Anti-Cancer Drugs 1994, **5**, pp. 567–572

Chemopreventive action by an extract from *Ocimum sanctum* on mouse skin papillomagenesis and its enhancement of skin glutathione S-transferase activity and acid soluble sulfydryl level

Ritu Prashar, Ashok Kumar, S Banerjee[1] and AR Rao[1]

Radiation and Cancer Biology Laboratory, Department of Zoology, University of Rajasthan, Jaipur 302 004, India. Tel: (+91) 141 512158; Fax: (+91) 141 41763. [1] Cancer Biology Laboratory, School of Life Sciences, Jawaharalal Nehru University, New Delhi, India.

We report the chemopreventive property of an ethanolic extract of the leaves of *Ocimum sanctum* (a traditional medicinal plant) on 7,12-dimethylbenz[*a*]anthracene induced skin papillomagenesis in male Swiss albino mice. A significant reduction in the values of tumor incidence, average number of tumors per tumor bearing mice and the cumulative number of papillomas was observed in mice treated topically with the leaf extract of *O. sanctum* at either the peri-initiational, post-initiational stages or continuously at peri- and post-initiational stages of papillomagenesis as compared to the corresponding control group. Topical application of *Ocimum* leaf extract for 15 days resulted in significant 2-fold elevation of reduced glutathione content in the skin of mice ($p < 0.05$). Similarly, glutathione S-transferase activity was also observed to be significantly elevated by 25% compared with the control group ($p < 0.05$) following *Ocimum* extract treatment.

Key words: Chemoprevention, glutathione, glutathione S-transferase, *Ocimum sanctum*, skin papillomagenesis.

Introduction

Ocimum sanctum L. (Sacred basil, green Tulsi) is a traditional medicinal plant. Its leaves on steam distillation yield a bright yellow oil possessing a pleasant odor characteristic of the plant. The oil is reported to possess antibacterial and antifungal properties.[1] The juice of leaves possess diaphoretic, antiperiodic, stimulating and expectorant properties. It is used in bronchitis, and applied to the skin in ringworm and other cutaneous diseases.[2] Although eugenol is the active principle,[2] other constituents of the plant extract, though minor ingredients, play a major role in determining the overall biologic activity of the medicinal plants.[3]

Financial assistance from the Council of Scientific and Industrial Research (CSIR), New Delhi, is gratefully acknowledged.

Correspondence to A Kumar

Our recent studies have shown that ethanolic extract of *Ocimum* leaves has a modulatory influence on mouse hepatic and extrahepatic biotransformation enzyme activity and glutathione level (unpublished data). Those findings stimulated us to study the possible chemopreventive action of *O. Sanctum* on chemical carcinogenesis.

The present study evaluates the influence of topical application of *Ocimum* leaf extract on the activity of glutathione S-transferase (GST) and reduced glutathione (GSH) content in mouse skin for its presumptive role in chemoprevention of 7,12-dimethylbenz[*a*]anthracene (DMBA) induced skin papillomagenesis.

Materials and methods

Animals

Random-bred, male Swiss albino mice (7–8 weeks old) were obtained from the Animal Facility (JNU, New Delhi). The animals were provided with standard mice feed (Hindustan Lever, India) and tap water *ad libitum*. The dorsal skin of the animals in the interscapular area was shaved 3 days before the commencement of the experiment and only those animals in the resting phase of the hair cycle were chosen for the study.

Chemicals

DMBA, croton oil, 1-chloro-2, 4-dinitrobenzene (CDNB), 5′-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione and bovine serum albumin (BSA) were procured from Sigma (St Louis, MO). DMBA was dissolved in acetone at a concentration of 50 μg/50 μl. Croton oil was diluted in acetone to give a 1% dilution.

Preparation of *O. sanctum leaf* extract

Briefly, this consisted of distilling the dried leaf powder of *Ocimum* in a round-bottom flask using absolute alcohol. The procedure was repeated thrice for 13 h duration and the leftover residue after the third distillation was filtered and the remaining alcohol was allowed to evaporate. The thick paste obtained after appropriate dilution in acetone was applied topically over the shaven area of the skin of mice at a dose level of 5.0 mg/kg body weight.

For estimation of reduced GSH and GST activity, 0.04 g of leaf extract was dissolved in 1.2 ml acetone. From this stock solution, 150 µl was applied on to each mouse.

Preparation of homogenate and supernatant fraction

Animals were killed by cervical dislocation, and the skin was carefully removed, trimmed free of extraneous collagen material attached underneath by scraping with a scalpel blade and cut into small pieces. It was then weighed, blotted dry and homogenized in ice-cold Tris–KCl buffer (pH 7.4) to yield a 10% (w/v) homogenate. A 0.5 ml aliquot of this homogenate was used for assaying reduced glutathione while the remainder was centrifuged at 800 *g* for 10 min. The supernatant was transferred into pre-cooled polypropylene centrifugation tubes and centrifuged again at 15 000 *g* for 30 min in a high speed refrigerated centrifuge (model: Dupont RC5C). The supernatant fraction after discarding any floating lipid layer was used for assaying GST enzyme activity.

Assay methods

Determination of GST activity. The cytosolic GST activity was determined spectrophotometrically at 37°C according to the procedure of Habig *et al.*[4] The reaction mixture (3 ml) contained 1.7 ml of 100 mM phosphate buffer (pH 6.5), 0.1 ml of 30 mM GSH and 0.1 ml of 30 mM CDNB. After preincubating the reaction mixture at 37°C for 5 min, the reaction was started by the addition of 0.1 ml and the absorbance was followed for 5 min at 340 nM. The reaction mixture without the enzyme was used as the blank. The specific activity of GST is expressed as µmol of GSH–CDNB conjugate formed per min per mg protein using an extinction coefficient of 9.6 mM^{-1} cm^{-1}.

The protein content was measured according to Bradford's method using BSA as a standard.[5]

Determination of GSH content. Tissue levels of GSH were determined by the method as described by Moron *et al.*[6] Homogenates were immediately precipitated with 0.1 ml of 25% trichloroacetic acid and the precipitate was removed after centrifugation. Free SH groups were assayed in a total 3 ml volume by the addition of 2 ml of 0.6 mM DTNB prepared in a 0.2 M phosphate buffer (pH 8.0) to 0.1 ml of the supernatant and the absorbance was read at 412 nm using a Shimadzu UV-160 spectrophotometer. GSH was used as a standard to calculate µmol GSH/g tissue.

Statistical analysis

Statistical significance of differences between control and experimental groups was determined by Student's *t*-test.

Experimental design

Effect of *Ocimum* extract on DMBA-induced skin papillomagenesis

The following treatment groups were compared.

Group I (n = 10). A single dose of 50 µg of DMBA in 50 µl of acetone was applied topically over the shaven area of the skin of the mice. Two weeks later croton oil (100 µl of 1% croton oil in acetone) was applied three times per week until the end of the experiment (15 weeks).

Group II (n = 10). Animals received a topical treatment (on the shaven area of the skin of mice) of an ethanolic extract of the leaves of *O. sanctum* (5.0 mg/kg body weight) in 50 µl acetone 7 days before and 7 days after the application of DMBA. Croton oil was given as in group I.

Group III (n = 10). Animals received a topical treatment of *Ocimum* extract (5.0 mg/kg body weight) in 50 µl acetone, starting from the time of croton oil treatment till the end of the 15 weeks of the experiment. DMBA was given as in group I.

Group IV (n = 10). Animals were treated topically with *Ocimum* extract (5.0 mg/kg body weight) throughout the experimental period, i.e. before

and after DMBA application and also at the promotional stage. Croton oil was given as in group I. The experiment was carried out for 15 weeks.

Group V (n = 10). Animals received only croton oil treatment which was given as in group I.

Group VI (n = 10). Animals received *Ocimum* extract (5 mg/kg body weight) in 50 µl acetone topically and croton oil was applied as in group I. The animals were not treated with DMBA.

Group VII (n = 10). Animals received DMBA treatment as in group I but they did not receive either *Ocimum* extract or croton oil treatment.

Group VIII (n = 10). Animals were topically treated with *Ocimum* extract (5.0 mg/kg body weight) throughout the experimental period and were given DMBA as in group I, but were not treated subsequently with croton oil.

Group IX (n = 10). Animals group were only given a topical treatment of *Ocimum* extract (5.0 mg/kg body weight) for 15 weeks.

During the 15 weeks of experiment, mice of groups I–IX were weighed weekly and also at the time of autopsy. They were carefully examined once a week for the presence of skin papillomas and the number of papillomas on each affected mouse was recorded. Skin papillomas were defined as lesions with a diameter greater than 1 mm that were present for at least two consecutive observations.

Reduced GSH and GST activity

The effect of *Ocimum* extract on reduced GST activity and GST activity in mouse skin was investigated in the following two groups.

Group A (n = 8). Mice were put on a normal diet and the hair on the dorsal surface of the animal was clipped off; 3 days later acetone (150 µl) treatment was started and it was given topically once a day for 15 days.

Group B (n = 8). Animals were put on a normal diet and the hair on the dorsum clipped off; 3 days later 150 µl *Ocimum* extract (0.04 g dissolved in 1.2 ml acetone) treatment was started and it was given topically once a day for 15 days. The body weights of mice were recorded initially and at the end of the experiment.

Results

Figure 1 shows the cumulative number of papillomas in the experimental and control groups recorded during the observation period. Figure 2 depicts the average number of tumors per tumor bearing mouse in control and experimental groups. Figure 3 depicts the percentage of mice with papillomas in control and experimental groups.

In the control group (group I) in which a single topical application of DMBA was followed, 2 weeks later, by repeated applications of croton oil, skin papillomas appeared in all (100%) animals and the cumulative number of papillomas induced during the observation period was 30. The mean number of tumors per effective mouse was observed to be 3.0 ± 0.8 and the average tumor weight was 162.5 mg. Animals of groups II, which received *Ocimum* extract treatment at the peri-initiational phase of tumorigenesis, showed only 30% tumor incidence and the cumulative number of papillomas was only 5. The mean number of tumors per effective mouse was reduced to 0.5 ± 0.28 and the average tumor weight was observed to be 5.2 mg. All animals in group III, which were given *Ocimum* extract treatment at the promotional stage of tumorigenesis, showed 40% tumor incidence and the cumulative number of papillomas was observed to be 10. The mean number of tumors was observed to be 1.0 ± 0.47 and the average tumor weight was 24.3 mg. Mice of group IV, given a continuous treatment of *Ocimum* extract at peri- as well as at the post-initiational phases, showed a significant reduction in the incidence of tumors (30%) as well as the cumulative number of papillomas (6) and the mean number of tumors per effective mouse (0.6 ± 0.35). The average tumor weight was 4.1 mg. Animals in the rest of the groups did not show any papilloma occurrence during the 15 weeks of the observation period.

Topical application of *Ocimum* leaf extract for 15 days resulted in significant elevation of GSH content in the skin of mice ($p < 0.05$). Similarly, GST activity was also observed to be significantly elevated by 1.40-fold more than in the control group ($p < 0.05$) following *Ocimum* extract treatment (Table 1).

Discussion

Our data indicate a chemopreventive effect of ethanolic leaf extract of *O. sanctum* on DMBA induced skin papillomagenesis in male Swiss albino mice. In the animals which were given a single topical ap-

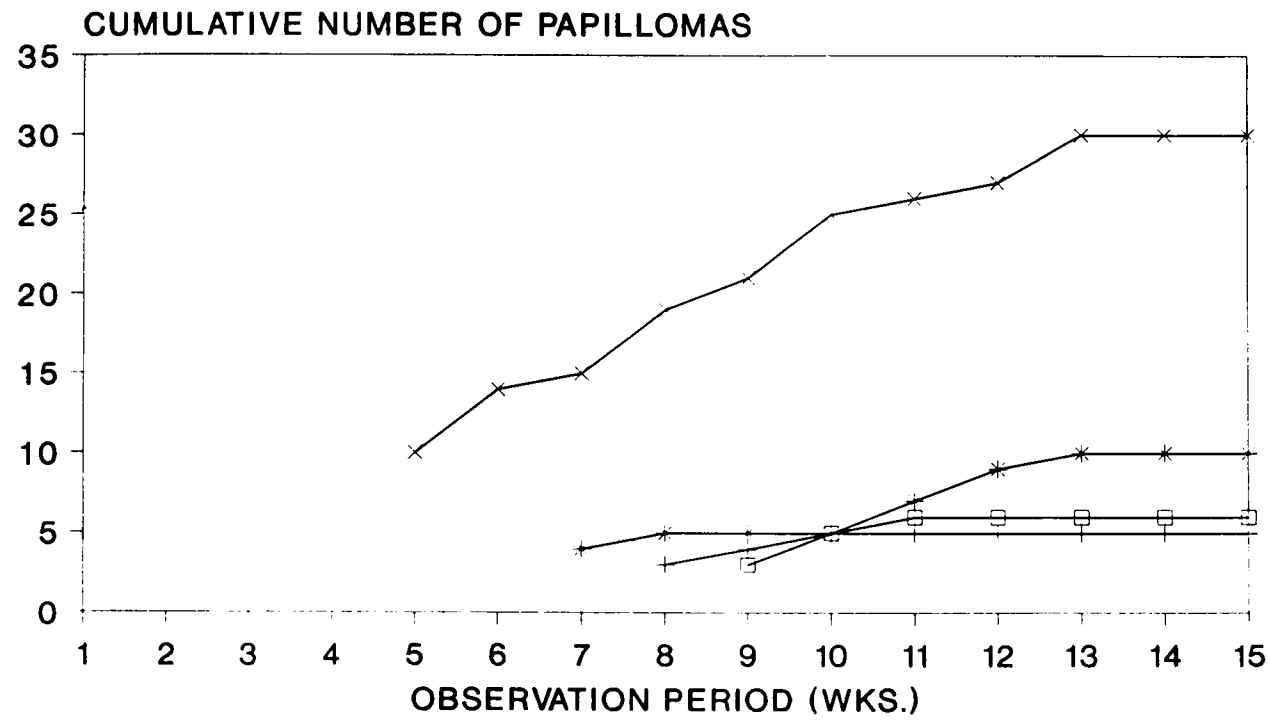

Figure 1. Cumulative number of papillomas in control [group I(x)] and experimental groups [groups II (+), III (*) and IV (□)] recorded during the observation period. Group I: initiator + promoter; group II: initiator + promoter + modifier (*Ocimum* given at the peri-initiational phase of papillomagenesis); group III: initiator + promoter + modifier (*Ocimum* treatment given at the promotional stage); group IV: initiator + promoter + modifier (*Ocimum* treatment given both at peri- as well as post-initiational phases).

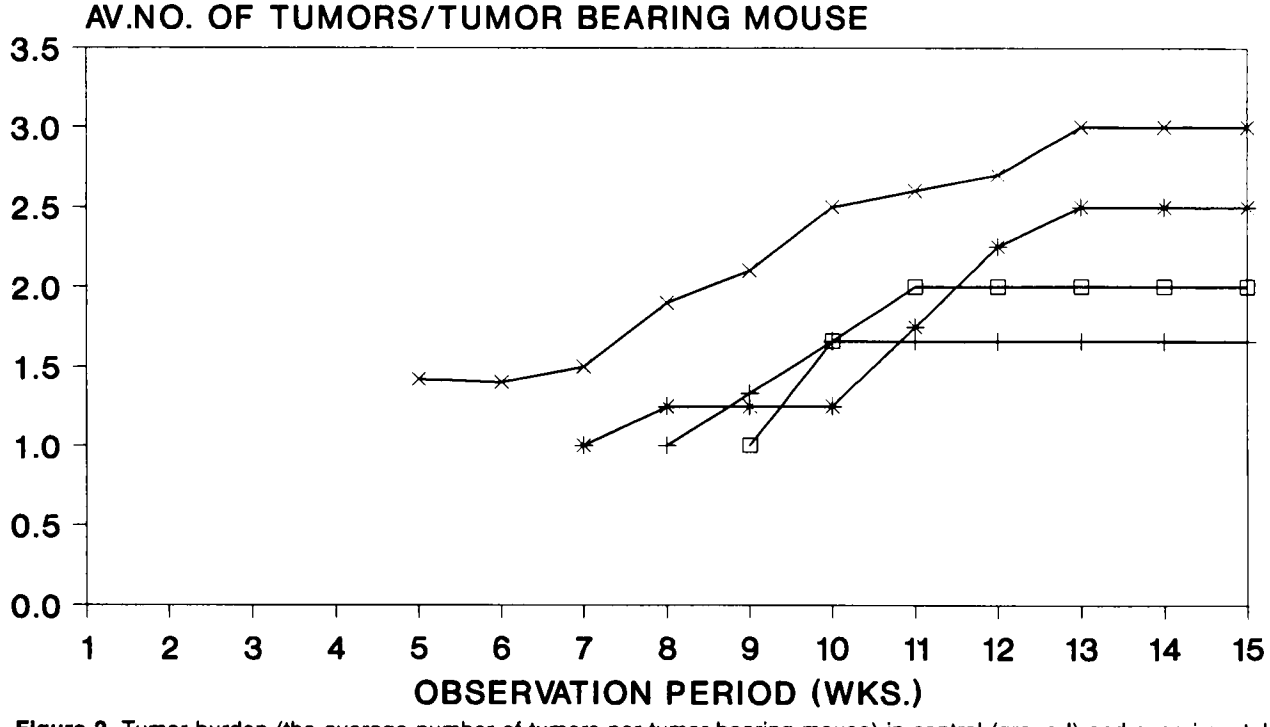

Figure 2. Tumor burden (the average number of tumors per tumor bearing mouse) in control (group I) and experimental groups (groups II–IV). See Figure 1 for a description of treatment groups and symbols.

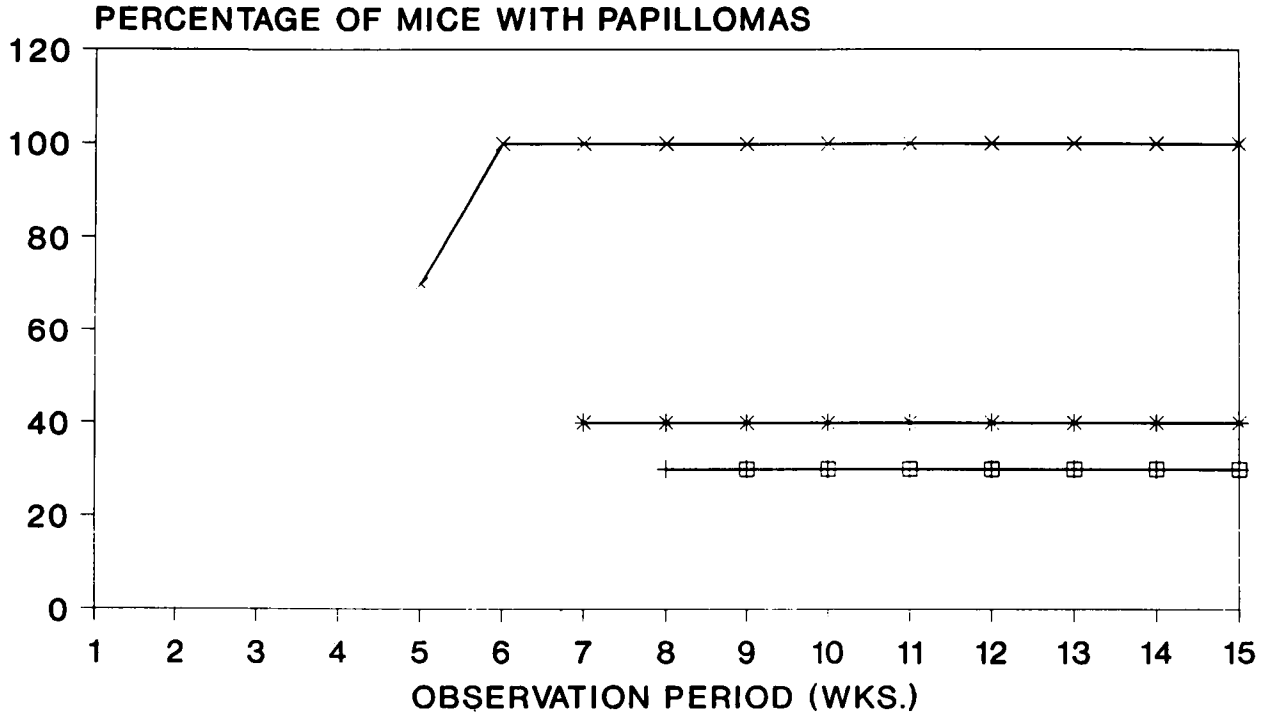

Figure 3. The percentage of mice with papillomas in control (group I) and experimental groups (groups II–IV). See Figure 1 for a description of treatment groups and symbols.

Table 1.

Groups	Treatment	GSH concentration[a]	GST activity[b]
A	Acetone	0.02 ± 0.0	0.04 ± 0.01
B	*Ocimum* extract in acetone	0.04 ± 0.0	0.05 ± 0.01

Ratio of GSH for group B/group A is significant ($p < 0.005$). Also, the ratio (1.25) of GST for group B/group A is significant ($p < 0.005$).

[a] GSH level expressed as μmol/g tissue.

[b] GST activity was measured using CDNB as substrate and expressed as μmol CDNB–GSH conjugated/min/mg protein.

plication of DMBA (50 μg/50 μl of acetone) followed by repeated applications of croton oil (1% in acetone, three times per week) after 2 weeks, the cumulative number of papillomas and the value of tumor burden were both significantly higher than in mice which were also treated with *Ocimum* extract. *Ocimum* treatment was equally effective in group I and III, where it was given at the peri-initiational stage of papillomagenesis and at all the stages continuously.

Many plant materials are known to influence the hepatic biotransformation enzyme profiles that are involved in activation and detoxication of xenobiotic chemicals including chemical carcinogens.[7–9]

In the present study, wherein CDNB was used as a non-specific substrate for assaying total cytosolic GST, it was noticed that topical application of *Ocimum* leaf extract for 15 days significantly enhanced the GST activity in the skin of mice. The elevation of cytosolic GST implies a protective role against the cytotoxic or carcinogenic effect of many compounds which are activated by the biotransformation enzymes of the endoplasmic reticulum, by scavenging the electrophilic metabolites.[10–13]

Eugenol, the active principle in *Ocimum* leaf extract, has been shown to induce detoxification enzymes conjugating many ultimate carcinogens.[14] Furthermore, no evidence of carcinogenicity due to eugenol has been observed in male or female rats.[15] Moreover, a decrease in mutagenic response by microsomes from eugenol treated rats has been observed.[16]

Although eugenol is the active principle,[2] other constituents of the plant extract, though minor ingredients, play a major role in determining the overall biologic activity of the medicinal plants.[3] Therefore, the whole crude extract was studied for its possible chemopreventive action and not eugenol alone.

By acting in the proposed manner, *Ocimum* extract induced increase in GST activity might offer *in*

vivo protection to tissues wherein carcinogenicity or gentoxicity are mediated by reactive electrophilic metabolites.

The present study also demonstrates that topical application with *Ocimum* extract significantly elevates the level of SH groups in the skin of mice. Cellular acid soluble SH content, which almost entirely consists of GSH, functions in protecting the cells against free radicals generated during oxidative metabolism as well as from cellular lethality following exposure to drugs or radiation and acting as an acceptor of electrophilic molecules involved in cancer initiation.[17–19]

Recently we carried out a study on the modulatory influence of an ethanolic leaf extract of *Ocimum* on the activities of cytochrome P-450, cytochrome b_5 and arylhydrocarbon hydroxylase enzymes in liver, and GST and GSH glutathione level in the liver, lung and stomach of mouse. We observed that oral treatment of 400 and 800 mg/kg body weight for 15 days caused significant elevation in all the above mentioned parameters, which play an important role in the detoxification of carcinogens as well as mutagenes (unpublished data).

Thus *Ocimum* leaf extract presumably renders the mice resistant to harmful oxygen species and chemical carcinogenic action.

Hence, from a mechanistic point of view it is possible that *Ocimum* extract may be augmenting detoxification of carcinogens in the skin by increasing GST activity. Further, it is also possible that the reactive oxygen intermediates generated by the phorbol ester present in croton oil may be scavenged by the SH groups elevated in the skin by *O. sanctum*.

This protective effect of *Ocimum* is reflected in the decreased values of tumor burden, tumor incidence and also the cumulative number of papillomas.

Conclusions

In conclusion, our results provide evidence for the first time that an ethanolic leaf extract of *O. sanctum* has a modulatory influence on mouse skin GST activity and acid soluble sulfydryl levels, and exhibits a chemopreventive action on DMBA-induced skin papillomagenesis.

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(Received 31 March 1994; received in revised form 9 May 1994; accepted 14 June 1994)