) and upregulating antioxidant defence mechanisms [2]. 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 [3, 4].

Carcinoma of the stomach, the second most common cancer worldwide, is a major cause of mortality and morbidity in India [5]. Accumulating evidence supports the hypothesis that medicinal plants and phytochemicals may offer chemoprotection during experimental gastric carcinogenesis induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) [6–8]. Recently, we documented the tumour inhibitory effects of garlic and lycopene in the MNNG model [9, 10].

Azadirachta indica A Juss, commonly known as neem, a large evergreen tree that grows throughout the Indian subcontinent has attained worldwide prominence because of its 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 have been used traditionally for the treatment of inflammation, infections, skin diseases and dental disorders. Neem preparations have been reported to exert antimicrobial, antipyretic, antihypertensive, antidiabetic and immunostimulant effects [11–13]. In previous reports, we documented the antioxidant and hepatoprotective properties of aqueous extracts of neem leaf in the MNNG model [14–16]. We have also demonstrated the chemopreventive potential of aqueous neem leaf extract on 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch carcinogenesis and MNNG-induced gastric carcinogenesis [9, 17].

The alcoholic extracts of neem leaf are almost fifty times more effective than aqueous extracts and exhibit a wide range of pharmacological properties including hypoglycemic, antifertility and anti-inflammatory effects [18–20]. Most notably, alcoholic extracts of neem leaf have been reported to protect against gastric ulcer, a premalignant lesion and inhibit infection by *Helicobacter pylori*, an important etiological agent in gastric carcinogenesis [21, 22]. Furthermore, neem leaf is reported to contain azadirachtin, which has inhibitory effects on cell proliferation [23, 24]. We therefore undertook the present investigation to evaluate possible *in vivo* protective effects of ethanolic extract of neem leaf at three different concentrations on MNNG-induced changes in the extent of lipid peroxidation and the status of the antioxidants superoxide dismutase (SOD), catalase (CAT), GSH, GPx and GST in the stomach, liver and erythrocytes of Wistar rats.

2. Investigations and results

2.1. 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. [25] and in erythrocytes by the method of Buege and Aust [26]. The pink coloured chromogen formed by the reaction of 2-thiobarbituric acid with the breakdown products of lipid peroxidation was read at 535 nm. SOD was assayed by the method of Kakkar et al. [27] 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 [28] based on the utilization of hydrogen peroxide by the enzyme. GSH was determined by the method of Anderson [29] based on the development of a yellow colour when DTNB is added to compounds containing sulfhydryl groups. GPx activity was assayed by the method of Rotruck *et al.* [30] with modifications. A known amount of enzyme preparation was incubated with hydrogen peroxide in the presence of GSH for 10 min. The amount of hydrogen peroxide utilized was determined by estimating GSH content by the method of Anderson [29]. The activity of glutathione S-transferase (GST) was determined as described by Habig et al. [31] 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. [32].

2.2. 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 $P < 0.05$.

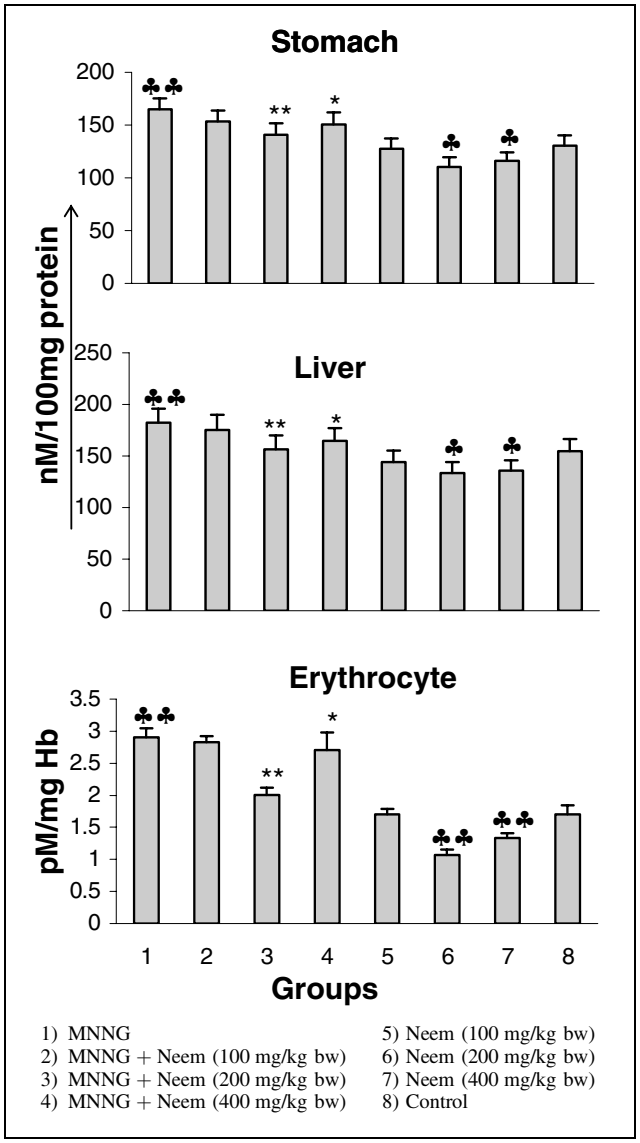

Fig. 1: The effects of pre-treatment with three doses of ethanolic neem leaf extract on MNNG-induced lipid peroxidation in the stomach, liver and erythrocytes.
♣ Significantly different from group 8 ($p < 0.05$)
♣♣ Significantly different from group 8 ($p < 0.001$)
* Significantly different from group 1 ($p < 0.05$)
** Significantly different from group 1 ($p < 0.001$)

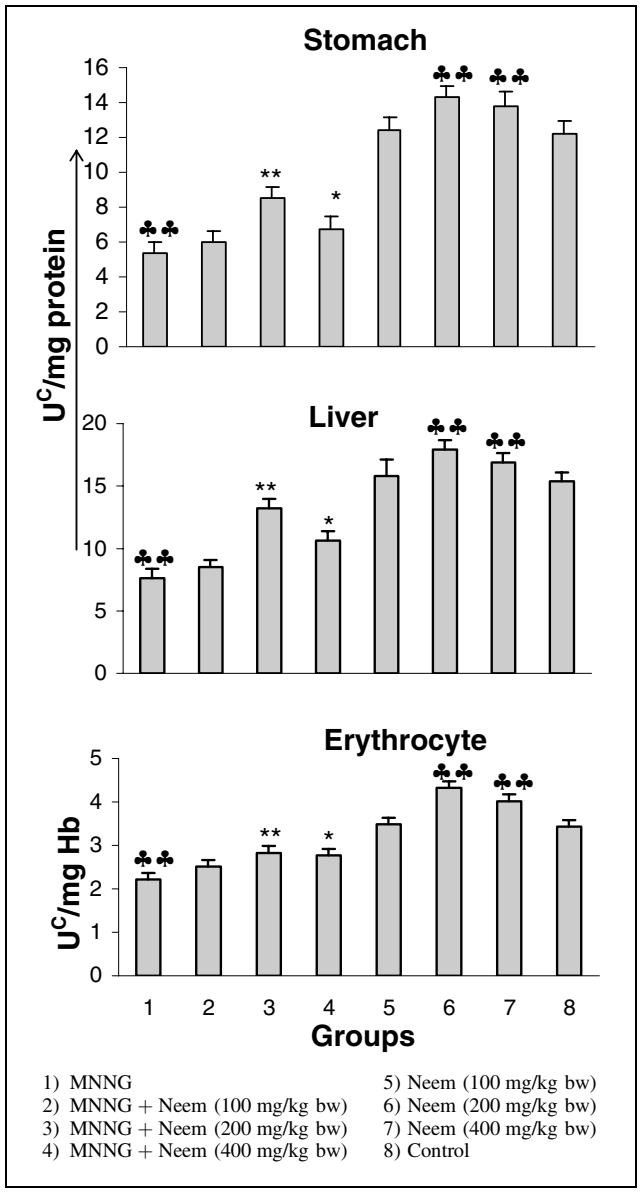

Fig. 2: The influence of pre-treatment with three doses of ethanolic neem leaf extract on SOD activity in the stomach, liver and erythrocytes.
♣ Significantly different from group 8 ($p < 0.05$)
♣♣ Significantly different from group 8 ($p < 0.001$)
* Significantly different from group 1 ($p < 0.05$)
** Significantly different from group 1 ($p < 0.001$)
C – Amount of enzyme required to give 50% inhibiton of NBT reduction

2.3. Results

Figure 1 illustrates the effects of pretreatment with three doses of ethanolic neem leaf extract on MNNG-induced lipid peroxidation in the stomach, liver and erythrocytes. The extent of lipid peroxidation was significantly enhanced by MNNG (group1) and significantly lowered by administration of ethanolic neem leaf extract at doses of 200 and 400 mg/kg bw (groups 6 and 7) compared to controls. Pretreatment with 200 and 400 mg/kg bw (groups 3 and 4) ethanolic neem leaf extract significantly reduced MNNG-induced lipid peroxidation compared to group 1.

Figures 2–6 illustrate the influence of pretreatment with ethanolic neem leaf extract on GSH concentration and activities of SOD, CAT, GPx and GST in the stomach, liver and erythrocytes. While MNNG administration (group 1) significantly lowered GSH, SOD, CAT, GPx and GST, administration of 200 and 400 mg/kg bw ethanolic neem leaf extract (groups 6 and 7) significantly enhanced the antioxidant status compared to untreated controls (group 8). Pretreatment with 200 and 400 mg/kg bw ethanolic neem leaf extract to animals administered MNNG (groups 3 and 4) significantly increased all the antioxidants compared to group 1.

3. Discussion

Excessive OFR generation with consequent oxidative stress has been implicated in the pathogenesis of gastric mucosal damage and diseases of the gut [33, 34]. The stomach is exposed to a wide range of OFR arising from various sources. In particular, MNNG-like carcinogens are formed in the stomach through lumenal nitrosylation of naturally occurring guanidine compounds such as L-arginine and creatine by dietary nitrite in the presence of gastric acid [35].

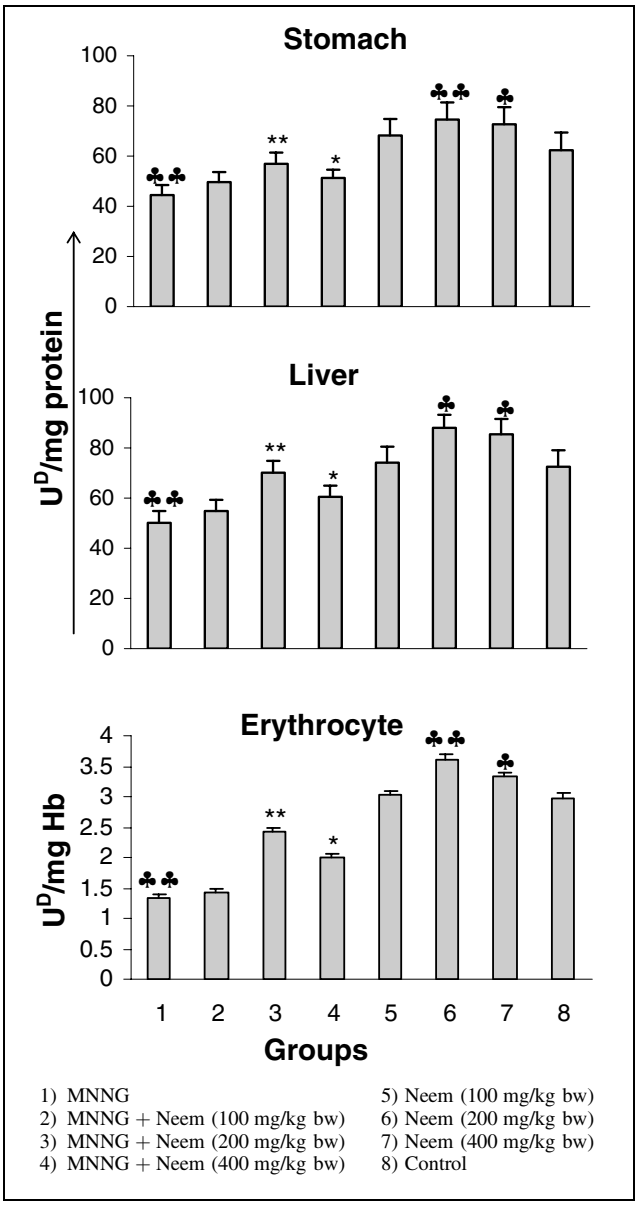

Fig. 3: The influence of pre-treatment with three doses of ethanolic neem leaf extract on CAT activity in the stomach, liver and erythrocytes.
♣ Significantly different from group 8 ($p < 0.05$)
♣♣ Significantly different from group 8 ($p < 0.001$)
* Significantly different from group 1 ($p < 0.05$)
** Significantly different from group 1 ($p < 0.001$)
D – μmoles of H_2O_2 utilised/sec

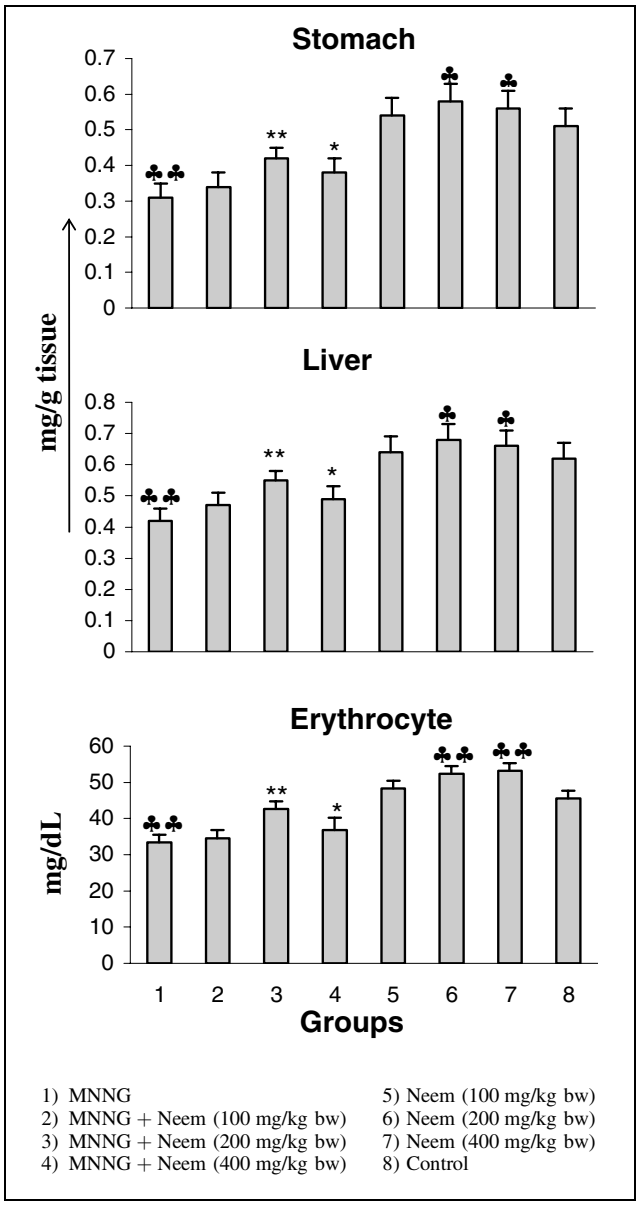

Fig. 4: The influence of pre-treatment with three doses of ethanolic neem leaf extract on GSH concentration in the stomach, liver and erythrocytes.
♣ Significantly different from group 8 ($p < 0.05$)
♣♣ Significantly different from group 8 ($p < 0.001$)
* Significantly different from group 1 ($p < 0.05$)
** Significantly different from group 1 ($p < 0.001$)

OFR have been suggested to play a key role in the mutagenic and carcinogenic effects of MNNG. MNNG is known to generate MNNG radicals when exposed to acidic pH such as that prevailing in the stomach. Furthermore, reaction of MNNG with H_2O_2 generates highly toxic, unstable and reactive hydroxyl radicals that can cause oxidative damage to the gastric mucosa [36]. Of particular concern, however, is the ability of hydroxyl radicals to traverse cell membranes causing deleterious effects at sites far from the target tissue. In particular, the erythrocytes are prone to oxidative damage due to high content of iron and polyunsaturated fatty acids and their role as O_2 transporters [37]. Although the liver has a rich supply of antioxidants, an increase in the levels of TBARS was observed when hepatocytes were exposed to MNNG [38]. Thus enhanced lipid peroxidation in the stomach, liver and erythrocytes of MNNG-treated rats can be attributed to excessive OFR production exacerbated by deficient antioxidant defences.

Reduced activities of SOD and CAT seen in MNNG-treated rats can cause accumulation of superoxide anion and H_2O_2 with deleterious consequences including DNA strand breaks and conformational changes in proteins. GSH in conjunction with GPx and GST plays a crucial role in maintaining the integrity of the gastric mucosa as well as the liver and erythrocytes when challenged by toxic agents [39, 40]. MNNG-induced depletion of GSH and GSH-dependent enzymes seen in the present study may shift the redox status of these tissues with adverse impact on critical –SH groups of functional proteins.

Pretreatment with ethanolic extract of neem leaf effectively reduced MNNG-induced lipid peroxidation and enhanced antioxidant levels in the stomach, liver and erythrocytes with significant effects at the concentration of 200 mg/kg body weight. Chemopreventive agents are recognized to induce antioxidants and GSH-dependent detoxification enzymes at various sites in addition to the target organs. The results of the present study substantiate

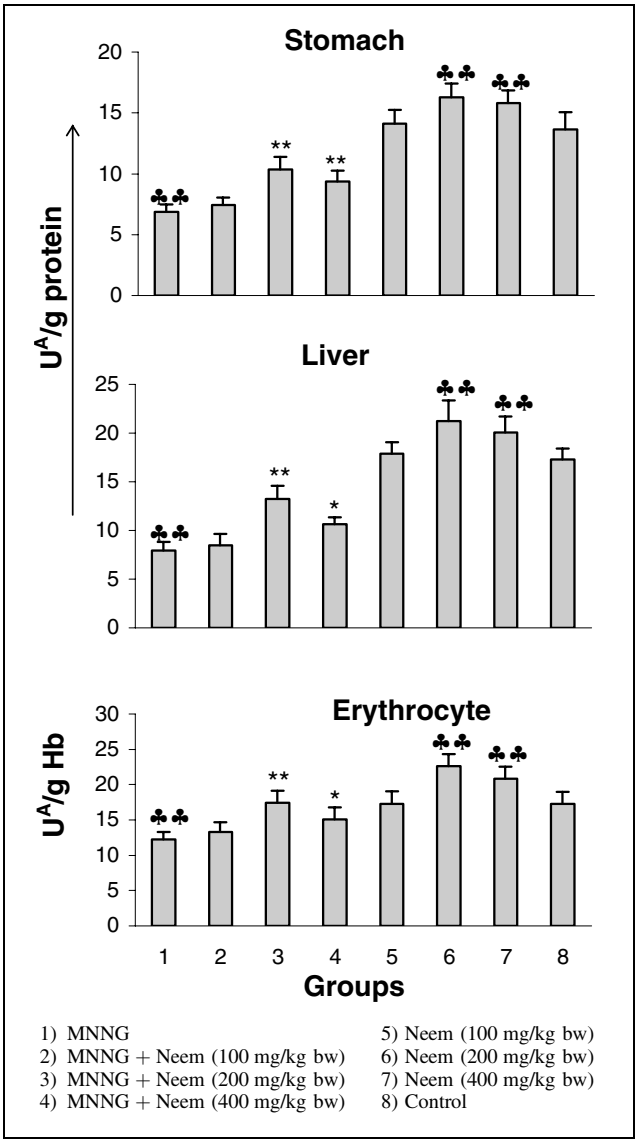

Fig. 5: The influence of pre-treatment with three doses of ethanolic neem leaf extract on GPx activity in the stomach, liver and erythrocytes.
♣ Significantly different from group 8 ($p < 0.05$)
♣♣ Significantly different from group 8 ($p < 0.001$)
* Significantly different from group 1 ($p < 0.05$)
** Significantly different from group 1 ($p < 0.001$)
A – μmoles of GSH utilised/min

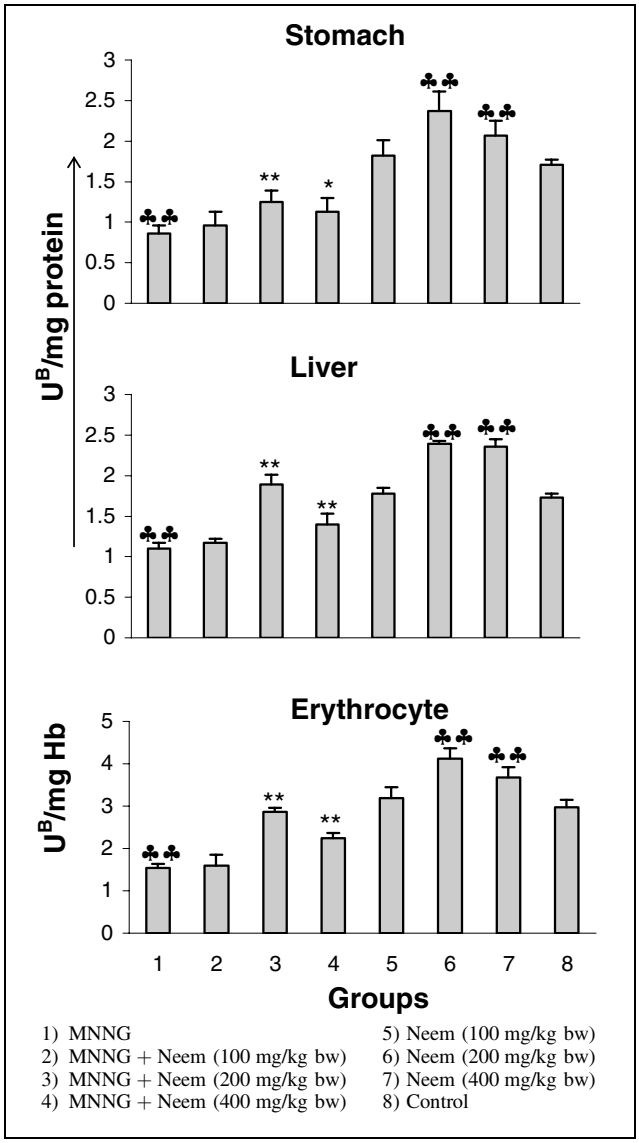

Fig. 6: The influence of pre-treatment with three doses of ethanolic neem leaf extract on GST activity in the stomach, liver and erythrocytes.
♣ Significantly different from group 8 ($p < 0.05$)
♣♣ Significantly different from group 8 ($p < 0.001$)
* Significantly different from group 1 ($p < 0.05$)
** Significantly different from group 1 ($p < 0.001$)
B – μmoles of CDNB-GSH conjugate formed/min

the radical scavenging properties of neem preparations documented by other workers. Neem leaf is reported to decrease the extent of lipid peroxidation [19]. Labadie et al. [41] demonstrated the inhibitory action of neem stem bark on superoxide anion production. Neem flowers are known to induce phase II enzymes such as GST in rats [42].

Alcoholic neem leaf extract contains a number of potent antioxidants and anticarcinogens that modulate OFR-induced lipid peroxidation including β-carotene, flavonoids and various limonoids [43]. β-Carotene is known to inhibit lipid peroxidation by trapping peroxyl radicals [44]. Quercetin, a highly ethanol soluble bioflavonoid and potent antioxidant, has been reported to decrease MNNG-induced DNA damage and induce phase II enzymes [45, 46]. Although quantitation and characterization of individual components was not attempted, the results of the present investigation indicate that ethanolic neem leaf extract by inducing SOD, CAT and GSH-dependent biotransformation enzymes, provides *in vivo* protection against MNNG-induced oxidative stress not only to the stomach and liver but presumably to other organs through the circulation. These studies strengthen the observation that medicinal plants have potential inhibitory effects on chemical mutagenesis and carcinogenesis. Further studies on the biochemical and molecular mechanisms of chemoprevention by alcoholic extract of neem leaf on MNNG-induced gastric carcinogenesis are however warranted.

4. Experimental

4.1. 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) were purchased from Sigma Chemical Company, St. Louis, USA. MNNG of purity $\geq$97% was obtained from Fluka-Chemika-Biochemika, Buchs, Switzerland.

4.2. Animals

All the experiments were carried out with male Wistar rats aged 6–8 weeks obtained from the Central Animal House, Annamalai University, India. The animals were housed six in a polypropylene cage and provided food and water *ad libitum*. All animals were fed standard pellet diet (Mysore Snack Feed Ltd, Mysore, India) and maintained under standard conditions of temperature and humidity with an alternating 12 hours light/dark cycle. The animals 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 of Annamalai University.

4.3. Collection of plant material

Fresh matured leaves of *A. indica* were collected locally during March-April 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.

4.4. Preparation of neem leaf extract

The ethanolic extract of neem leaf was prepared according to the procedure described by Chattopadhyay [19]. Air-dried powder (1 kg) of *A. indica* leaves was mixed with 3 L of 70% ethyl alcohol and kept at room temperature for 36 h. The slurry was stirred intermittently for 2 h 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 48 g/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 final concentrations of 10, 20 and 40 mg/mL and used for the experiment.

4.5. Treatment schedule

The animals were randomized into experimental and control groups and divided into eight groups of six animals each. Rats in group 1 were given MNNG (40 mg/kg body weight) by intragastric intubation [47]. Animals in groups 2, 3 and 4 received intragastric administration of ethanolic neem leaf extract at a concentration of 100, 200 and 400 mg/kg body weight respectively for 5 days followed by MNNG 1.5 h after the final feeding. Groups 5, 6 and 7 were given neem leaf extract alone (100, 200 and 400 mg/kg body weight respectively) for 5 days. Group 8 received the same volume of normal saline and served as control. The animals were sacrificed by cervical dislocation 27 h after the carcinogen exposure.

4.6. Preparation of tissue homogenate

Tissue samples were weighed and homogenized using appropriate buffer in an all glass homogeniser with Teflon pestle.

4.7. Preparation of hemolysate

Blood samples were collected in heparinised tubes and plasma was separated by centrifugation at 1000 *g* for 15 min. After centrifugation, the buffy coat was removed and the packed cells washed thrice 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.

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References

1 Kucuk, O.: Cancer Invest. **20**, 237 (2002)
2 Weisburger, J. H.: Mutat. Res. **480–481**, 23 (2001)
3 Ketterer, B.: Mutat. Res. **202**, 343 (1988)
4 Singh, R. P.; Padmavathi, B.; Rao, A. R.: Mol. Cell. Biochem. **213**, 99 (2000)
5 Sethi, S.; Annamma, M. O.; Preetha, K.: Indian J. Pathol. Microbiol. **42**, 73 (1999)
6 Nishikawa, A.; Furukawa, F.; Imazawa, T.; Hasegawa, T.; Takahash, M.: Food Chem. Toxicol. **33**, 21 (1995)
7 Kim, D. J.; Ahn, B.; Han, B. S.; Tsuda, H.: Cancer Lett. **112**, 203 (1997)
8 Uedo, N.; Tatsuta, M.; Iishi, H.; Baba, M.; Sakai, N.; Yano, H.; Otani, T.: Cancer Lett. **137**, 131 (1999)
9 Arivazhagan, S.: Balasenthil, S.; Nagini, S.: GI Cancer. **3**, 375 (2001)
10 Velmurugan, B.; Bhuvaneswari, V.; Usha, K. B.; Nagini, S.: Eur. J. Cancer Prev. **11**, 19 (2001)
11 van der Nat, J. M.; van der Sluis, W. G.; de Silva, K. T..; Labadie, R. P.: **35**, 1 (1991)
12 Puri, H. S.: (Ed): Neem. p. 87 Harwood Academic Publishers, Singapore 1999
13 Biswas, K.; Chattopadhyay, I.; Banerjee, R. K.; Bandyopadhyay, U.: Current Science **82**, 1336 (2002)
14 Arivazhagan, S.; Balasenthil, S.; Nagini, S.: Med. Sci. Res. **27**, 527 (1999)
15 Arivazhagan, S.; Balasenthil, S.; Nagini, S.: Phytother. Res. **14**, 291 (2000)
16 Arivazhagan, S.; Balasenthil, S.; Nagini, S.: Cell Biochem. Funct. **18**, 17 (2000)
17 Balasenthil, S.; Arivazhagan, S.; Ramachandran, C. R.; Ramachandran, V.; Nagini, S.: J. Ethnopharmacol. **67**, 189 (1999)
18 Chattopadhyay, R. N.; Maitra, S. K.; Chattopadhyay, R. R.: Fitoterapia **64**, 332 (1993)
19 Chattopadhyay, R. R.: Indian J. Exp. Biol. **36**, 418 (1998)
20 Parshad, O.; Gardner, M. T.; The T. L.; Williams, L. A..; Fletcher, C. K.: Phytother. Res. **168** (1997)
21 Garg, G. P.; Nigam, S. K.; Ogle, C. W.: Planta. Med. **59**, 215 (1992)
22 Febry, W.; Okema, P.; Ansorg, R.: Chemotherapy **42**, 315 (1996)
23 Gogate, S. S.: Natl. Med. J. India **9**, 297 (1996)
24 Schaaf, O.; Jarvis, A. P.; Van der Esch, S. A.; Giagnacovo, G.; Oldham, N. J.: J. Chromatogr. *A* **886**, 89 (2000)
25 Ohkawa, H.; Ohisi, N.; Yagi, K.: Anal. Biochem. **95**, 351 (1979)
26 Buege, J. A.; Aust, S. D.: Methods Enzymol. **52**, 302 (1987)
27 Kakkar, P.; Das, B.; Viswanathan, P. N.: Indian J. Biochem. Biophys. **21**, 130 (1984)
28 Sinha, K. A.: Anal. Biochem. **47**, 389 (1972)
29 Anderson, M. E. (Ed.): Methods in enzymology. p. 548, Academic Press, New York 1985
30 Rotruck, J. T.; Pope, A. L.; Ganther, H. E.; Swanson, A. B.; Hafeman, D. G.; Hoekstra, W. G.: Science **179**, 588 (1973)
31 Habig, W. H.; Pabst, M. J.; Jakoby, W. B.: J. Biol. Chem. **249**, 7130 (1974)
32 Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J.: J. Biol. Chem. **193**, 265 (1951)
33 Yoshikawa, T.; Takahashi, S.; Kondo, M.: Exs. **62**, 353 (1992)
34 Naya, M. J.; Pereboom, D.; Ortego. J.; Alda, J. O.; Lanas, A.: Gut **40**, 175 (1997)

35 Sobala, G. M.; Pignatelli, B.; Schorah, C. J.; Bartsch, H.; Sanderson, M.; Dixon M. F.; Shires, S.; King, R. G. F.; Axon, A. T. R.: Carcinogenesis **12**, 193 (1991)
36 Mikuni, T.; Tatsuta, M.; Kamachi, M.: Cancer Res. **45**, 6442 (1985)
37 Hebbel, R. P.: J. Lab. Clin. Med. **107**, 401 (1986)
38 Reitnan, F. A.; Shertzer, H. G.; Berger, M. L.; Biochem. Pharmacol. **37**, 3183 (1988).
39 Meister, A.: Cancer Res. **54**, 1969 (1994)
40 Goldin, E.; Ardite, E.; lizalde, J. I.; Odriozola, A.; Panes, J.; Pique, J. M.; Fernandez-Checa, J. C.: Gastroenterology **112**, 855 (1997)
41 Labadie, R. P.; Van der Nat, J. M.; Simons, J. M.; Kroes, S.; Kosasi, S.; Van der Berg, A. J. J.; Hart, L. A.; Van der Sluis, W. G.; Abeysekra, A.; Bamanurachchi, A.; De Silva, K. T. D.: Proc. Of XI Asian Symposium on Medicinal Plants and Spices, Bandung, Indonesia. **24**–**28**, p. 407 (1989)
42 Kusamran, W. R.; Ratanavila, A.; Tepsuwan, A.: Food Chem. Toxicol. **36**, 475 (1998)
43 Govindachari, T. R.: Current Science **63**, 117 (1992)
44 Miller, N. J.; Sampson, J.; Candeias, L. P.; Bramley, P. M.; Rice-Evans, C. A.: FEBS Lett. **417**, 261 (1996)
45 Yuting, C.; Rongliang, Z.; Zhongjian, J.; Yong, J.: Free Radical Biol. Med. **9**, 19 (1990)
46 Blasiak, J.; Trzeciak, A.; Gasiorowska, A.; Drzewoski, J.; Malecka-Panas, E.: Plant Foods Hum. Nutr. **57**, 53 (2002)
47 Abraham, S. K.: Food Chem. Toxicol. **27**, 787 (1989)