JPP Journal of Pharmacy and Pharmacology

JPP 2004, 56: 1291–1298

Received May 6, 2004
Accepted July 5, 2004
DOI 10.1211/0022357044373
ISSN 0022-3573

Modulatory effect of *Morus indica* against two-stage skin carcinogenesis in Swiss albino mice: possible mechanism by inhibiting aryl hydrocarbon hydroxylase

Lakshmi Prasad, Tajdar H. Khan, Anuradha Sehrawat and Sarwat Sultana

Abstract

The modulatory effect of the methanolic extract of *Morus indica* on 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) induced oxidative stress and 7,12-dimethylbenz(a)anthracene induced and croton oil (0.5% per mouse/0.2 mL acetone, v/v) promoted skin tumourigenesis in Swiss albino mice was studied. The efficacy of the *M. indica* extract was also evaluated in-vitro by studying the inhibition of the activity and level of aryl hydrocarbon hydroxylase, cytochrome P450, DNA sugar damage in calf thymus DNA and Fe^{++}/ascorbate-induced lipid peroxidation in microsomes of mice. Significant increases in the activity of antioxidant enzymes ($P < 0.001$) and a concomitant decrease ($P < 0.001$) in the cutaneous malondialdehyde level were observed at three doses of plant extract (2.5, 5.0 and 7.5 mg kg^{-1}). Application of *M. indica* 1 h before each application of croton oil showed inhibitory effects on tumour promotion in terms of a reduction in the number of tumours/mouse and percentage of mice with tumours. It was also accompanied by an extension of the tumour latency period. TPA, which resulted in a rapid and transient stimulation of mouse epidermal ornithine decarboxylase activity ($P < 0.001$), was inhibited dose dependently by pre-treatment with *M. indica* extract ($P < 0.001$). The results suggest that *M. indica* extract may be useful as a therapeutic agent for cancer control as it blocks or suppresses events associated with chemical carcinogenesis.

Introduction

Skin carcinogenesis involves three stages: initiation, promotion and progression. Initiation is a reversible stage in which changes occur in the genes controlling growth. The genetic machinery of the cell is damaged at this stage. The second stage involves exposure to promoting agents and deals with the increased cell proliferation (Chatterjee et al 1999). During the progression stage, there is a loss of growth control and an evasion from host defence mechanisms (Sun 1990). There is substantial evidence that free radicals, particularly oxygen radicals, play an important role in the complex course of multi-step carcinogenesis. The involvement of reactive oxygen species has been implicated in the promotion stage of carcinogenesis. Reactive oxygen species produce oxidative stress in the tissue and provide a mitogenic stimulus leading to cellular proliferation (Troll & Wiesner 1985). These reactive oxygen species, generated as a result of exposure to pesticides, drugs, tobacco or other pollutants, pose a serious threat to cells and are probably responsible for cellular damage, tissue damage, protein and DNA modification, leading to carcinogenesis (Chance et al 1979) and many other diseases (Cross et al 1987). The DNA damage caused by free radicals is in the form of base damage, single-strand breaks, double-strand breaks, cross-linking between DNA and chromosomal aberrations. Some of the drug metabolizing enzymes, for example cytochrome P450 and aryl hydrocarbon hydroxylase that are represented in an inducible form, are used for the identification of protective compounds.

The antioxidant effects of fruit, vegetables, beverages and some herbs play a crucial role in the aetiology of human cancer. Cancer may be prevented by a surprisingly large number of chemical compounds. This strategy for the prevention of cancer through the use of naturally occurring or synthetic chemical agents is known as chemoprevention. Chemopreventive agents function towards DNA reactive carcinogens through the

Section of Chemoprevention and Nutrition Toxicology, Department of Medical Elementology and Toxicology, Jamia Hamdard (Hamdard University), Hamdard Nagar, New Delhi 110062, India

Lakshmi Prasad, Tajdar H. Khan, Anuradha Sehrawat, Sarwat Sultana

Correspondence: Sarwat Sultana, Department of Medical Elementology and Toxicology, Faculty of Science, Jamia Hamdard (Hamdard University), Hamdard Nagar, New Delhi 110062, India. E-mail: sarwat786@rediffmail.com

Funding: Sarwat Sultana is grateful to the Indian Council of Medical Research, New Delhi, for providing funds to carry out this research.

inactivation of parent compounds or their metabolites by direct binding, by the inhibition of enzymatic activation and by inducing detoxifying (phase 2) enzymes (Stoner et al 1977).

Morus indica Linn. (Moracea), commonly known as white mulberry, is valued for its foliage and constitutes the main food for mulberry silk worms (*Bombyx mori* Linn.). The plant is eaten as is or made into preserves or syrup. It is often used to treat diarrhoea, ulcerated intestines, small pox, lumbago, as a gargle in inflammation and thickening of vocal cords, and it also heals cracks in the soles of feet. Chatterjee et al (1983) reported that the extract of *M. indica* has an anti-inflammatory effect in exudative, proliferative and chronic phases of inflammation, and it also possesses antipyretic and anti-hyperglycaemic action. The phytochemical investigation of *M. indica* shows the presence of the isoprenoid flavones, morusinol and isoquercitrin, which possess antioxidant and anti-inflammatory activity. The anti-inflammatory response of isoquercitrin has been studied in rats with carrageenan-induced inflammation (Morikawa et al 2003). Three different phytoalexins, moracin C, moracin N and chalcomoracin, which possess free radical scavenging activity, are also reported to be present in the mulberry leaves (Sharma et al 2001).

We hypothesized that *M. indica* may suppress the cutaneous oxidative stress and toxicity of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), which promotes tumourigenesis through the elaboration of oxidative stress. It stimulates the production of reactive oxygen metabolites such as $O_2^{-}\cdot$, H_2O_2 and $OH\cdot$ in the inflammatory cells, most notably in phagocytes and neutrophils (Weiss & Lobuglio 1982; Cerutti 1985; Kensler & Taffe 1986). The treatment of TPA has been reported to induce a variety of changes in murine skin, including dark basal keratinocytes and sustained epidermal hyperplasia, reactive oxygen species formation in epidermis, elevated epidermal cycloxygenase, lipoxygenase activities and elevated epidermal ornithine decarboxylase (ODC) activity leading to increase in polyamine biosynthesis (Katiyar et al 1996).

In the present study, we studied the modulatory effect of the methanolic extract of *Morus indica* on TPA-induced oxidative stress and 7,12-dimethylbenz(a)anthracene (DMBA) induced and croton oil promoted skin tumourigenesis in mice. The efficacy of the *M. indica* extract was also evaluated in-vitro by studying the inhibition of the activity and level of aryl hydrocarbon hydroxylase, cytochrome P450, DNA sugar damage in calf thymus DNA and Fe^{++}/ascorbate-induced lipid peroxidation in microsomes of mice.

Materials and Methods

Chemicals

Reduced glutathione (GSH), oxidized glutathione, nicotinamide adenine dinucleotide phosphate reduced (NADPH), bovine serum albumin, 1,2-dithio-bis-nitrobenzoic acid (DTNB), 1-chloro-2, 4-dinitro benzene (CDNB), phenylmethyl sulfonylfluoride (PMS), pyridoxal-phosphate, DMBA, croton oil, calf thymus DNA and TPA were all obtained from Sigma Chemical Co (St Louis, MO). DL-[^{14}C]ornithine was purchased from Amersham Corporation (Little Chalfont, UK). All other chemicals were of the highest purity commercially available.

Plant material

Morus indica was collected from the herbal garden of Jamia Hamdard, New Delhi. Professor Mohammed Iqbal (Medicinal Plant Division, Department of Environmental Botany, Hamdard University) verified the identity of the plant material. Freshly collected plant material was chopped, shade-dried and coarsely powdered to a mesh size of 1 mm as described by Antonio & Souza Britto (1998).

Preparation of extract

The extraction procedure was as described by Didry et al (1998). Powdered plant material was repeatedly extracted in a 4000-mL round-bottomed flask with 2000 mL solvents of increasing polarity starting with petroleum ether, benzene, ethyl acetate, acetone, methanol and double-distilled water. The reflux time for each solvent was 4 h. The methanolic extract was cooled at room temperature, filtered and evaporated to dryness under reduced pressure in a rotatory evaporator (Buchi Rotavapor, Flawil, Switzerland). The condensed fraction obtained was used for further study after preliminary in-vitro tests (lipid peroxidation, DNA sugar damage, cytochrome P450 and aryl hydrocarbon hydroxylase). The other fractions showed no efficacy in the in-vitro assessment and were therefore rejected.

Animals

Adult female Swiss albino mice (8 weeks old, 20–25 g), obtained from the Central Animal House Facility of Hamdard University, were housed 10 per cage, and food and water were available *ad libitum*. The dorsal skin of each mouse was shaved with electrical clippers followed by the application of hair removing cream (Anne French; Geoffrey Manners & Co. Ltd, Bombay, India) at least 2 days before the treatment. Only mice that did not show signs of hair re-growth were used.

Treatment regimen

To study the effect of pre-treatment of mice with *M. indica* on TPA mediated cutaneous oxidative stress, 36 male mice were randomly allocated to six groups of six mice each. Group 1 mice served as a control and received a topical application of acetone (0.2 mL per mouse). Group 2 were treated with TPA (20 nmol per mouse/0.2 mL acetone) only. Groups 3, 4 and 5 received topical applications of *M. indica* at doses of 2.5, 5.0 and 7.5 mg kg^{-1} in 0.2 mL acetone for 3 consecutive days. Group 4 received only topical application of *M. indica* at a higher dose of 7.5 mg kg^{-1} in 0.2 mL acetone. At 1 h after *M. indica* treatment, the mice in groups

3, 4 and 5 received a single topical application of TPA (20 nmol per mouse/0.2 mL acetone). At 12 h after TPA treatment, all these mice were killed by cervical dislocation, their skins were quickly removed and processed for subcellular fractionation. For all biochemical estimations, postmitochondrial supernatant, cytosol or microsomes were used.

To study the effect of *M. indica* on TPA mediated induction of cutaneous ODC, the groups were formed exactly as described for oxidative stress. At 1 h after *M. indica* treatment, the mice in groups 2, 3, 4 and 5 received topical application of TPA. In the case of ODC activity, the mice were killed 6 h after TPA treatment and were then further processed for the subcellular fractionation.

Experiments were conducted according to the two-stage initiation promotion protocol of tumourigenesis for tumour studies. Only mice in the resting phase of the hair cycle were used and they were divided into four groups of 20 mice each. Initiation was achieved with a single topical application of 40 μg DMBA per mouse/0.2 mL acetone. At 10 days after initiation, all of the mice were treated twice weekly with a topical application of croton oil (phorbol ester) (0.5% per mouse/0.2 mL acetone, v/v) for 25 weeks. *M. indica* extracts at a dose of 2.5, 5.0 and 7.5 mg kg^{-1} were topically applied before each TPA treatment. Mice in all the groups were observed for any apparent signs of toxicity and mortality during the entire period of study. The data are expressed as the percentage of mice with tumours and the number of tumours per mouse and are plotted as a function of weeks on test.

Tissue preparation

After the desired time period, control and treated mice were killed by cervical dislocation. For biochemical studies, a known amount of tissue was minced and homogenized in a polytron homogenizer (Kinematica A.G., Littau, Switzerland) and subjected to subcellular fractionation to obtain postmitochondrial supernatant (PMS) and microsomes for biochemical estimations.

Biochemical estimations for in-vitro study

Estimation of lipid peroxidation

To assess the potential of *M. indica* to inhibit lipid peroxide formation by quenching ·OH radicals in an in-vitro study, three different concentrations of plant extract were selected. The whole assay system was done according to the method of Wright et al (1981). Group 1 served as a control in which the reaction mixture consisted of 0.58 mL phosphate buffer (0.1 M, pH 7.4), 0.2 mL microsomes, 0.2 mL ascorbic acid (100 mM) and 0.02 mL ferric chloride (100 mM). This reaction mixture was incubated at 37°C in a shaking water bath for 1 h. The reaction was stopped by the addition of 1 mL of TCA (10%). Following addition of 1.0 mL thiobarbituric acid (0.67 %), all the tubes were placed in a boiling water bath for 20 min. The tubes were shifted to an ice bath and then centrifuged at 2500 *g* for 10 min. In groups 2, 3 and 4, in addition to the complete control reaction mixture, different concentrations of plant extract were also added (30, 40 and 50 μg, respectively). The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm. The results were expressed as nmol MDA formed h^{-1} (g tissue)$^{-1}$ at 37°C by using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Assay of DNA sugar damage

Hydroxyl radical scavenging was measured by studying the competition between deoxyribose and the test compound (*M. indica* extract) for hydroxyl radicals generated from the Fe^{3+}/ascorbate radical generating system. The DNA sugar damage was assayed as follows. The reaction mixture consisted of 0.5 mL calf thymus DNA (1 mg mL^{-1} of 0.15 M NaCl), 0.5 mL phosphate buffer (0.1 M, pH 7.4) and 0.05 mL of $FeCl_3$ (100 μM in final concentration). It was incubated for 1 h at 37°C in a shaking water bath. After incubation, 1 mL thiobarbituric acid (0.67%) was added to the reaction mixture and then it was kept in a boiling water bath for 25 min. The thiobarbituric acid reacting species generated form an adduct showing a characteristic absorption at 535 nm, which was monitored using a spectrophotometer (Model-21 D Milton Roy, Rochester, NY).

Determination of cytochrome P450 content

The cytochrome P450 content was determined by the method of Omura & Sato (1964). Briefly, a pinch of sodium dithionate was added to a reaction mixture containing 0.2 mL microsomes and 0.8 mL of phosphate buffer. This was then divided equally between two matched cuvettes. The contents of the test cuvette were gently bubbled with carbon monoxide for about 1 min and then the optical density was simultaneously taken at 450 nm and 490 nm. In groups 2, 3 and 4, in addition to the above reaction mixture, 30, 40 and 50 μg of plant extract was added.

Aryl hydrocarbon hydroxylase

The activity of aryl hydrocarbon hydroxylase was determined by slight modification of the fluorimetric method described by Nebert & Gelbion (1968). Group 1 served as a control in which the incubation mixture consisted of 250 μL phosphate buffer (0.1 M, pH 7.4), 70 μL NADPH (71 mM), 30 μL $MgCl_2$ (0.1 M), 100 μL PMS, and 550 μL d-water in a total volume of 1 mL. The reaction was initiated by adding 50 μL benzo(a)pyrene (2 mM) and the mixture was then incubated at 37°C in a shaking water bath for 1 h. The reaction was stopped by the addition of 1 mL cold acetone and 3 mL hexane. The reaction mixture was vortexed thoroughly and centrifuged at 3000 *g* for 5 min. Aliquots (2 mL) of the organic phase were transferred to a fresh tube containing 2 mL NaOH (1 M). Aliquots of the aqueous alkaline phase were determined by the fluorescence of the sample in a fluorimeter at an excitation wavelength of 520 nm and emission wavelength of 520 nm. Groups 2, 3 and 4, in which plant material (30, 40 and 50 μg) was added, were similarly treated. Data was expressed in terms of 3-OH-benzo(a)pyrene min^{-1} (mg protein)$^{-1}$.

Biochemical estimations for in-vivo study

Estimation of GSH
The GSH in skin was determined by the method of Jollow et al (1974). A 1.0-mL volume of PMS (10%) was mixed with 1.0 mL sulfosalicylic acid (4%). The samples were incubated at 4°C for at least 1 h and then centrifuged at 1200 *g* for 15 min at 4°C. The reaction mixture contained 0.4 mL of the filtered sample, 2.2 mL phosphate buffer (0.1 M, pH 7.4) and 0.4 mL DTNB (4 mg mL) in a total volume of 3.0 mL. The yellow colour developed was read immediately at 412 nm on a spectrophotometer (Milton Roy Model-21 D). The GSH concentration was calculated as nmol GSH (g tissue)$^{-1}$.

Assay for glutathione S-transferase (GST) activity
GST activity was assayed by the method of Habig et al (1974). The reaction mixture consisted of 2.5 mL phosphate buffer (0.1 M, pH 6.5), 0.2 mL GSH (1 mM), 0.2 mL CDNB (1 mM) and 0.1 mL of the cytosolic fraction (10%) in a total volume of 3.0 mL. The changes in absorbance were recorded at 340 nm, and enzymatic activity was calculated as nmol CDNB conjugate formed min^{-1} (mg protein)$^{-1}$ using a molar extinction coefficient of $9.6 \times 10^3\ \text{M}^{-1}\,\text{cm}^{-1}$.

Assay for glutathione reductase activity
Glutathione reductase activity was assayed by the method of Carlberg & Mannervick (1975). The reaction mixture consisted of 1.65 mL phosphate buffer (0.1 M, pH 7.6), 0.1 mL EDTA (0.5 mM), 0.05 mL oxidized glutathione (1 mM), 0.1 mL NADPH (0.1 mM) and 0.1 mL PMS (10%) in a total volume of 2.0 mL. Enzyme activity was quantitated at 25°C by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized min^{-1} (mg protein)$^{-1}$ using a molar extinction coefficient of $6.22 \times 10^3\ \text{M}^{-1}\,\text{cm}^{-1}$.

Assay for lipid peroxidation
Lipid peroxidation was determined by the method of Wright et al (1981) as described above.

Assay for catalase activity
Catalase activity was assayed by the method of Claiborne (1985). Briefly, the reaction mixture consisted of 2.0 mL phosphate buffer (0.1 M, pH 7.4), 0.95 mL hydrogen peroxide (0.019 mM) and 0.05 mL PMS (10%) in a final volume of 3.0 mL. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated as nmol H_2O_2 consumed min^{-1} (mg protein)$^{-1}$.

Assay for glutathione peroxidase activity
Glutathione peroxidase activity was measured by the method of Mohandas et al (1984). The reaction mixture consisted of 1.44 mL phosphate buffer (0.1 M, pH 7.4), 0.1 mL EDTA (1 mM), 0.1 mL sodium azide (1 mM), 0.05 mL glutathione reductase (1 IU mL^{-1}), 0.05 mL GSH (1 mM), 0.1 mL NADPH (0.2 mM), 0.01 mL H_2O_2 (0.25 mM) and 0.1 mL 10% PMS in a total volume of 2 mL. The disappearance of NADPH at 340 nm was recorded at 25°C. Enzyme activity was calculated as nmol NADPH oxidized min^{-1} (mg protein)$^{-1}$ using a molar extinction coefficient of $6.22 \times 10^3\ \text{M}^{-1}\,\text{cm}^{-1}$.

Assay for glucose 6-phosphate dehydrogenase activity
The activity of glucose 6-phosphate dehydrogenase was assayed by the modified method of Zaheer et al (1965). The reaction mixture consisted of 0.3 mL Tris-HCl buffer (0.05 M, pH 7.6), 0.1 mL NADP (0.1 mM), 0.1 mL glucose 6-phosphate (0.8 mM), 0.1 mL $MgCl_2$ (8 mM), 0.3 mL 10% PMS and 2.1 mL distilled water in a total volume of 3 mL. The changes in absorbance were recorded at 340 nm and enzyme activity was calculated as nmol NADPH oxidized min^{-1} (mg protein)$^{-1}$ using a molar extinction coefficient of $6.22 \times 10^3\ \text{M}^{-1}\,\text{cm}^{-1}$.

Quinone reductase activity
The quinone reductase activity was measured by the method of Benson et al (1980). The assay mixture consisted of 0.1 mL cytosolic fraction (10%), 0.7 mL of bovine serum albumin (0.1%), 0.02 mL Tween 20 (1%), 0.1 mL of FAD (150 μM), 0.02 mL of NADPH (0.2 mM), 0.05 mL of 2,6-dichloro-indophenol (0.29%) and 2 mL of Tris-HCl buffer (25 mM, pH 7.4) with a final volume of 3 mL. The optical density was read at 600 nm for 3 min. The enzyme activity was calculated as nmol 2,6-dichloro-indophenol reduced min^{-1} (mg protein)$^{-1}$.

Ornithine decarboxylase activity
ODC activity was determined using 0.4 ml cutaneous 105 000 *g* supernatant fraction per assay tube by measuring the release of CO_2 from DL-[1-^{14}C] ornithine by the method of O'Brien et al (1975). The skin was homogenized in Tris-HCl buffer (pH 7.5, 50 mM) containing EDTA (0.4 mM), pyridoxal phosphate (0.32 mM), PMSF (0.1 mM), 2-mercaptoethanol (1.0 mM), dithiothreitol (4.0 mM) and Tween 80 (0.1%) at 4°C using a polytron homogenizer (Kinematica AGPT 3000). In brief, the reaction mixture contained 400 µl enzymes and 0.095 ml co-factor mixture containing pyridoxal phosphate (0.32 mM), EDTA (0.4 mM), dithiothreitol (4.0 mM), ornithine (0.4 mM), Brij 35 (0.02%) and DL-[1-^{14}C] ornithine (0.05 µCi) in a total volume of 0.495 ml. After adding buffer and co-factor mixture to blank and other tubes, the tubes were closed immediately with a rubber stopper containing 0.2 ml ethanolamine and methoxyethanol mixture (2:1) in the central well and kept in a water-bath at 37°C. After 1 hour of incubation, the enzyme activity was arrested by injecting 1.0 ml citric acid solution (2.0 M) along the sides of the glass tubes and the solution was continued for one hour to ensure complete absorption of CO_2. Finally, the central well was transferred to a vial containing 2 ml ethanol and 10 ml toluene-based scintillation fluid. Radioactivity was counted in a liquid scintillation counter (LKB Wallace-1410). ODC activity was expressed as picomole CO_2 released h^{-1} (mg protein)$^{-1}$.

Protein estimation
The protein concentration in all samples was determined by the method of Lowry et al (1951).

Table 1 Effect of *Morus indica* extract on lipid peroxidation, DNA sugar damage, cytochrome P450 and aryl hydrocarbon hydroxylase in an in-vitro study

Group	Lipid peroxidation (nmol MDA formed (g tissue)$^{-1}$ h^{-1})	DNA sugar damage (nmol MDA formed (mg DNA)$^{-1}$)	Cytochrome P450 (nmol (mg protein)$^{-1}$)	Aryl hydrocarbon hydroxylase (μmol 3-OH benzo(a)pyrene min^{-1} (mg protein)$^{-1}$)
Control	2.59 ± 0.001	1.32 ± 0.01	4.92 ± 0.11	0.15 ± 0.001
Complete control + *M. indica* (30 μg)	2.44 ± 0.02***	1.19 ± 0.01***	3.53 ± 0.22***	0.0093 ± 0.001*
Complete control + *M. indica* (40 μg)	2.25 ± 0.05***	1.17 ± 0.01***	3.17 ± 0.26***	0.0068 ± 0.0009***
Complete control + *M. indica* (50 μg)	1.56 ± 0.21***	1.13 ± 0.01***	2.92 ± 0.32***	0.0049 ± 0.0021***

Each value represents the mean ± s.e. of five independent trials. *$P < 0.05$ and ***$P < 0.001$, significantly different compared with control.

Statistical analysis

The level of significance between different groups was based on analysis of variance followed by Dunnett's test.

Results

The central findings of our study were that *M. indica* suppressed TPA mediated cutaneous oxidative stress, DNA damage and membrane lipid peroxidation. It inhibited the tumour promoting effect of croton oil (phorbol ester) in mice initiated with DMBA, and reversed the induction in cutaneous ODC activity. It also helped in delaying the conversion of DMBA into its more electrophilic form by inhibiting the activity of aryl hydrocarbon hydroxylase and cytochrome P450.

The free radicals generated by the iron/ascorbate system were inhibited by the addition of plant extract at doses of 30, 40 and 50 μg in mice microsomes. Maximum inhibition of 39% was shown at a concentration of 50 μg plant extract. Addition of *M. indica* plant extract to deoxyribose assay also caused dose-dependent ($P < 0.001$) degradation of the sugar into a malondialdehyde-like compound, which formed a chromogen with thiobarbituric acid. A significant reduction in the level of cytochrome P450 activity of 29%, 35% and 59% was observed at the three different concentrations of plant extract. Maximum inhibition of 67% in aryl hydrocarbon hydroxylase activity was found in the case of 50 μg plant extract (Table 1).

The in-vivo induction of lipid peroxidation of 228% was attained by the pre-treatment of mice with TPA alone. Lipid peroxidation was significantly inhibited (195%, 154% and 124%) by prophylactic treatment of mice with *M. indica* at doses of 2.5, 5.0 and 7.5 mg kg^{-1}, respectively. Single topical application of TPA resulted in the depletion of cutaneous glutathione and a decrease in the activities of GST, quinone reductase, catalase, glutathione peroxidase and glucose 6-phosphate dehydrogenase to a level of 80%, 53%, 56%, 29%, 78% and 39%, respectively, of the acetone treated control group. This depletion in the level of glutathione and its metabolizing enzyme was recovered by pre-treatment of the mice with the plant extract ($P < 0.001$). The protection provided by *M. indica* was dose dependent (Tables 2 and 3).

Table 2 Effect of pre-treatment of mice with *Morus indica* extract on TPA mediated depletion of cutaneous glutathione and on the activity of antioxidant molecules

Treatment group	Glutathione (nmol GSH (g tissue)$^{-1}$)	Glutathione reductase (nmol NADPH oxidized min^{-1} (mg protein)$^{-1}$)	Glutathione peroxidase (nmol NADPH oxidized min^{-1} (mg protein)$^{-1}$)	Catalase (nmol H_2O_2 consumed min^{-1} (mg protein)$^{-1}$)
Acetone treated control	38.50 ± 0.26	76.88 ± 2.31	179.8 ± 1.71	37.5 ± 3.97
TPA (20 nmol/0.2 mL acetone/mouse)	$30.90 \pm 0.84^{\dagger\dagger}$	$48.21 \pm 2.24^{\dagger\dagger}$	$141.3 \pm 7.12^{\dagger\dagger}$	$10.7 \pm 1.83^{\dagger\dagger}$
M. indica (2.5 mg kg^{-1}) + TPA (20 nmol/0.2 mL acetone/mouse)	34.20 ± 0.08**	52.48 ± 1.13	145.97 ± 4.18	16.8 ± 2.4
M. indica (5 mg kg^{-1}) + TPA (20 nmol/0.2 mL acetone/mouse)	35.10 ± 0.04***	65.61 ± 2.79***	157.7 ± 6.94	20.5 ± 2.88*
M. indica (7.5 mg kg^{-1}) + TPA (20 nmol/0.2 mL acetone/mouse)	36.80 ± 0.14***	68.81 ± 0.50***	162.40 ± 2.61*	27.2 ± 4.96**
M. indica (7.5 mg kg^{-1})	36.70 ± 0.20	7.40 ± 1.52	177.20 ± 1.20	36.12 ± 2.12

Each value represents mean ± s.e., n = 6. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significantly different compared with the corresponding value for the TPA alone treated control group. $^{\dagger\dagger}P < 0.01$, significantly different compared with the corresponding value for the acetone treated control group.

Table 3 Effect of prophylactic treatment of mice with *Morus indica* on TPA mediated cutaneous glutathione *S*-transferase, glucose 6-phosphate dehydrogenase and quinone reductase

Treatment group	Glutathione *S*-transferase (nmol CDNB conjugate formed min^{-1} (mg protein)$^{-1}$)	Glucose 6-phosphate dehydrogenase (nmol NADP reduced min^{-1} (mg protein)$^{-1}$)	Quinone reductase (nmol DCPIP reduced min^{-1} (mg protein)$^{-1}$)
Acetone treated control	77.51 ± 2.74	18.30 ± 2.3	81.57 ± 9.88
TPA alone (20 nmol/0.2 mL acetone/mouse)	$41.09 \pm 13.0^{\dagger\dagger}$	$7.12 \pm 2.3^{\dagger\dagger}$	$46.46 \pm 1.48^{\dagger\dagger}$
M. indica (2.5 mg kg^{-1}) + TPA (20 nmol/0.2 mL acetone/mouse)	55.55 ± 0.89	9.41 ± 2.19	52.95 ± 3.77
M. indica (5 mg kg^{-1}) + TPA (20 nmol/0.2 mL acetone/mouse)	63.42 ± 1.58***	11.40 ± 1.78	60.60 ± 0.83***
M. indica (7.5 mg kg^{-1}) + TPA (20 nmol/0.2 mL acetone/mouse)	67.26 ± 0.81***	14.7 ± 2.40*	66.10 ± 0.69***
M. indica (7.5 mg kg^{-1})	75.15 ± 2.42	17.20 ± 2.12	74.0 ± 1.50

Each value represent mean ± s.e., n = 6. *$P < 0.05$ and ***$P < 0.001$, significantly different compared with the corresponding value for the TPA alone treated control group. $^{\dagger\dagger}P < 0.01$, significantly different compared with the corresponding value for the acetone treated control group.

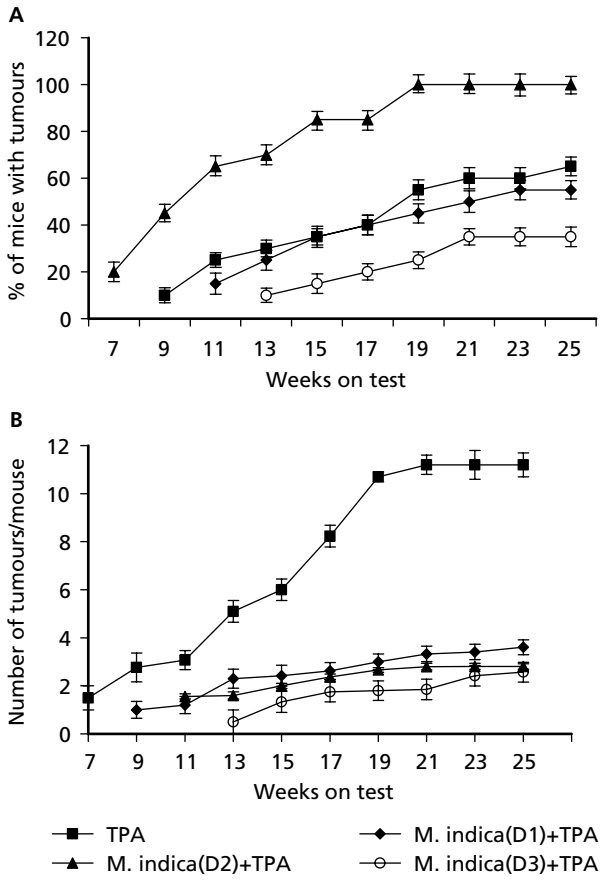

Figure 1 Effect of pre-treatment with *Morus indica* extract on croton oil mediated tumour promotion in DMBA initiated skin of mice. A. Percentage incidence of tumours plotted as a function of weeks on test. (B) Number of tumours per mouse plotted as a function of number of weeks on test. Each group had 20 mice. D1, D2 and D3 represent the application of 2.5, 5.0 and 7.5 mg kg^{-1} *Morus indica* extract, respectively.

The inhibition of skin tumourigenesis was evident when tumour data were considered as the percentage of mice with tumours (Figure 1A) and the number of tumours per mouse (Figure 1B). The appearance of skin papilloma was noticed at the end of Week 7 in the DMBA initiated and croton oil promoted mice. The number of tumours at Week 13 in the DMBA and croton oil treated group was 5.1, with 70% incidence of tumour-bearing mice, while the number of tumours at the end of Week 25 was 11.2, with 100% incidence of tumour-bearing mice. In the pre-treated group in which the mice were given topical application of *M. indica* extract (30, 40 and 50 μg) before the application of croton oil twice every week, the appearance of first tumour was prolonged to Weeks 9, 11 and 13, respectively. The percentage of tumour-bearing mice was 30% and 10% at Week 13, and 65% and 35% at Week 25, with the lower and higher doses, respectively. The total number of tumours per week at Week 25 decreased dose dependently to 3.61 and 2.57 in the lower and higher dose groups, respectively.

Figure 2 shows that treatment of mice with TPA alone induced the activity of ODC by 2-fold compared with the acetone treated control group. This increase in the activity of cutaneous ODC by TPA was dose dependently reduced by treatment with *M. indica* extract, ranging from 51% to 103% for lower and higher doses, respectively.

Discussion

In our day-to-day life, we are constantly exposed to an array of chemicals that act as free radical generators. A promising way to control chemical mutagenesis and carcinogenesis is to prevent the formation of reactive metabolites by the monooxygenases (Miller & Miller 1971). Drug metabolizing enzymes such as cytochrome P450 and aryl hydrocarbon hydroxylase, which are critical determinants of the carcinogenicity induced by various

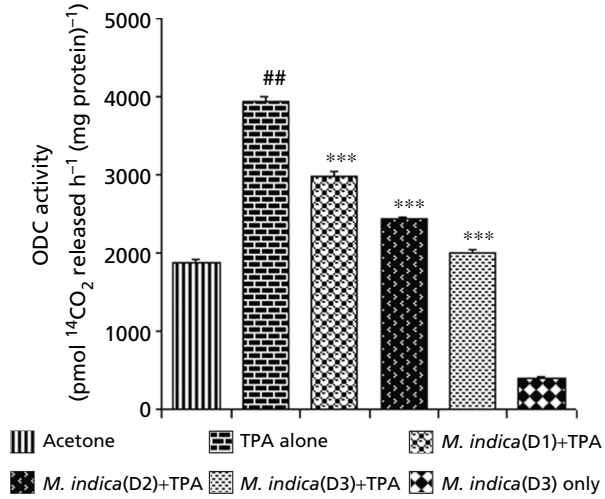

Figure 2 Effect of pre-treatment of mice with *Morus indica* extract on cutaneous ornithine decarboxylase (ODC) activity.

chemicals, play a major role in the bioactivation and detoxification of xenobiotics and carcinogens. Their oxidative activity transforms inert compounds into reactive metabolites that are able to interact and bind covalently with DNA, RNA and proteins of cells (Jerina et al 1979). In the present study, we used benzo(a)pyrene, the carcinogenic property of which is correlated with the induction of aryl hydrocarbon hydroxylase (Conney 1982; Gooderham & Mannering 1985). Increasing concentrations of *M. indica* extract added to the incubation mixture caused a dose-dependent inhibition of epidermal aryl hydrocarbon hydroxylase activity. It may be postulated that compounds that modulate the activity of the enzymes involved in the bioactivation of various carcinogens could reduce the risk of tumour development. The importance of this enzyme in carcinogenesis has led to a continuing search for inhibitors that may be useful in modifying the cancer-causing effects of polycyclic aromatic hydrocarbons. The deoxyribose assay shows that on interaction of free radicals with transition metals, hydroxyl radicals are produced by Fentons reaction and are responsible for DNA damage. Characterization of this damage to DNA has indicated that both the sugar and the base moieties are susceptible to oxidation, causing single-strand breakage and cross-linking to protein. Many naturally occurring chemopreventive agents cause an induction of GST by decreasing the potential of DNA damaging entities and converting them into excretable metabolites (Coles & Ketterer 1990). There is also evidence that agents that block the elaboration of hydroxyl radicals can inhibit DNA damage, mutation and malignant transformation induced by oxygen species in cell free and cellular systems (Aruoma et al 1989). The effects of *M. indica* on in-vitro parameters suggest that *M. indica* may combat against TPA mediated toxicity and DMBA initiated and croton oil promoted two-stage skin carcinogenesis.

TPA and some other phorbol esters have been reported to act as strong tumour promoters through an oxygen mediated mechanism (Sun 1990), and oxygen radicals are the critical components of the tumour promotion process (Sun 1990; Sultana et al 1997). Since tumour promotion is closely related to oxidative stress, compounds that exhibit anti-inflammatory and antioxidant properties may also show chemopreventive activity. Our study showed that *M. indica* extract ameliorated TPA induced lipid peroxidation and restored the depleted levels of glutathione. It also reversed TPA mediated inhibition of antioxidant enzymes such as catalase, glucose 6-phosphate dehydrogenase, glutathione peroxidase and GST, restoring most of them approximately to control levels. Dysfunction of these antioxidant enzymes has been reported in many diseases, including rheumatoid arthritis, reperfusion injury, cardiovascular diseases and immune injury, as well as cancer (Gonzalis et al 1984). Further, *M. indica* extract restored the depleted levels of quinone reductase, which is an essential characteristic of anti-tumour promoting chemopreventive agents.

ODC, an appropriate target for chemoprevention, is used extensively as a biochemical marker to evaluate the tumour promoting potential of a test agent. We assessed the effect of *M. indica* on TPA induced cutaneous ODC activity. A sharp decrease in TPA mediated induction in ODC activity with pre-treatment of *M. indica* suggests the antitumour promoting potential of the plant extract.

Croton oil, which is mainly composed of phorbol ester, is a well-known tumour promoter in murine skin. Its tumour promoting potential has been related to the presence of TPA. The present study showed a delay in the onset of tumourigenesis by increasing the latency period and decreasing the tumour incidence in mice pre-treated with *M. indica* in DMBA initiated and croton oil promoted mice. This activity of *M. indica* may be attributed to the presence of antioxidant flavonoids (isoquercitrin) and moracins, which act as strong superoxide radical scavengers and singlet oxygen quenchers. Isoquercitrin, a polyphenolic antioxidant is present in a number of fruits and vegetables such as *Peucedanum japonicum*, *Juniperus chinensis*, *Chrysophyllum cainito* and Rooibos tea. The high content of isoquercitrin in these plants is reflected in their antioxidant activity, in alleviating allergies and in the treatment of a number of dermatological diseases such as photosensitive dermatitis (Luo et al 2002). The results of the present study indicate that *M. indica* may be used as a chemopreventive agent against cutaneous oxidative stress and carcinogenesis, as evident by the alteration in marker enzymes of oxidative stress and tumour development.

Conclusion

The effects of *M. indica* against two-stage skin carcinogenesis were studied. The anti-tumour promoting effect of *M. indica* may be linked to its antioxidant nature and its ability to inhibit the activity of ODC. The efficacy of the plant extract is thought to be due to the presence of

flavonoids (isoquercitrin) and phytoalexins (moracin C, moracin N and chalcomoracin), which have proven chemopreventive effects.

References

Antonio, M. A., Souza Britto, A. R. (1998) Oral anti-inflammatory and anti-ulcerogenic activities of a hydroalcoholic extract and partitioned fraction of *Turnera ulmifora* (Turneraceae). *J. Ethnopharmacol*. **61**: 215–228

Aruoma, O. I., Halliwell, B., Gajewski, E., Dizdaroglu, M. (1989) Damage to the bases in DNA induced by hydrogen peroxide and ferric ion on chelates. *J. Biol. Chem*. **264**: 20 509–20 512

Benson, A. M., Hunkeler, M. J., Talalay, P. (1980) Increase of NADPH : quinone reductase activity by dietary antioxidant. Possible role in protection against carcinogenesis and toxicity. *Proc. Natl Acad. Sci. USA* **77**: 5216–5220

Carlberg, I., Mannervick, B. (1975) Glutathione reductase level in rat brain. *J. Biol. Chem*. **250**: 5575–5580

Cerutti, P. (1985) Prooxidant states and tumor promotion. *Science* **227**: 375–381

Chance, B., Sies, H., Boveris, A. (1979) Hydrogen peroxide metabolism in mammalian organs. *Physiol. Rev*. **59**: 527–605

Chatterjee, G. K., Burman, T. K., Nagchaudhuri, A. K., Pal, S. P. (1983) Antiinflammatory and antipyretic activities of *Morus indica*. *Planta Med*. **48**: 116–119

Chatterjee, M., Katiyar, S. K., Mohan, R. R., Agarwal, R. A. (1999) Flavanoid antioxidant, silymarin, affords exceptionally high protection against tumor promotion in the SENCAR mouse skin tumorigenesis model. *Cancer Res*. **59**: 622–632

Claiborne, A. (1985) Catalase activity In: Greenwald, R. A (ed.) *CRC Handbook of methods in oxygen radical research*. CRC Press, Boca Raton, pp 283–284

Coles, P., Ketterer, B. (1990) The role of glutathione and glutathione transferase in chemical carcinogenesis. *Crit. Rev. Biochem. Mol. Biol*. **25**: 47–50

Conney, A. H. (1982) Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons. GHA Clowes Memorial Lecture. *Cancer Res*. **42**: 4875–4917

Cross, C. E., Halliwell, B., Borish, E. T., Pryor, W. A., Ames, B. N., Saul, R. L., Mc Cord, J. M., Harman, D. (1987) Oxygen radicals and human disease. *Ann. Intern. Med*. **197**: 526–545

Didry, N., Dubereuil, L., Trotin, F., Pinkas, M. (1998) Antimicrobial activity of aerial parts of *Drossera pelitata* Smith on oral bacteria. *J. Ethnopharmacol*. **60**: 91–96

Gonzalis, R., Auclair, C., Voisin, E., Gautero, H., Dhermy, D., Boivin, P. (1984) Superoxide dismutase, glutathione peroxidase and catalase in red blood cells from patients with malignant diseases. *Cancer Res*. **44**: 4137–4139

Gooderham, N. J., Mannering, G. J. (1985) Depression of the hepatic cytochrome P450 monooxygenase system by treatment of mice with the anti-neoplastic agent, 5-azacytidine. *Cancer Res*. **45**: 1569–1572

Habig, W. H., Pabst, M. J., Jokoby, W. B. (1974) GST: the first enzymatic step in mercapturic acid formation. *J. Biol. Chem*. **249**: 7130–7139

Jerina, D. M., Yagi, H., Thakker, D. R., Karley, J. M., Mak, H. D., Boyd, D. R., Gadaginamath, D., Wood, A. W., Buening, M., Chang, R. L., Levin, W., Conney, A. H. (1979) Stereoselective metabolic activation of polycyclic aromatic hydrocarbons. *Adv. Pharmacol. Ther*. **9**: 53–62

Jollow, D. J., Mitchell, J. R., Zampaglione, N., Gillete, J. R. (1974) Bromobenzene induced liver necrosis: protective role of glutathione and evidence for 3,4, bromobenzene oxide as the hepatotoxic intermediate. *Pharmacology* **11**: 151–169

Katiyar, S. K., Agarwal, R., Mukhtar, H. (1996) Inhibition of tumor promotion in Sencarmouse skin by ethanolic extract of *Zingiber officinale* rhizome. *Cancer Res*. **56**: 1023–1030

Kensler, T. W., Taffe, B. G. (1986) Free radicals and tumor promotion. *Adv. Free Radic. Biol. Med*. **2**: 347–387

Lowry, O. M., Rosenbrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with folin phenol reagent. *J. Biol. Chem*. **193**: 265–275

Luo, X. D., Basile, M. J., Kennelly, E. J. (2002) Polyphenolic antioxidants from the fruits of *Chrysophyllum cainito* L. (star apple) *J. Agric. Food Chem*. **5**: 1379–1382

Miller, J. A., Miller, E. C. (1971) Chemical carcinogenesis: mechanism and approaches to its control. *J. Natl Cancer Inst*. **47**: V–XIV

Mohandas, J., Marshall, J. J., Duggin, G. G., Horvath, J. S., Tiller, D. (1984) Differential distribution of glutathione and glutathione related enzymes in rabbit kidney. *Cancer Res*. **44**: 5086–5091

Morikawa, K., Nonaka, M., Narahara, M., Torii, I., Kawaguchi, K., Yoshikawa, T., Kumazawa, Y., Morikawa, S. (2003) Inhibitory effect of quercetin on carrageenan-induced inflammation in rats. *Life Sci*. **74**: 709–721

Nebert, D. W., Gelbion, H. V. (1968) Substrate inducible microsomal aryl hydrocarbon hydrolase in mammalian cell culture: assay and properties of induced enzyme. *J. Biol. Chem*. **243**: 6242–6249

O'Brien, T. G., Simsiman, R. C., Boutwell, R. K. (1975) Induction of the polyamine biosynthesis enzymes in mouse epidermis by tumor promoting agents. *Cancer Res*. **35**: 1662–1670

Omura, T., Sato, R. (1964) The carbon monoxide binding pigment of liver microsome. *J. Biol. Chem*. **239**: 2370–2378

Sharma, R., Sharma, A., Shono, T., Takasugi, M., Shirata, A., Fugimura, T. (2001) Mulberry moracins: scavengers of UV stress-generated free radicals. *Biosci. Biotechnol. Biochem*. **65**: 1402–1405

Stoner, G. D., Morse, M. A., Kellhoff, G. J. (1977) Perspectives in cancer chemoprevention. *Environ. Health Perspect*. **105**: 945–954

Sultana, S., Shahid, P., Iqbal, M., Athar, M. (1997) Crude extracts of hepatoprotective plants *Solnum nigrum* and *Cichorium intybus* inhibit free radical mediated DNA damage. *J. Ethnopharmacol*. **45**: 189–192

Sun, Y. (1990) Free radicals, antioxidant enzymes and carcinogenesis. *Free Radic. Biol. Med*. **8**: 583–599

Troll, B. G., Wiesner, R. (1985) The role of oxygen radicals as a possible mechanism of tumor promotion. *Ann. Rev. Pharmacol. Toxicol*. **25**: 509–528

Weiss, S. J., Lobuglio, A. F. (1982) Biology of disease: phagocyte-generated oxygen metabolites in cellular injury. *Lab. Invest*. **47**: 5–18

Wright, J. R., Colby, H. D., Miles, P. R. (1981) Cytosolic factors that affect microsomal lipid peroxidation in lung and liver. *Arch. Biochem. Biophys*. **206**: 296–304

Zaheer, N., Tiwari, K. K., Krishnan, P. S. (1965) Exposure and solubilization of hepatic mitochondrial shunt dehydrogrogenases. *Arch. Biochem. Biophys*. **109**: 646–648