Proliferation, angiogenesis and apoptosis-associated proteins are molecular targets for chemoprevention of MNNG-induced gastric carcinogenesis by ethanolic *Ocimum sanctum* leaf extract

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ABSTRACT

Introduction: This study was designed to evaluate the chemopreventive effects of ethanolic *Ocimum sanctum* (OS) leaf extract on cell proliferation, apoptosis and angiogenesis during N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced gastric carcinogenesis.

Methods: The rats were divided into four groups of ten each. Rats in group one were given MNNG (150 mg/kg body weight) by intragastric intubation three times, with a two-week interval between treatments. Rats in group two were administered MNNG as in group one, and in addition, they received intragastric intubation of ethanolic OS extract (300 mg/kg body weight) three times per week, starting on the day following the first exposure to MNNG. The intubation of ethanolic OS extract continued until the end of the experimental period. Rats in group three were given ethanolic OS leaf extract only. Group four served as controls. All the rats were killed after an experimental period of 26 weeks.

Results: Intragastric administration of MNNG-induced well-differentiated squamous cell carcinomas that showed increased cell proliferation, and angiogenesis with evasion of apoptosis, as revealed by the upregulation of proliferating cell nuclear antigen (PCNA), glutathione S-transferase-pi (GST-pi), Bcl-2, cytokeratin (CK) and vascular endothelial growth factor (VEGF) and with downregulation of Bax, cytochrome C and caspase 3 protein expression. Administration of ethanolic OS leaf extract reduced the incidence of MNNG-induced gastric carcinomas. This was accompanied by decreased expression of PCNA, GST-pi, Bcl-2, CK and VEGF, and overexpression of Bax, cytochrome C, and caspase 3.

Conclusion: This study provides evidence that, in MNNG-induced gastric carcinogenesis, the key proteins involved in the proliferation, invasion, angiogenesis and apoptosis, are viable molecular targets for chemoprevention using ethanolic OS leaf extract.

Keywords: apoptosis, cell proliferation, chemoprevention, gastric cancer, N-methyl-N'-nitro-N-nitrosoguanidine, *Ocimum sanctum*

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INTRODUCTION

Gastric cancer, the second most common cause of cancer death worldwide, is a major cause of mortality in Chennai, India. The development of stomach cancer is associated with sustained genetic mutations that leads to excessive cell proliferation, dysregulation of cellular differentiation, evasion of apoptosis, as well as sustained angiogenesis.[1] Proteins that play a major role in each of these processes have therefore assumed significance as surrogate endpoint biomarkers (SEBs) for chemoprevention.

Chemoprevention is a promising approach to primary gastric cancer prevention. However, it is essential to screen the chemopreventive efficacy of putative agents in experimental animal tumour models before embarking on clinical trials.[2] Gastric tumours

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induced by the administration of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in Wistar rats are ideal for the analysis of stomach cancer development and the effect of chemoprevention. MNNG-induced gastric tumours in Wistar rats simulate many of the features observed in human stomach cancer.

The major risk factors associated with human stomach cancer, such as high salt and *Helicobacter pylori* infection, were found to promote MNNG-induced gastric carcinogenesis.[3] Overexpression of Bcl-2 and mutations in the tumour suppressor gene p53 have been documented in humans, as well as in MNNG-induced gastric cancer.[4] Like human stomach tumours, MNNG-induced rat stomach carcinomas also exhibit infiltrative capacity and loss of differentiation.[5] The similarities between human stomach cancer and MNNG-induced gastric tumours provided the basis for analysing the effect of putative chemopreventive agents in the MNNG model. Previous studies from this laboratory have demonstrated the chemopreventive potential of several dietary agents and medicinal plants using the MNNG model.[6,7]

Of late, medicinal plants rich in antioxidant phytochemicals are being explored for chemopreventive potential. *Ocimum sanctum* Linn. (OS) commonly known as holy basil, has attracted the focus of attention owing to its medicinal properties. All parts of OS, namely, leaves, flowers, stem, roots, seeds, and even the whole plant, have been used in traditional medicine. In particular, the medicinal properties of the OS leaf have generated the most interest. OS leaf extracts have been reported to possess immunomodulatory, antiulcer, anti-inflammatory and anticarcinogenic properties.[8]

Recently, we demonstrated the protective effects of OS leaf extract against 7,12-dimethylbenz[a]anthracene (DMBA)-induced genotoxicity, oxidative stress and imbalances in xenobiotic-metabolising enzymes.[9] Several studies have documented the inhibitory effects of OS leaf extracts against chemically-induced tumours in experimental animals.[10,11] However, data concerning the chemopreventive potential and molecular mechanisms of ethanolic OS leaf extract on MNNG-induced gastric carcinogenesis have not been documented. The protective effects of OS against gastric ulcer, a major risk factor for stomach cancer, suggest efficacious potential in chemoprevention of gastric cancer. The present study was designed to evaluate the chemopreventive potential of ethanolic OS leaf extract against MNNG-induced gastric carcinogenesis, by immunolocalisation of key proteins involved in proliferation (proliferating cell nuclear antigen, PCNA; glutathione S-transferase-pi, GST-pi), infiltration (Cytokeratin, CK), angiogenesis (vascular endothelial growth factor; VEGF) and apoptosis (Bcl-2, Bax, cytochrome C and caspase 3).

METHODS

MNNG was obtained from Fluka-Chemika-Biochemika, Buchs, Switzerland. All other reagents used were of analytical grade. Male Wistar rats aged 6–8 weeks, weighing 80–100g, 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 standard pellet diet (Mysore Snack Feed Ltd, Mysore, India) and water *ad libitum*. The rats were maintained in accordance with the guidelines of the Indian Council of Medical Research, India.

Fresh, matured leaves of OS collected locally during October–December were identified by a pharmacognosy expert. These leaves were dried in the shade and reduced to powder form. The powders were then used for the extraction. Voucher specimens were deposited at the herbarium of the Botany Department, Annamalai University. Air-dried powder (1 kg) of OS leaves was mixed with 3 L of 70% ethamol and kept at room temperature for 36 hours. 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 40°C) and finally dried in a vaccum dessicator. The residue collected (yield 38 g/kg of OS 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 30 mg/ml and used for the experiment.

The rats were randomised into experimental and control groups and divided into four groups of ten rats each. Rats in group one were given MNNG (150 mg/kg body weight) by intragastric intubation three times with a gap of two weeks in between the treatments.[12] Rats in group two were administered MNNG as in group one, and in addition, received intragastric intubation of ethanolic OS extract (300 mg/kg body weight) three times per week starting on the day following the first exposure to MNNG. The administration of OS extract subsequently continued until the end of the experimental period. The dose administered in the present study (300 mg/kg body weight) was based on a previous report on the protective effects of OS on DMBA-induced genotoxicity and carcinogenicity.[9] Group three rats were given ethanolic OS leaf extract alone as in group two but without MNNG. Group four received basal diet and tap water throughout the experiment and served as the untreated control. The experiment was terminated at 26 weeks and all the rats were sacrificed by cervical dislocation after an overnight fast. Tissues were fixed in 10% formalin, embedded in paraffin and mounted on

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

Group	Treatment	PCNA[a]	GST-pi[b]				Bcl-2[b]				Bax[b]				Cytochrome C[b]				Caspase[b]				Cytokeratin[c]			VEGF[b]			
			0	I	2	3	0	I	2	3	0	I	2	3	0	I	2	3	0	I	2	3	0	I	II	0	I	2	3
1.	MNNG	77.20±3.48[e]	0♠	1	1	8♠	0♠	1	2	7♠	9♠	1	0	0	8♠	2	0	0	9	1	0	0	1	1♠	8♠	0	0	3♠	7♠
2.	MNNG+OS	62.97±3.10[f]	5*	3	2	0*	4*	2	4	0*	0*	1	1	8*	0*	1	7*	2	1*	2	2	5*	2	7*	1*	1	7*	2	0*
3.	OS	42.50±3.62	7	3	0	0	8	2	0	0	5	5	0	0	6	4	0	0	4	6	0	0	0	9	1	4	3	3	0
4.	Control	43.10±3.95	8	2	0	0	8	2	0	0	6	4	0	0	5	5	0	0	5	5	0	0	0	9	1	1	2	7	0

[a] Labelling index for PCNA, expressed as the number of cells with positive staining per 100 counted cells.

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

[c] 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.

[e] Significantly different from group 4 ($p < 0.001$) ANOVA followed by LSD.

[f] Significantly different from group 1 ($p < 0.001$) ANOVA followed by LSD.

♠ Significantly different from group 4 by χ^2 test ($p < 0.05$).

* Significantly different from group 1 by χ^2 test ($p < 0.05$).

polylysine-coated glass slides. The sections were used for immunohistochemical staining.

The tissue sections were deparaffinised by heat at 60°C for ten 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 five 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_2O_2 in distilled water for 15 minutes to inhibit endogenous peroxidase activity. Nonspecific antibody binding was reduced by incubating the sections with normal goat serum for 20 minutes. The sections were then incubated with mouse monoclonal antibodies PCNA, CK AE1/AE3, Bcl-2 (Dako, Carprinteria, CA, USA), caspase 3, cytochrome C (NeoMarkers, CA, USA), VEGF (Santa Cruz Biotechnology, CA, USA), rabbit polyclonal antibodies GST-pi (BioGenex, San Ramon, CA, 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, Carprinteria, CA, USA) followed by streptavidin-biotin-peroxidase for 30 minutes each at room temperature.

The immunoprecipitate was visualised by treating the sections with 3,3'-diaminobenzidine (Dako, Carprinteria, CA, USA) and counterstaining with haematoxylin. For negative controls, the primary antibody was replaced with TBS. Positive controls for each antibody was also processed simultaneously. The labelling indices for PCNA were calculated as the number of cells with positive staining per 100 counted cells in three high power fields. The expression of GST-pi, 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, II = positive staining throughout the basal and/or suprabasal region.

DEVD-specific caspase 3 activity was assayed using 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 a 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 substrate is calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined pNA solutions.

The tumour incidence and grading of GST-pi, Bcl-2, Bax, cytochrome C, caspase 3, CK and VEGF were statistically compared using the χ^2 test. Statistical analysis on the data for tumour burden was carried out using the Student's *t* test. PCNA labelling index and data for colorimetric assay of caspase 3 were analysed using ANOVA followed by LSD. The results were considered

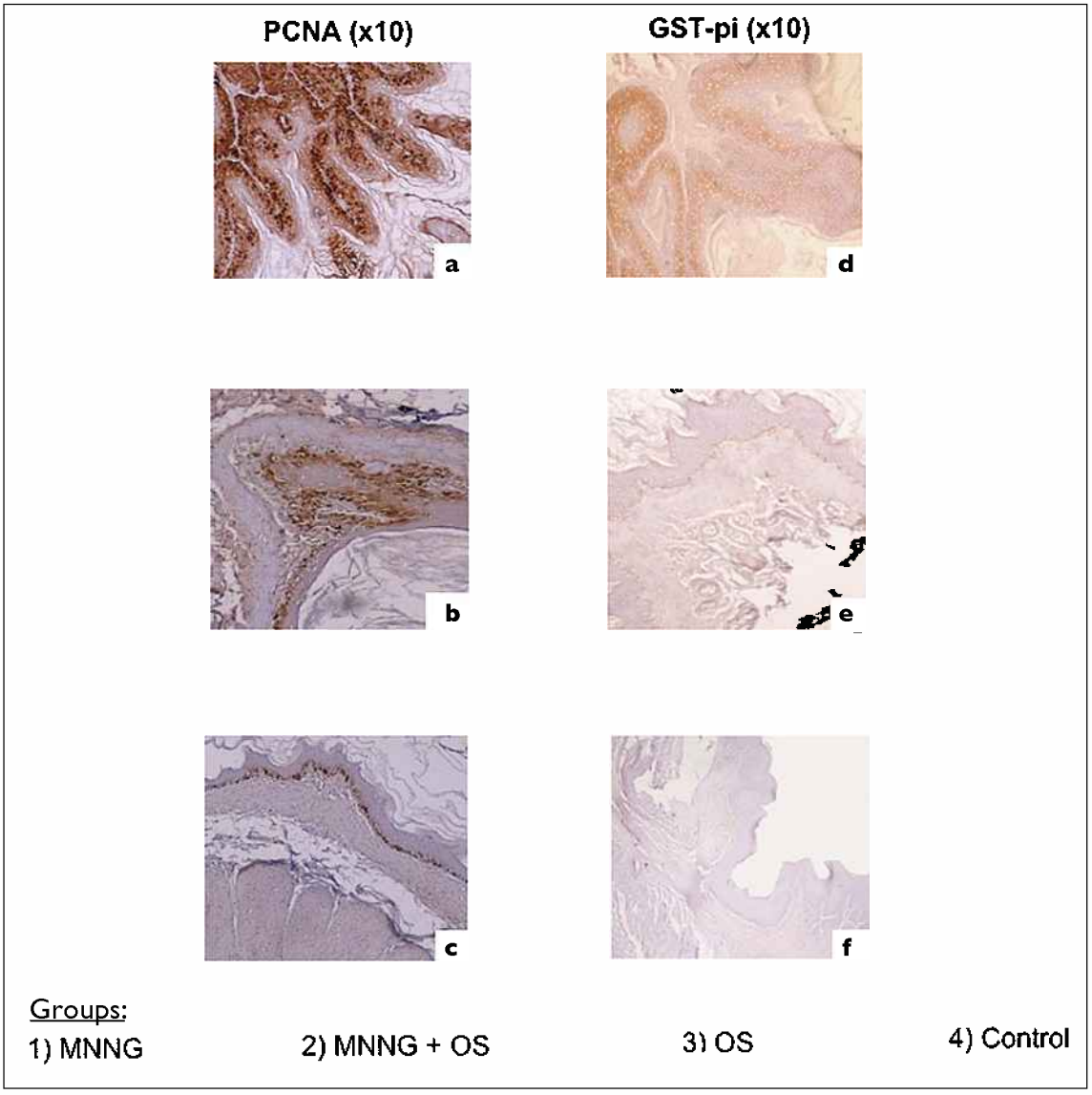

Fig. 1 Representative photomicrographs of immunohistochemical staining of PCNA and GST-pi expression in experimental and control rats (Mean ± SD; n = 10) (Avidin-biotin peroxidase method, Haematoxylin counter stain).
(a) Overexpression of PCNA in group 1 rats (× 10)
(b) Decreased expression of PCNA in group 2 rats (× 10)
(c) PCNA expression in rats administered OS alone and control rats: Groups 3 and 4 (× 10)
(d) Overexpression of GST-pi in group 1 rats (× 10)
(e) Decreased expression of GST-pi in group 2 rats (× 10)
(f) GST-pi expression in rats administered OS alone and control rats: Groups 3 and 4 (× 10)

statistically significant if the p-value was < 0.05.

RESULTS

In rats administered MNNG alone (group one), the incidence of gastric tumours was 100% with a mean tumour burden of 124.4 mm^3. No tumours were observed in groups two to four. Table I shows the effect of ethanolic OS leaf extract on expression of proliferation, infiltration, angiogenesis and apoptosis markers in the stomach of the experimental and control rats. In MNNG-treated rats (group one), the expression of PCNA, CK, Bcl-2 and VEGF was significantly higher and that of GST-pi, Bax, cytochrome C and caspase 3 was significantly lower than in control rats (group four). Administration of ethanolic OS leaf extract significantly decreased CK, PCNA, Bcl-2 and VEGF expression, and significantly increased the expression of GST-pi, Bax, cytochrome C and caspase 3 compared to group one. No significant changes in protein expression were observed in group three rats. While immunostaining of PCNA showed nuclear localisation, the remaining proteins were found in the

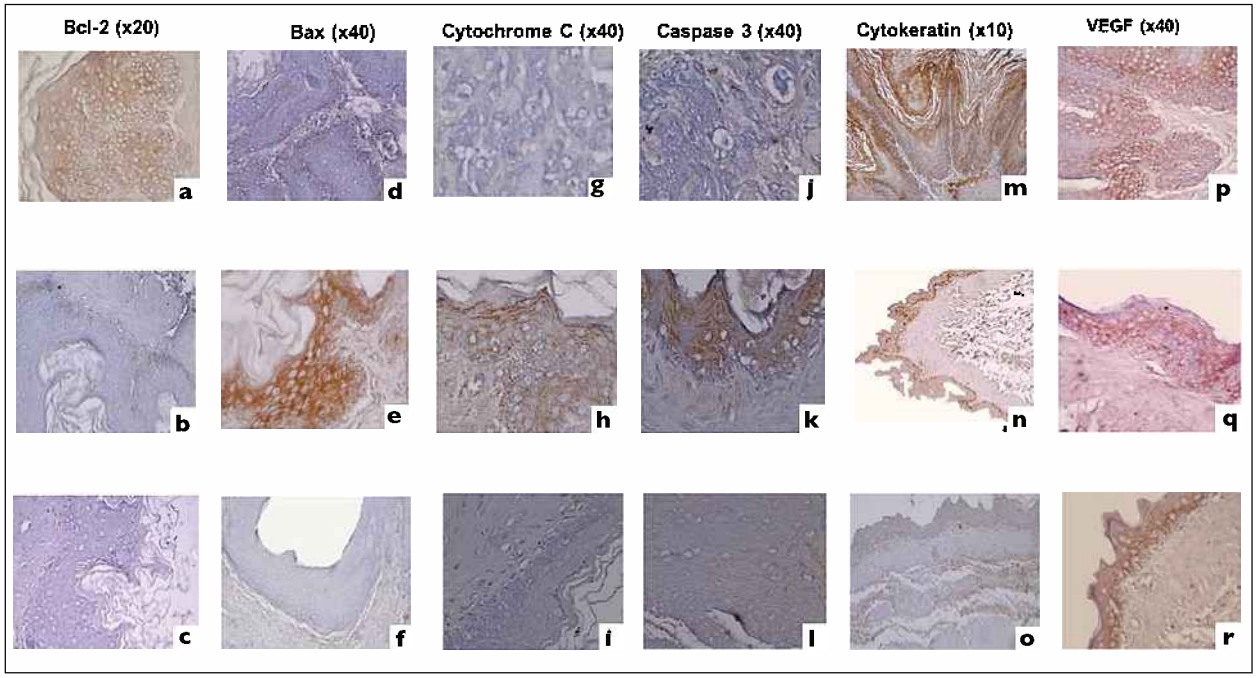

Fig. 2 Representative photomicrographs of immunohistochemical staining of Bcl-2, Bax, cytochrome C, caspase 3, CK and VEGF expression in experimental and control rats (Mean ± SD) (Avidin-biotin peroxidase method, Haematoxylin counter stain).
(a) Overexpression of Bcl-2 in group 1 rats (× 20)
(b) Decreased expression of Bcl-2 in group 2 rats (× 20)
(c) Bcl-2 expression in rats administered OS alone and control rats: Groups 3 and 4 (× 20)
(d) Decreased expression of Bax in group 1 rats (× 40)
(e) Overexpression of Bax in group 2 rats (× 40)
(f) Bax expression in rats administered OS alone and control rats: Groups 3 and 4 (× 40)
(g) Decreased expression of cytochrome C in group 1 rats (× 40)
(h) Overexpression of cytochrome C in group 2 rats (× 40)
(i) Cytochrome C expression in rats administered OS alone and control rats: Groups 3 and 4. (× 40)
(j) Decreased expression of caspase 3 in group 1 rats (× 40)
(k) Overexpression of caspase 3 in group 2 rats (× 40)
(l) Caspase 3 expression in rats administered OS alone and control rats: Groups 3 and 4 (× 40)
(m) Overexpression of CK in group 1 rats (× 10)
(n) Decreased expression of CK in group 2 rats (× 10)
(o) CK expression in rats administered OS alone and control rats: Groups 3 and 4 (× 10)
(p) Overexpression of VEGF in group 1 rats (× 40)
(q) Decreased expression of VEGF in group 2 rats (× 40)
(r) VEGF expression in rats administered OS alone and control rats: Groups 3 and 4 (× 40)

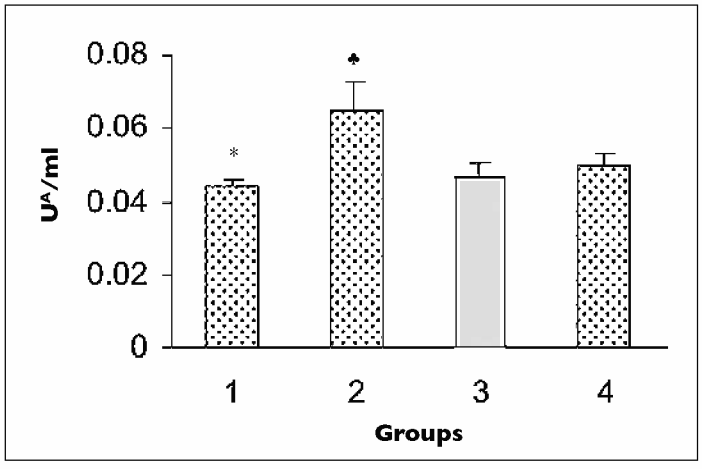

Fig. 3 Activity of caspase 3 in the stomach tissue of experimental and control rats (Mean ± SD; n = 10).
* Significantly different from group 4 ($p < 0.01$) ANOVA followed by LSD
♣ Significantly different from group 1 ($p < 0.001$)
A μmoles of pNA formed/min
Groups 1: MMNG; 2: MMNG + OS; 3: OS; 4: Control.

cytoplasmic region. Representative photomicrographs of immunostaining are shown in Figs. 1 and 2.

Fig. 3 shows the activity of DEVD-specific caspase 3 in the stomach tissue of experimental and control rats. In MNNG-treated rats (group one), caspase 3 activities were significantly reduced as compared with the control rats (group four). Treatment with ethanolic OS leaf extract significantly increased enzyme activity in group two rats as compared with group one. In rats administered ethanolic OS leaf extract alone (group three), the activity of caspase 3 was not significantly different from that in the controls.

DISCUSSION

MNNG, a nitroso compound and a potent mutagen, has been widely used as a gastric carcinogen. MNNG is known to induce erosions of the gastric mucosa, an initial precancerous change that may play a significant role in the initiation of forestomach carcinogenesis.[13] Saito et al demonstrated that MNNG induces gastric ulcers at the initial stage, followed by tumours at later stages in beagle dogs.[14] In the present study, MNNG induced well-differentiated squamous cell carcinomas with high tumour burden. These tumours displayed increased cell proliferation, infiltrative and angiogenic potential, coupled with apoptosis evasion, as revealed by the upregulation of PCNA, GST-pi, Bcl-2, CK and VEGF and corresponding downregulation of Bax, cytochrome C and caspase 3. PCNA, a pleiotropic protein expressed in the nuclei of proliferating cells, regulates several cellular processes including DNA replication, repair and cell proliferation, while GST-pi confers a proliferative advantage on tumours by protecting against cytotoxic free radicals.[15-17] Both PCNA and GST-pi also inhibit apoptosis by interacting with proapoptotic proteins. The shift in the balance of antiapoptotic Bcl-2 and proapoptotic Bax, as evidenced by an 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. Furthermore, these tumours exhibited enhanced expression of CK and VEGF, which are reliable indicators of invasion and angiogenesis respectively. Increased cell proliferation, together with apoptosis evasion, infiltration and neovascularisation may confer a selective growth advantage on MNNG-induced gastric tumours.

Administration of ethanolic OS leaf extract exerted significant chemopreventive effects against MNNG-induced stomach tumours. Gastric ulcer, a major risk factor for stomach cancer, has been reported to predispose rats exposed to non-carcinogenic doses of MNNG to carcinoma of the stomach.[18] Both methanolic and ethanolic extracts of OS have been documented to inhibit gastric ulcers.[19,20] The protective effects of ethanolic OS leaf extract against gastric ulcers may suggest efficacious potential in the chemoprevention of gastric cancer, seen in the present study. The results of the present study also substantiate the anticarcinogenic activities of OS preparations previously reported by other workers. OS has been reported to inhibit benzo(a)pyrene induced stomach tumours and 3'-methyl-4-dimethyl aminobenzene-induced hepatomas in rats.[11] Karthikeyan et al demonstrated that oral administration, as well as topical application, of aqueous and ethanolic OS leaf extract delayed the incidence of DMBA-induced HBP carcinomas.[10]

To our knowledge, this is the first report to demonstrate the effects of OS leaf extract on markers of cell proliferation, invasion, angiogenesis and apoptosis. An interesting finding in the present study is the differential sensitivities of gastric carcinoma and normal stomach tissue to growth control and apoptosis induction by ethanolic OS leaf extract. Administration of ethanolic OS leaf extract selectively induced apoptosis in MNNG-treated rats but not in normal rats. Our results support the hypothesis that natural products, and their constituent phytochemicals that drive tumour cells to undergo apoptosis, but direct normal cells towards survival pathways, are ideal chemopreventives.[21]

The chemopreventive potential of ethanolic OS leaf extract may be ascribed to the presence of one or more constituent phytochemicals such as eugenol, ursolic acid, apigenin, luteolin and carvacrol. Eugenol, ursolic acid and carvacrol have been reported to inhibit cell proliferation in vitro.[22-24] Eugenol, ursolic acid and apigenin were found to induce apoptosis by influencing Bcl-2/Bax ratio and cytochrome C mediated caspase 3 activation.[25-27] Luteolin has been documented to inhibit the invasive potential of HepG2 cells.[28] Thus, OS contains a rich array of phytochemicals, each of which influences distinct molecular pathways of chemoprevention. Use of OS leaf extract is a strategic approach to administer a combination of bioactive phytochemical entities that could modulate aberrant signal transduction pathways in cancer. Furthermore, interactions among the phytochemicals may have synergistic effects that could reduce the toxicity of individual agents and enhance efficacy.

Traditionally, OS preparations have been used in different systems of medicine to treat a variety of illnesses.[8] However, it has become increasingly important to subject the OS leaf to the rigours of modern ethnopharmacological research. The results of the present study demonstrate that the OS is a promising candidate for cancer chemoprevention studies. Extensive investigations on the metabolism, tissue distribution,

synergistic pharmacodynamic interactions of individual phytochemicals, as well as the molecular mechanisms of chemoprevention, are warranted for the potential development of modern drugs from the OS leaf.

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