

# Combinatorial chemopreventive effect of *Azadirachta indica* and *Ocimum sanctum* on oxidant-antioxidant status, cell proliferation, apoptosis and angiogenesis in a rat forestomach carcinogenesis model

Manikandan P, Vidjaya Letchoumy P, Prathiba D, Nagini S

## ABSTRACT

**Introduction:** We investigated the combinatorial chemopreventive efficacy of *Azadirachta indica* (AI) and *Ocimum sanctum* (OS) against N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced gastric carcinogenesis, based on changes in oxidant-antioxidant status, cell proliferation, apoptosis and angiogenesis.

**Methods:** Male Wistar rats were assigned to four groups. Rats in groups 1 and 2 received MNNG (150 mg/kg body weight i.g.) three times with a gap of two weeks in between the treatment. Group 2 rats additionally received ethanolic AI (100 mg/kg body weight i.g.) and OS (150 mg/kg body weight i.g.) leaf extract three times per week for 26 weeks. Group 3 animals were given AI and OS leaf extract alone, whereas group 4 served as the control.

**Results:** Lipid and protein oxidation and status of the antioxidants, superoxide dismutases, catalase, reduced glutathione (GSH) and GSH-dependent enzymes together with markers of proliferation (proliferating cell nuclear antigen [PCNA], glutathione S-transferase-Pi [GST-P]), invasion (cytokeratin [CK]), angiogenesis (vascular endothelial growth factor [VEGF]) and apoptosis (Bcl-2, Bax, cytochrome C and caspase-3) were used to biomonitor chemoprevention. Rats administered MNNG developed forestomach carcinomas that displayed low lipid and protein oxidation coupled to enhanced antioxidant activities, and overexpression of PCNA, GST-P, CK, VEGF and Bcl-2 with downregulation of Bax, cytochrome C and caspase-3. Coadministration of AI and OS extract suppressed MNNG-induced gastric carcinomas accompanied by modulation of the oxidant-antioxidant status, inhibition of cell proliferation and angiogenesis, and induction of apoptosis.

**Conclusion:** The results of the present study suggest that chemoprevention by AI and OS combination may be mediated by their antioxidant, antiangiogenic, antiproliferative and apoptosis inducing properties.

**Keywords:** antioxidants, apoptosis, *Azadirachta indica*, cell proliferation, chemoprevention, gastric cancer, *Ocimum sanctum*, oxidant-antioxidant status, rat forestomach carcinogenesis model

*Singapore Med J 2008;49(10):814-822*

## INTRODUCTION

Chemoprevention has received growing consideration as a potential means to control the incidence of gastric cancer, the second most common malignancy worldwide and a major cause of mortality in Chennai, India.<sup>(1)</sup> Since multiple signaling pathways are dysfunctional in gastric cancer and new oncogenic mutations accumulate with carcinogenic progression, chemoprevention can be most effectively achieved with combination regimens that address multiple targets.<sup>(2)</sup> However, identification of effective chemoprevention cocktails requires conclusive evidence in a site-specific animal tumour model before embarking on clinical trials. Gastric cancer induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in Wistar rats that shows similarities to human gastric tumours is an ideal model for investigating the effects of intervention by chemopreventive agents. In previous studies from this laboratory, we have demonstrated the chemopreventive potential of several dietary agents and medicinal plants in the MNNG model.<sup>(3-5)</sup>

Recently, medicinal plants have emerged as attractive candidates for cancer chemoprevention because of their safety, relative to cytotoxic synthetic agents.<sup>(6)</sup> In particular, the leaves of neem (*Azadirachta indica* [AI]) and tulsi (*Ocimum sanctum* [OS]) offer promise in chemoprevention of gastric cancer because of their antioxidant, anti-inflammatory and antiproliferative

Department of  
Biochemistry and  
Biotechnology,  
Faculty of Science,  
Annamalai University,  
Annamalainagar  
608002,  
Tamil Nadu,  
India

Manikandan P, MSc,  
MPhil  
Research Scholar

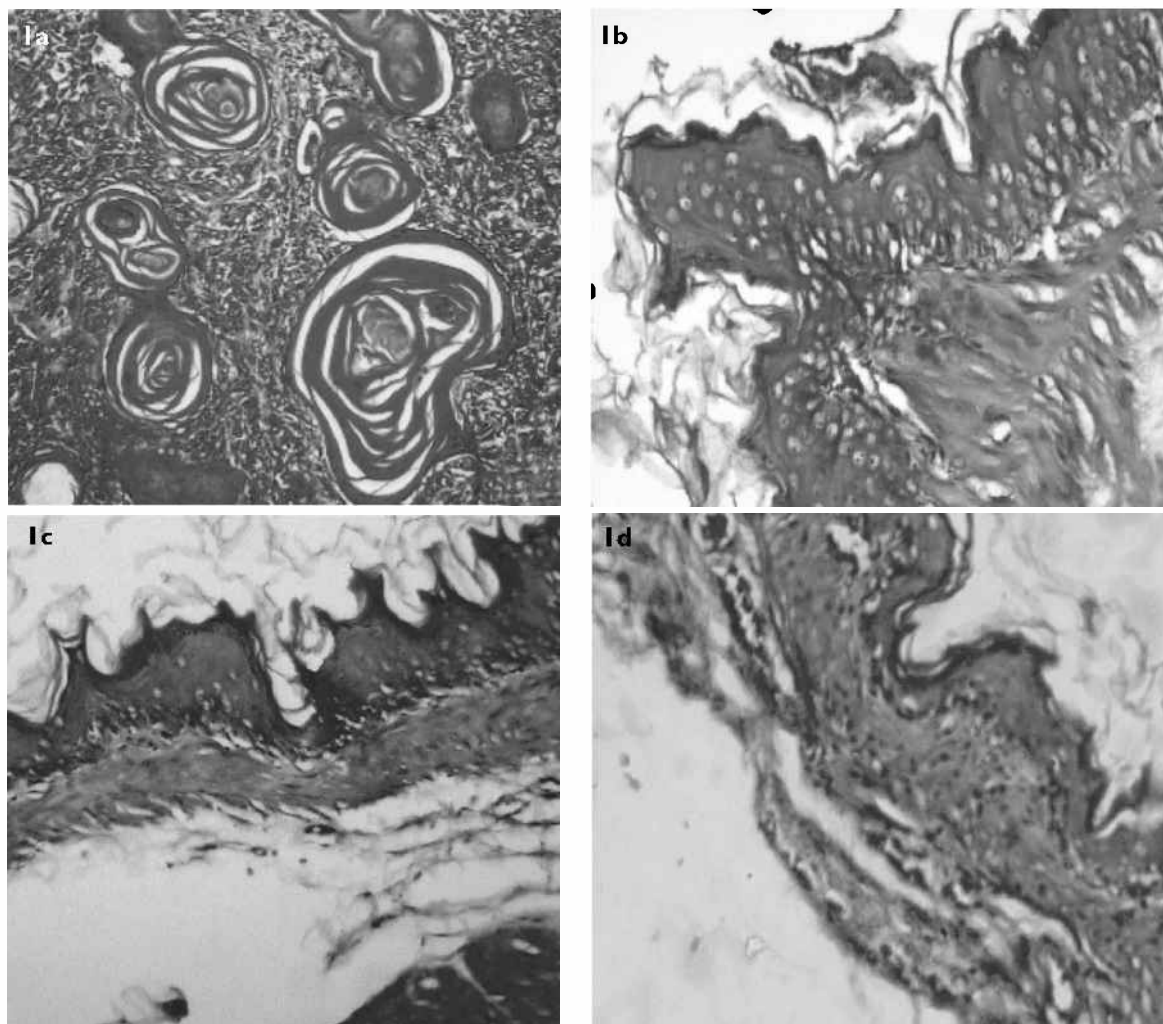
Vidjaya Letchoumy P,  
MSc, MPhil  
Research Scholar

Nagini S, MSc, PhD  
Professor

Department of  
Pathology,  
Sri Ramachandra  
Medical College and  
Research Institute,  
Chennai 600116,  
India

Prathiba D, MBBS,  
MD  
Professor

Correspondence to:  
Prof Siddavaram  
Nagini  
Tel: (91) 4144 239 842  
Fax: (91) 4144 238 145  
Email: s\_nagini@  
yahoo.com; snlabau@  
gmail.com



**Fig. 1** Photomicrographs of histopathological changes in the stomach tissues of the control and experimental animals show (a) squamous cell carcinoma with extensive infiltration in group 1 rats; (b) mild dysplasia in the forestomach epithelium of group 2 rats; (c) stomach epithelium showing normal histology in group 3 rats; and (d) stomach tissue showing normal keratinised stratified squamous epithelium in group 4 rats. (Haematoxylin & eosin,  $\times 20$ ).

properties.<sup>(7-9)</sup> Most notably, extracts of AI and OS are recognised to inhibit gastric ulcer and *Helicobacter pylori* infection, important risk factors for gastric carcinogenesis.<sup>(10-12)</sup> Previously, we documented the inhibitory effects of an AI and OS against MNNG-induced rat forestomach carcinogenesis.<sup>(5,13)</sup> However, the effect of an AI and OS combination on experimental gastric carcinogenesis has not been investigated.

Of late, combination chemoprevention has attracted the focus of research attention due to its high potency and reduced toxicity. Further, medicinal plants in combination are shown to interact synergistically with high efficacy and have a broader spectrum of action.<sup>(14)</sup> The present study was therefore designed to investigate the combinatorial chemopreventive effects of ethanolic AI and OS leaf extract on MNNG-induced gastric carcinogenesis. The extent of lipid and protein oxidation and the status of the antioxidants, superoxide dismutases (SODs), catalase (CAT), reduced glutathione (GSH) and

GSH-dependent enzymes in the stomach, were used to biomonitor chemoprevention. In addition, the expression of markers of proliferation (proliferating cell nuclear antigen [PCNA] and glutathione S-transferase-Pi [GST-P]), invasion (cytokeratin [CK]), angiogenesis (vascular endothelial growth factor [VEGF]) and apoptosis (Bcl-2, Bax, cytochrome C, and caspase-3) was analysed in the stomach tissue by immunohistochemical localisation, and the activity of caspase-3 was assayed by a colorimetric method.

## METHODS

Bovine serum albumin, 2-thiobarbituric acid, 2,4-dinitrophenylhydrazine, GSH, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and nicotinamide adenine dinucleotide phosphate reduced (NADPH) were purchased from Sigma Chemical Company, St. Louis, MO, USA. MNNG was obtained from Fluka-Chemika-Biochemika, Buchs, Switzerland. All other reagents used

**Table I. Body weight, tumour incidence and histopathological changes in control and experimental animals (mean  $\pm$  SD; n = 10).**

Group	Treatment	Body weight (g)		Tumour incidence	Tumour multiplicity <sup>a</sup>	Tumour burden <sup>b</sup> (mm <sup>3</sup> )	Keratosis	Hyperplasia	Dysplasia	SCC
		Initial	Final							
1.	MNNG	97.70 $\pm$ 6.49	108.12 $\pm$ 10.10 <sup>c</sup>	10/10 (100)	2.70 $\pm$ 0.94	126.40 $\pm$ 11.30	+++ (10/10)	+++ (10/10)	+++ (10/10)	10
2.	MNNG + AI + OS	95.20 $\pm$ 8.21	122.00 $\pm$ 8.79 <sup>d</sup>	–	–	–	++ (3/10)	+ to ++ (6/10)	+ (1/10)	–
3.	AI + OS	94.60 $\pm$ 9.10	123.00 $\pm$ 10.76	–	–	–	–	–	–	–
4.	Control	94.90 $\pm$ 8.12	121.00 $\pm$ 11.17	–	–	–	–	–	–	–

+: mild; ++: moderate; +++: severe; – no change; data in parentheses indicate no. of animals.

<sup>a</sup> Tumour multiplicity: number of tumours per rat.

<sup>b</sup> Mean tumour burden was calculated by multiplying the mean tumour volume ( $4/3\pi r^3$ ) with the mean number of tumours ( $r = 1/2$  tumour diameter in mm).

<sup>c</sup> Significantly different from group 4 by Student's *t*-test. ( $p < 0.05$ ).

<sup>d</sup> Significantly different from group 1 by Student's *t*-test. ( $p < 0.05$ ).

**Table II. PCNA labelling index and expression of GST-P, Bcl-2, Bax, cytochrome C, caspase-3, cytokeratin, and VEGF in experimental and control animals (n = 10).**

Group	Treatment	PCNA <sup>a</sup>	GST-P <sup>b</sup>				Bcl-2 <sup>b</sup>				Bax <sup>b</sup>				Cytochrome C <sup>b</sup>				Caspase <sup>b</sup>				Cytokeratin <sup>c</sup>			VEGF <sup>b</sup>			
			0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	0	1	II	0	1	2	3				
1.	MNNG	78.94 $\pm$ 7.03 <sup>e</sup>	0*	2	1	7*	0*	2	1	7*	8*	2	0	0	9*	1	0	0	8	2	0	0	1	2*	7*	0	0	2*	8*
2.	MNNG + AI + OS	63.63 $\pm$ 5.32 <sup>f</sup>	6*	2	2	0*	5*	3	2	0*	0*	1	2	7*	0*	2	6*	2	1*	2	2	5*	2	7*	1*	1	8*	1	0*
3.	AI + OS	43.33 $\pm$ 3.64	8	2	0	0	8	2	0	0	4	6	0	0	6	4	0	0	4	6	0	0	0	9	1	4	4	2	0
4.	Control	44.00 $\pm$ 4.14	9	1	0	0	9	1	0	0	6	4	0	0	5	5	0	0	5	5	0	0	0	9	1	1	2	7	0

<sup>a</sup> Labelling index for PCNA expressed as the number of cells with positive staining per 100 counted cells.

<sup>b</sup> Expression scored as 0: negative; 1: focal and mildly intense; 2: one-third and two-thirds of cells stained moderately; and 3: majority of cells (> two-thirds) stained intensely.

<sup>c</sup> Expression graded as 0: failure to detect the keratin; I: staining confined either to the basal area or some evidence of suprabasal staining; II: positive staining throughout the basal and/or suprabasal region.

<sup>e</sup> Significantly different from group 4 ( $p < 0.05$ ) ANOVA followed by LSD

<sup>f</sup> Significantly different from group 1 ( $p < 0.05$ ) ANOVA followed by LSD

\* Significantly different from group 4 by  $\chi^2$ -test ( $p < 0.05$ )

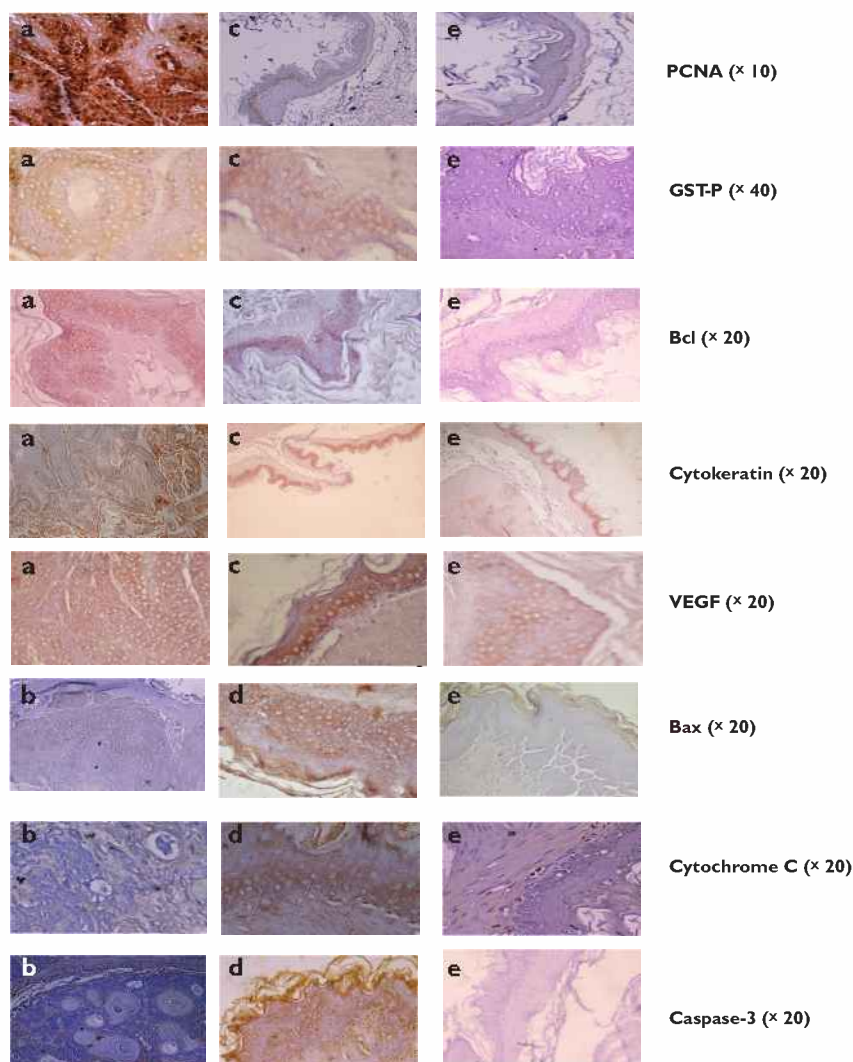
\* Significantly different from group 1 by  $\chi^2$ -test ( $p < 0.05$ )

were of analytical grade. Male Wistar rats aged 6–8 weeks, weighing 80–100 g, obtained from the Central Animal House, Annamalai University, India, were used for the study. They were maintained under standard conditions of temperature and humidity with an alternating 12-h light/dark cycle and provided a standard pellet diet (Mysore Snack Feed Ltd, Mysore, India) and water *ad libitum*. All animals were maintained in accordance with the guidelines of the Indian Council of Medical Research, India.

Fresh matured leaves of AI and OS collected locally during October–December were identified by a pharmacognosy expert. These leaves were dried in shade, powdered, and the powder was used for the extraction. Voucher specimens were deposited at the herbarium of the Botany Department, Annamalai University. The procedure for the preparation of both ethanolic AI and OS leaf extract was same as described previously.<sup>(5,13)</sup> Air-dried powder (1 kg) of AI and OS leaves was mixed separately with 3 L of 70% ethyl alcohol and kept at room temperature for 36 hours. The slurry was stirred intermittently for two hours and left overnight. The mixture was then filtered and the filtrate was concentrated under reduced pressure (bath temperature 40°C) and finally dried in a vacuum

dessicator. The residue collected (yield 48 g and 38 g/kg of AI and OS leaf powder, respectively) was a thick paste, green in colour and gummaceous in nature. The AI and OS leaf extracts were dissolved in normal saline to obtain a final concentration of 10 mg and 15 mg/ml respectively and used for the experiment.

The animals were randomised into experimental and control groups and divided into four groups of ten animals each. Rats in group 1 were given MNNG (150 mg/kg body weight) by intragastric intubation three times with a gap of two weeks in between the treatments.<sup>(15)</sup> Rats in group 2 were administered MNNG as in group 1, and in addition received intragastric intubation of ethanolic AI (100 mg/kg body weight) and OS (150 mg/kg body weight) leaf extract three times per week starting on the day following the first exposure to MNNG and continued until the end of the experimental period. The doses of AI and OS were half that of the effective doses for maximum chemoprevention used in our previous studies, because combined administration would be expected to attenuate the risk of adverse effects by decreasing the dose of the individual agents.<sup>(5,13)</sup> Group 3 animals were given ethanolic AI and OS leaf extract alone as in group 2 but without MNNG.



**Fig. 2** Representative photomicrographs of immunohistochemical staining of PCNA, GST-P, Bcl-2, Bax, cytochrome C, caspase-3, cytokeratins and VEGF expression in experimental and control animals (Mean  $\pm$  SD; n = 10), show (a) overexpression of PCNA, GST-P, Bcl-2, cytokeratins, and VEGF in animals administered MNNG (Group 1); (b) downregulation of Bax, cytochrome C and caspase-3 in animals administered MNNG (Group 1); (c) downregulation of PCNA, GST-P, Bcl-2, cytokeratins, and VEGF in animals administered MNNG+AI and OS combination (Group 2); (d) overexpression of Bax, cytochrome C and caspase-3 in animals administered MNNG+AI and OS combination (Group 2); and (e) PCNA, GST-P, 4-HNE, Bcl-2, Bax, cytochrome C, caspase-3, cytokeratins, and VEGF expression in control animals.

Group 4 received a basal diet and tap water throughout the experiment and served as the untreated control. The experiment was terminated at 26 weeks and all animals were killed by cervical dislocation after an overnight fast. The stomach tissues were subdivided and variously processed for distribution to each experiment.

Fresh tissues were used for biochemical estimations. After weighing, the stomach tissues were homogenised in an all glass homogeniser with Teflon pestle and stored in ice until use. Tissues were immediately fixed in 10% neutral buffered formalin, embedded in paraffin, and mounted on polylysine-coated slides and stained with haematoxylin and eosin. Basal cell hyperplasia, dysplasia, and squamous cell carcinoma (SCC) were diagnosed. Hyperplasia of forestomach epithelium was indicated by an increased number of basal cells. Irregular epithelial

stratification, increased number of mitotic figures, increased nuclear-to-cytoplasmic ratio and loss of polarity of basal cells characterised the dysplastic lesions. SCC was diagnosed by the invasion of underlying tissues, nuclear pleomorphism and increased mitoses.

The tissue sections were deparaffinised by heat at 60°C for 10 minutes, followed by three washes in xylene. After gradual hydration through graded alcohol, the slides were incubated in citrate buffer (pH 6.0) for two cycles of 5 minutes in a microwave oven for antigen retrieval. The sections were allowed to cool for 20 minutes and then rinsed with Tris-buffered saline (TBS), and treated with 3% H<sub>2</sub>O<sub>2</sub> in distilled water for 15 minutes to inhibit the endogenous peroxidase activity. Nonspecific antibody binding was reduced by incubating the sections with normal goat serum for 20 minutes. The sections were then

**Table III. Lipid peroxidation and protein carbonyl content in the stomach of experimental and control animals (mean  $\pm$  SD; n = 10).**

Group	Treatment	TBARS (nmol/mg protein)	LOOH (mmol/mg protein)	CD (mg/mg protein)	Protein carbonyl (pmol/mg protein)
1.	MNNG	7.60 $\pm$ 0.60*	135.30 $\pm$ 12.16*	115.50 $\pm$ 9.46*	36.31 $\pm$ 3.72*
2.	MNNG+ AI+OS	8.73 $\pm$ 0.85*	153.26 $\pm$ 13.33*	129.63 $\pm$ 11.12*	54.76 $\pm$ 2.90*
3.	AI+OS	11.21 $\pm$ 0.97*	158.32 $\pm$ 16.72*	135.97 $\pm$ 13.15*	69.89 $\pm$ 4.68*
4.	Control	12.33 $\pm$ 1.13	173.76 $\pm$ 14.14	146.75 $\pm$ 11.97	73.57 $\pm$ 6.83

\* Significantly different from group 4 ( $p < 0.05$ ) ANOVA followed by LSD\* Significantly different from group 1 ( $p < 0.05$ )

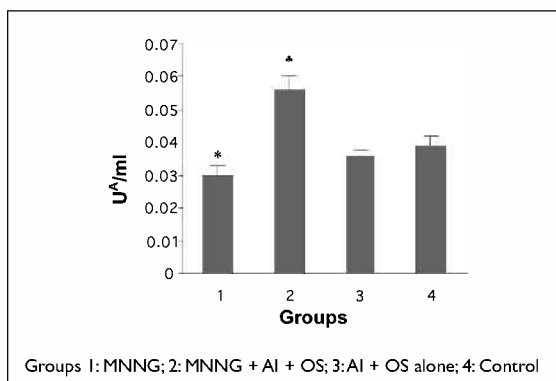
incubated with mouse monoclonal antibodies PCNA, CK AE1/AE3, Bcl-2 (Dako, Carpinteria, CA, USA), caspase-3, cytochrome C (NeoMarkers, USA) and VEGF (Santa Cruz Biotechnology, CA, USA) and rabbit polyclonal antibodies GST-P (BioGenex, USA) and Bax (Santa Cruz Biotechnology, CA, USA) at room temperature for one hour. The slides were washed with TBS and then incubated with anti-rabbit and anti-mouse biotin-labelled secondary antibody (Dako, Carpinteria, CA, USA) followed by streptavidin-biotin-peroxidase for 30 minutes each at room temperature.

The immunoprecipitate was visualised by treating with 3,3'-diaminobenzidine (Dako, Carpinteria, CA, USA) and counterstaining with haematoxylin. For negative controls, the primary antibody was replaced with TBS. Positive controls for each antibody were also processed simultaneously. The labelling indices for PCNA were calculated as the number of cells with a positive staining per 100 counted cells in three high power fields. The expression of GST-P, Bcl-2, Bax, cytochrome C, caspase-3 and VEGF was regarded as negative (0) when there was no staining; weak (1) when the staining was focal and mildly intense; moderate (2) when between one-third and two-third of cells stained moderately; and strong (3) when the majority of cells (> two-thirds) stained intensely. The CK expression was graded as, 0: failure to detect the keratin; I: staining confined either to the basal area or some evidence of suprabasal staining; and II: positive staining throughout the basal and/or suprabasal region.

Lipid peroxidation was estimated as evidenced by the formation of thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LOOH) and conjugated dienes (CD). TBARS were assayed in the stomach tissue by the method of Ohkawa et al.<sup>(16)</sup> LOOH were estimated by the method of Jiang et al.<sup>(17)</sup> and CD by the method of Rao and Recknagel.<sup>(18)</sup> Protein oxidation was measured by the method of Levine et al.<sup>(19)</sup> based on the reaction of the carbonyl group with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone. Total SOD and Mn-SOD activities were assayed as described by Oberley

and Spitz,<sup>(20)</sup> based on the half-maximal inhibition of nitrobluetetrazolium (NBT) reduction. Cu-Zn SOD activity was calculated by deducting the activity of Mn-SOD from total SOD activity. The activity of CAT was assayed by the method of Sinha,<sup>(21)</sup> based on the utilisation of H<sub>2</sub>O<sub>2</sub> by the enzyme. GSH was determined by the method of Anderson,<sup>(22)</sup> by measurement of the yellow colour that develops when DTNB is added to compounds containing sulfhydryl groups. Oxidized glutathione (GSSG) was estimated following the oxidation of NADPH using glutathione reductase (GR) at 340 nm according to the method of Srivastava and Beutler.<sup>(23)</sup> Selenium-dependent glutathione peroxidase (Se-GPx) activity was assayed by following the utilisation of H<sub>2</sub>O<sub>2</sub> according to the method of Rotruck et al.<sup>(24)</sup> Se-independent GPx activity was assayed following the method described by Lawrence and Burk,<sup>(25)</sup> using cumene peroxide as a substrate. The activity of GST was determined as described by Habig et al.<sup>(26)</sup> The activity of gamma-glutamyl transpeptidase (GGT) was assayed by the method of Fiala et al.<sup>(27)</sup> with gamma glutamyl p-nitroanilide as the substrate. GR activity was assayed by the method of Carlberg and Mannervik,<sup>(28)</sup> using GSSG as a substrate and flavin adenine dinucleotide as a cofactor. The protein content was estimated by the method of Lowry et al.<sup>(29)</sup> with bovine serum albumin as the standard.

DEVD-specific caspase-3 activity was assayed using a CASP-3-C colorimetric kit (Sigma Chemical Company, St Louis, MO, USA) according to the manufacturer's instructions. Cytosolic extracts were prepared by homogenising tissues in lysis buffer containing 50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethane sulphonic acid (HEPES) (pH 7.4), 5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) and 5 mM dithiothreitol (DTT). The supernatant was collected as an enzyme source. The caspase-3 colorimetric assay was based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-nitroanilide (Ac-DEVD-pNA) by caspase-3, resulting in release of the p-nitroaniline (pNA) moiety. The concentration of the pNA released from the



**Fig. 3** Bar chart shows the activity of caspase-3 in the stomach tissue of experimental and control animals (mean  $\pm$  SD; n = 10).

\* significantly different from group 4 ( $p < 0.05$ ) ANOVA followed by LSD

\* significantly different from group 1 ( $p < 0.05$ )

A  $\mu$ moles of pNA formed/ min

substrate was calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined pNA solutions.

The data was expressed as mean and standard deviation (SD). Body weights were analysed using Student's *t*-test. The grading of GST-P, Bcl-2, Bax, cytochrome C, caspase-3, CK, and VEGF were statistically compared using  $\chi^2$ -test. PCNA labelling index, data for colorimetric assay of caspase-3 and biochemical assays were analysed using analysis of variance (ANOVA), followed by the least significant difference test (LSD). The results were considered statistically significant if the *p*-value was  $< 0.05$ .

## RESULTS

Table I shows the mean body weight, and preneoplastic and neoplastic lesions in experimental and control animals. The mean final body weights of MNNG-treated animals (group 1) were significantly lower than that of controls (group 4). Treatment with combined ethanolic AI and OS leaf extract to MNNG-treated animals significantly increased the mean final body weight in group 2 animals compared to group 1. No significant differences in the body weights were observed in groups 3 and 4. In rats administered MNNG alone (group 1), the incidence of gastric tumours was 100% (10/10) with a mean tumour burden of 126.40 mm<sup>3</sup>. No tumours were observed in groups 2–4. Forestomach tumours induced by MNNG were SCCs with a number of epithelial keratin pearls and extensive infiltration. Tumour cells showed increased nuclear/cytoplasmic ratio, nuclear pleomorphism and hyperchromatism. Of the ten animals treated with MNNG and combined doses of ethanolic AI and OS leaf extract (group 2), three animals showed moderate keratosis, while

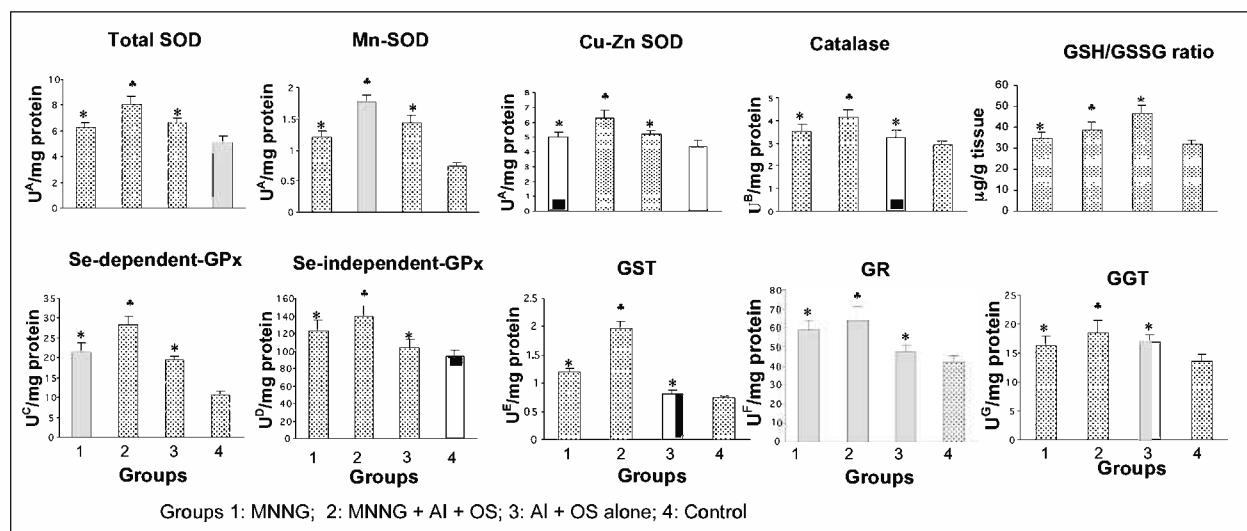
one animal showed mild dysplasia, and the remaining exhibited normal keratinised and stratified squamous epithelium with mild to moderate hyperplasia of the lining epithelium. The forestomach of rats in groups 3 and 4 showed normal lining of keratinised stratified squamous epithelium. Representative photomicrographs of the histopathological changes observed in the forestomach of control and experimental animals are shown in Fig. 1.

Table II shows the effect of coadministration of ethanolic AI and OS leaf extract on PCNA labelling index and expression of GST-P, Bcl-2, Bax, cytochrome C, caspase-3, CK and VEGF in the stomach of experimental and control animals. In MNNG-treated animals (group 1), the expression of PCNA, GST-P, Bcl-2, CK and VEGF was significantly higher, and that of Bax, cytochrome C and caspase-3 significantly lower, than in control animals (group 4). Coadministration of ethanolic AI and OS leaf extract significantly decreased PCNA, GST-P, Bcl-2, CK, and VEGF expression and significantly increased the expression of Bax, cytochrome C and caspase-3 compared to group 1. No significant changes in protein expression were observed in group 3 animals. While immunostaining of PCNA showed nuclear localisation, GST-P, Bcl-2, Bax, cytochrome C, caspase-3, CK, and VEGF were found in the cytoplasmic region. Representative photomicrographs of immunostaining are shown in Fig. 2.

Fig. 3 illustrates the activity of DEVD-specific caspase-3 in the stomach of control and experimental animals. In MNNG-treated animals (group 1), caspase-3 activity was significantly reduced as compared with the controls (group 4). Treatment with a combined dose of ethanolic AI and OS leaf extract significantly increased enzyme activity in group 2 animals as compared with group 1. In animals coadministered ethanolic AI and OS leaf extract alone (group 3), the activity of caspase-3 was not significantly different from that in the controls. Table III shows the effect of treatment with a combined dose of ethanolic AI and OS leaf extract on lipid and protein oxidation in the stomach. The extent of lipid peroxidation and the formation of protein carbonyl were significantly lower in MNNG-treated rats (group 1) compared to the controls. Coadministration of ethanolic AI and OS leaf extract (group 2) significantly increased MNNG-induced lipid and protein oxidation in the stomach compared to group 1. Combined administration of ethanolic AI and OS leaf extract alone significantly reduced lipid and protein oxidation in group 3 animals compared to the controls.

The activities of the antioxidant enzymes SOD (total SOD, MnSOD, and Cu-Zn SOD), CAT and GSH-dependent enzymes (GPxs, GST, GR and GGT) as well as the GSH/GSSG ratio in the stomach tissue of experimental





**Fig. 4** Bar charts show the activities of total SODs, catalase, GSH/GSSG ratio and GSH-dependent enzymes in the stomach tissues of experimental and control animals (mean  $\pm$  SD; n = 10).

\* significantly different from group 4 ( $p < 0.05$ ) ANOVA followed by LSD

<sup>A</sup> significantly different from group 1 ( $p < 0.05$ )

<sup>A</sup>: the amount of enzyme required to inhibit 50% NBT reduction

<sup>B</sup>:  $\mu$ moles of H<sub>2</sub>O<sub>2</sub> utilised/min

<sup>C</sup>:  $\mu$ moles of GSH utilised/min

<sup>D</sup>:  $\mu$ moles of NADPH utilised/min

<sup>E</sup>: CDNB-GSH conjugate formed/min

<sup>F</sup>:  $\mu$ moles of NADPH utilised/h

<sup>G</sup>:  $\mu$ moles of p-nitroaniline formed/h

and control animals are presented in Fig. 4. In MNNG-treated animals (group 1), the GSH/GSSG ratio as well as the activities of SODs, CAT and GSH-dependent enzymes were significantly increased compared to the controls (group 4). Coadministration of ethanolic AI and OS leaf extract to MNNG-treated animals (group 2) as well as controls (group 3) significantly enhanced the antioxidants compared to group 1 and group 4, respectively.

## DISCUSSION

Administration of MNNG induced well-differentiated forestomach SCCs that displayed low lipid and protein oxidation coupled to enhanced antioxidant activities and overexpression of PCNA and GST-P. Lipid peroxides are recognised to prolong the G1 phase of the cell cycle, whereas the antioxidant GSH, as well as PCNA, the cofactor for DNA polymerase  $\delta$ , are required for cell cycle progression.<sup>(30-32)</sup> PCNA and GST-P, reliable indicators of cell proliferation, have also been reported to inhibit apoptosis.<sup>(33,34)</sup> In addition, the increase in Bcl-2/Bax ratio with decreased expression of cytochrome C and caspase-3 provide evidence for apoptosis evasion in MNNG-induced gastric tumours. This was associated with overexpression of CK and VEGF, indicative of infiltrative and angiogenic potential of these tumours. Thus, modified oxidant-antioxidant status associated with overexpression of PCNA, GST-P, Bcl-2, CK and VEGF and downregulation of Bax, cytochrome C and caspase-3 confer a proliferative, infiltrative, angiogenic and apoptosis-resistant phenotype

on MNNG-induced stomach tumours.

To our knowledge, this is the first report on the combinatorial chemopreventive efficacy of AI and OS leaf extract on experimental gastric carcinogenesis. Coadministration of AI and OS modulated the susceptibility of the gastric mucosa to lipid and protein oxidation, while simultaneously enhancing the antioxidant status and downregulating PCNA and GST-P expression. These findings are in line with the antioxidant and antiproliferative effects of AI and OS preparations reported in the literature.<sup>(7,9,35-37)</sup> Downregulation of VEGF and CK is in accordance with similar observations with other chemopreventive agents.<sup>(38,39)</sup> The decrease in Bcl-2/Bax ratio, a reliable indicator of the overall propensity of a cell to undergo apoptosis coupled with the overexpression of cytochrome C and caspase-3, underscores the apoptosis-inducing potential of the AI-OS combination.<sup>(40)</sup> Taken together, these findings suggest that modulation of cellular redox status, inhibition of cell proliferation and angiogenesis and induction of apoptosis are major mechanisms through which AI-OS combination exerts its anticarcinogenic effects.

The combinatorial chemopreventive potential of AI and OS leaf extract seen in the present study may be ascribed to the rich array of constituent phytochemicals that are known to exhibit potent antiproliferative properties. The neem leaf is a "storehouse" of bioactive compounds such as nimbolide, nimbin, kaempferol and quercetin among several others.<sup>(7)</sup> The leaves of OS

contain apigenin, luteolin, eugenol and ursolic acid.<sup>(8)</sup> The use of the AI-OS combination is a strategic approach to administer a cocktail of phytochemical entities that could modulate multiple signal transduction pathways that are aberrant in cancer. Furthermore, synergistic interactions among the phytochemicals can ensure higher efficacy and potency in addition to overcoming problems of toxicity and resistance.

The results of the present study validate the hypothesis that medicinal plants with potent antioxidant, antiangiogenic, antiproliferative and apoptosis inducing properties, such as AI and OS, are effective chemopreventive agents especially in combination. A noteworthy consideration is the fact that the combination selectively induced apoptosis in MNNG-treated animals, but not in normal animals. In particular, dietary agents and medicinal plants that induce apoptosis are regarded as potential chemopreventive agents.<sup>(39,41)</sup> Traditionally, polyherbal preparations have been used to treat a variety of ailments. The results of the present study demonstrate that chemoprevention can be effectively achieved with a combination regimen that addresses multiple molecular targets. However, extensive studies on synergistic pharmacodynamic interactions of the phytochemicals as well as the effects on signal modulation are essential for the development of multiactive natural drugs from AI and OS for cancer chemoprevention in the future.

## REFERENCES

- Crew KD, Neugut AI. Epidemiology of gastric cancer. *World J Gastroenterol* 2006; 12:354-62.
- Zheng L, Wang L, Ajani J, Xie K. Molecular basis of gastric cancer development and progression. *Gastric Cancer* 2004; 7:61-77.
- Arivazhagan S, Balasenthil S, Nagini S. Attenuation by garlic and neem leaf extracts of sodium chloride enhanced gastric carcinogenesis induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in Wistar rats. *GI Cancer* 2001; 3:375-82.
- Velmurugan B, Mani A, Nagini S. Combination of S-allylcysteine and lycopene induces apoptosis by modulating Bcl-2, Bax, Bim and caspases during experimental gastric carcinogenesis. *Eur J Cancer Prev* 2005; 14:387-93.
- Subapriya R, Nagini S. Ethanolic neem leaf extract protects against MNNG-induced gastric carcinogenesis in Wistar rats. *Asian Pacific J Cancer Prev* 2003; 4:215-23.
- Park EJ, Pezzuto JM. Botanicals in cancer chemoprevention. *Cancer Metastasis Rev* 2002; 21:231-55.
- Subapriya R, and Nagini S. Medicinal properties of neem leaves: A review. *Curr Med Chem Anticancer Agents* 2005; 5:149-56.
- Gupta SK, Prakash J, Srivastava S. Validation of traditional claim of tulsi, *Ocimum sanctum* Linn. as a medicinal plant. *Indian J Exp Biol* 2002; 40: 765-73.
- Manikandan P, Murugan RS, Abbas H, Abraham SK, Nagini S. *Ocimum sanctum* Linn. (Holy Basil) ethanolic leaf extract protects against 7,12-dimethylbenz(a)anthracene-induced genotoxicity, oxidative stress, and imbalance in xenobiotic-metabolizing enzymes. *J Med Food* 2007; 10:495-502.
- Garg GP, Nigam SK, Ogle CW. The gastric antiulcer effects of the leaves of the neem tree. *Planta Med* 1993; 59:215-7.
- Goel RK, Sairam K, Dorababu M, Prabhu T, Rao CHV. Effect of standardized extract of *Ocimum sanctum* Linn. on gastric mucosal offensive and defensive factors. *Indian J Exp Biol* 2005; 43: 715-21.
- Febry W, Okema P, Ansory R. Activity of East African medicinal plants against *Helicobacter pylori*. *Chemotherapy* 1996; 42:315-7.
- Manikandan P, Vidjaya Letchoumy P, Prathiba D, Nagini S. Proliferation, angiogenesis and apoptosis-associated proteins are molecular targets for chemoprevention of MNNG-induced gastric carcinogenesis by ethanolic *Ocimum sanctum* leaf extract. *Singapore Med J* 2007; 48:645-51.
- Wang J, Guo LL, Wang YY. [Study on combination components and effectiveness of Chinese traditional herbal formulas]. *Zhongguo Zhong Yao Za Zhi* 2006; 31:5-9. Chinese.
- Saravanan K, Nagarajan B. Genotoxicity assessment in vivo in gastric carcinogenesis. *Med Sci Res* 1993; 21:119-21.
- Ohkawa H, Ohisi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; 95:351-8.
- Jiang ZY, Hunt JV, Wolff SP. Detection of lipid hydroperoxides using Fox method. *Anal Biochem* 1992; 202:384-9.
- Rao KS, Recknagel RO. Early onset of lipid peroxidation in rat liver after carbon tetrachloride administration. *Exp Mol Pathol* 1968; 9:271-8.
- Levine RL, Garland D, Oliver CN, et al. Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 1990; 186:464-78.
- Oberley LW, Spitz DR. Assay of superoxide dismutase activity in tumor tissue. *Methods Enzymol* 1984; 105:457-64.
- Sinha AK. Colorimetric assay of catalase. *Anal Biochem* 1972; 47:389-394.
- Anderson ME. Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol* 1985; 113:548-55.
- Srivastava SK, Beutler E. Accurate measurement of oxidized glutathione content of human, rabbit, and rat red blood cells. *Anal Biochem* 1968; 25:70-6.
- Rotruck JT, Pope AL, Ganther HE, et al. Selenium: biochemical role as a component of glutathione peroxidase. *Science* 1973; 179:588-90.
- Lawrence A, Burk RF. Glutathione peroxidase activity in selenium deficient rat liver. *Biochem Biophys Res Commun* 1976; 71:952-8.
- Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* 1974; 49:7130-9.
- Fiala S, Fiala AE, Dixon B. Gamma-glutamyl transpeptidase in transplantable, chemically induced rat hepatomas and "spontaneous" mouse hepatomas. *J Natl Cancer Inst* 1972; 48:1393-401.
- Carlberg I, Mannervik B. Glutathione reductase. *Methods Enzymol* 1985; 113:484-90.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RY. Protein measurement with the Folin-phenol reagent. *J Biol Chem* 1951; 193:265-75.
- Chajes V, Sattler W, Stranzl A, Kostner GM Influence of n-3 fatty acids on the growth of human breast cancer cells in vitro: relationship to peroxides and vitamin-E. *Breast Cancer Res Treat* 1995; 34:199-212.
- Poot M, Teubert H, Rabinovitch PS, Kavanagh TJ. De novo synthesis of glutathione is required for both entry into and progression through the cell cycle. *J Cell Physiol* 1995; 163:555-60.
- Jónsson ZO, Hindges R, Hübscher U. Regulation of DNA replication and repair proteins through interaction with the front side of proliferating cell nuclear antigen. *EMBO J* 1998;



- 17:2412-25.
33. Maga G, Hubscher U. Proliferating cell nuclear antigen (PCNA): a dancer with many partners, *J Cell Sci* 2003; 116:3051-60.
34. Zhao X, Ean Y, Shen J, Wu Y, Yin Z. Human glutathione S-transferase P1 suppresses MEKK1-mediated apoptosis by regulating MEKK1 kinase activity in HEK293 cells. *Mol Cell* 2006; 21:395-400.
35. Prakash J, Gupta SK, Singh N, Kochupillai V, Gupta YR. Antiproliferative and chemopreventive activity of *Ocimum sanctum* Linn. *Int J Med Biol Environ* 1999; 27: 165-72.
36. Uma Devi P. Radioprotective, anticarcinogenic and antioxidant properties of the Indian holy basil, *Ocimum sanctum* (tulsi). *Indian J Exp Biol* 2001; 39:185-90.
37. Kumar S, Suresh PK, Vijayababu MR, Arunkumar A, Arunakaran J. Anticancer effects of ethanolic neem leaf extract on prostate cancer cell line (PC-3). *J Ethnopharmacol*, 2005; 105:246-50.
38. Cao Y, Cao R. Angiogenesis inhibited by drinking tea. *Nature* 1999; 398:381.
39. Chandra Mohan KVP, Devaraj H, Prathiba D, Hara Y, Nagini S. Antiproliferative and apoptosis inducing effect of lactoferrin and black tea polyphenol combination on hamster buccal pouch carcinogenesis. *Biochim Biophys Acta* 2006; 1760:1536-44.
40. Skommer J, Wlodkowic D, Deptala A. Larger than life: Mitochondria and the Bcl-2 family. *Leuk Res* 2007; 31: 277-86.
41. Thatte U, Bagadey S, Dahanukar S. Modulation of programmed cell death by medicinal plants. *Cell Mol Biol* 2000; 46:199-214.

**I'M ONLINE. NOW.**  
LET'S TALK BOOKS.

eBooks on ScienceDirect offers users **24/7** remote access

There are over **4,800** eBooks on ScienceDirect

In addition to eBooks, ScienceDirect offers reference works, handbooks and book series online

Select eBook titles to be included in your institution's collection today!  
**www.elsevierbookselection.com**

*Get the broader perspective you need to make your research more productive with books on ScienceDirect.*  
<http://www.info.sciencedirect.com/content/books/eBooks/>

**ScienceDirect**  
makes sense.