

# Antimutagenicity of Herbal Detoxification Formula Smoke Shield Against Environmental Mutagens

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Smoke Shield is a formulation designed to reduce smoke related mutagenicity and toxicity in the population. Smoke Shield contains a dual extract of turmeric (*Curcuma longa*) obtained by supercritical CO<sub>2</sub> gas extraction and post-supercritical hydroethanolic extraction together with extracts of green tea and other spices, whose presence synergistically increases the activity of turmeric. In the present study we have shown its antimutagenic activity to various environmental mutagens *in vitro* and *in vivo*. Smoke Shield was found to produce significant inhibition of mutagenicity to *Salmonella typhimurium* induced by sodium azide and 4-nitro-0-phenylenediamine (NPD) at a concentration of 2 mg/plate while inhibition to N-methyl-N-nitro N-nitrosoguanidine was less significant. Inhibition was also found to depend upon the strain which was used. Smoke Shield was found to be more effective against mutagens needing metabolic activation such as 2-Acetamidofluorene (2-AAF) and benzo[a]pyrene. Smoke Shield was also found to significantly inhibit the mutagenicity induced by tobacco extract to *Salmonella typhimurium* TA102. Smoke Shield was also found to inhibit the urinary mutagenicity of rats treated with the benzo[a]pyrene and tobacco extract. Moreover, Smoke Shield administration was found to inhibit the urinary mutagenicity in smokers. These results indicate that Smoke Shield could inhibit mutagenic response *in vitro* and *in vivo* produced by several kinds of mutagens present in our atmosphere.

Key Words: Mutagenicity, Anti-mutagens, Smoke- Smoke Shield, Turmeric, Green tea

Smoke is one of the major public health hazards in the world. Smoke, whether coming from cigarettes, automobile exhaust or furnaces, or the barbequeing or charbroiling of meats etc. has been reported to have several mutagenic and carcinogenic substances wherein polyaromatic hydrocarbons and nitroso compounds predominate (1,2). These potential carcinogenic materials have been shown to get converted to their ultimate form by microsomal P450 enzymes which then bind with the guanine molecule in the DNA producing adducts and a mutagenic and carcinogenic response (3,4). In fact, the occurrence of lung and many other cancers has been shown to be directly related to cigarette smoking (5,6). Soot, which contains the particulate matter in the smoke, has been found to produce cancer at various sites. If the activation could be prevented it may be possible to inhibit the mutagenicity produced by PAHs (7).

Several plant extracts have been screened for their ability to inhibit carcinogenesis induced by various carcinogens and a few of them are undergoing clinical trial. Turmeric (*Curcuma longa*) has been shown to inhibit the carcinogenicity induced by several carcino-

genic substances and has been found to be non-toxic (8,9). Similar results have been obtained from green tea extract which has been shown to inhibit mutagenicity (10), carcinogenicity (11) and metastasis (12). Recently, New Chapter, USA has introduced a patent pending herbal formulation called Smoke Shield. Smoke Shield is designed to protect a person from health-damaging effects of all sources of smoke, such as from cigarette consumption, car exhaust fumes, and the charbroiling of meats. Smoke Shield is comprised of herbs supercritically extracted using compressed CO<sub>2</sub> gas in laboratory conditions. In addition, certain key herbs in the formula are separately hydroethanolicly extracted, which ensures the maximum representation of the protective plant constituents. The turmeric in Smoke Shield, for example, delivers both the value of the turmeric oils together with the protective action of the water-soluble curcuminoids. The quality of the herbs in Smoke Shield is assured for the potency of key marker compounds, and is devoid of any organic solvent residues. Other ingredients in Smoke Shield are green tea, clove, ginger, parsley, peppermint and rosemary.

In the present study, we have critically evaluated the effect of this preparation on mutagenicity induced by several mutagens, acting directly and those needing metabolic activation. We have also studied the effect of Smoke Shield on induced urinary mutagenicity in rats as well as in human smokers.

## Materials and Methods

'Smoke Shield' capsules were supplied by New Chapter, USA. Glucose-6-phosphate, L histidine, D biotin and NADP were purchased from Sisco Research Laboratories, Mumbai, India. 2-Acetamidofluorene (2AAF), benzo[*a*]pyrene (B[*a*]P), 4 nitro-*o*-phenylenediamine (NPD) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) were purchased from Sigma Chemicals, (St.Louis, MO, USA). All other chemicals were of analytical grade.

**Bacterial strain.** Histidine requiring strain of *Salmonella typhimurium* TA 98, TA 100, TA 102 and TA 1535 were kindly supplied by Prof. B.N. Ames, University of California, Berkley, USA. They were incubated in nutrient broth for 12 hrs and frozen permanents were prepared by freezing at -70°C in presence of 10% dimethyl sulfoxide. Fresh cultures used in the experiments were prepared by inoculating 40 µl of frozen permanents in 5 ml of nutrient broth and incubated for 12 hrs at 37°C and were used for the experiment.

**Preparation of mutagens.** All the chemical mutagens were dissolved in DMSO except sodium azide which was dissolved in water.

**Preparation of tobacco extract.** One hundred grams of tobacco were cut into small pieces and boiled in 500 ml of distilled water. This was evaporated to dryness under vacuum at 50°C.

**Animals.** Male Wistar rats aged 8-10 weeks, 250-300 gm were purchased from Kerala Agricultural University, Mannuthy. They were housed in well ventilated cages and fed with pelleted diet (Sai Durga Agencies, Bangalore) and kept in temperature controlled rooms. All the experiments were conducted according to the guidelines of Institutional Animal Ethics Committee.

**Determination of in vitro antimutagenicity.**

a) **Direct acting mutagens.** Antimutagenicity of

Smoke Shield was performed according to the method of Maron and Ames (13). Plate incorporation protocol was performed for direct acting mutagens. For this 2 ml of top agar containing 0.5 mM histidine/biotine was inoculated at mutagen concentrations given in the tables. Smoke Shield dissolved in DMSO at different concentration and freshly grown *Salmonella typhimurium* (0.1 ml having 10<sup>9</sup> cells approximately) were added to the top agar and the mixture was poured into minimal agar plates and incubated at 37°C for 48 hrs. After incubation the number of revertants were counted using a colony counter.

b) **Determination of antimutagenicity against mutagens needing activation.** Mutagens which require metabolic activation were processed by plate pre-incubation method of Matsushima et al (14). Liver microsomal fraction (S9) was prepared from Sprague Dawley rats treated with 0.1% phenobarbitol for 4 days (15). Activation mixture was prepared by mixing 50µl S9 mix with 0.25 ml phosphate buffer (0.2 M, pH 7.4), 20µl NADP (0.1 M), 2.5µl of glucose-6-phosphate and 10µl of MgCl<sub>2</sub>-KCl in the presence of mutagen in minimum volume of DMSO and various concentrations of Smoke Shield and incubated for 30 min. at 37°C. Further, it was mixed with top agar and poured onto minimal agar plates and incubated for 48 hrs at 37°C. After incubation, the number of revertants were counted using a colony counter.

Toxicity of Smoke Shield to bacterial strains were determined by incubating different concentrations of Smoke Shield without mutagen for 48 hrs. and checking the background lawn. Percentage inhibition of mutagenicity was determined by the formula:

$$\% \text{ inhibition of mutagenicity} = \frac{(R_1 - SR) - (R_2 - SR)}{R_1 - SR} \times 100$$

where R<sub>1</sub> is the number of revertants without Smoke Shield, R<sub>2</sub> is the number of revertants with each concentration of Smoke Shield, and SR is spontaneous revertants.

**Determination of antimutagenic activity of Smoke Shield in vivo.** Male Wistar rats were divided into three groups (6 nos/group). Group I was the control group which received vehicle (Ground nut oil, 0.5 ml). Group II animals were treated with benzo[*a*]pyrene (50 mg/kg, i.p) dissolved in ground nut oil. Group III animals were treated with Smoke Shield (0.5 gm/ kg body weight, p.o. for 15 days). 1h after the last dose of the drug administration, benzo[*a*]pyrene (50 mg/kg, i.p) in ground nut oil was given intraperitoneally as a single dose. After treatment with benzo[*a*]pyrene animals in the three groups were put in separate metabolic cages

and 24h urine samples were collected. Urine samples were frozen immediately at  $-70^{\circ}\text{C}$  until analysed.

A similar experiment was also conducted using tobacco extract. Tobacco extract (2.5 gm/kg p.o) was given to group of rats in the presence or in the absence of exposure to Smoke Shield (0.5 gm/kg body weight, p.o. 15 days). 24 hr urine samples were collected and were kept at  $-70^{\circ}\text{C}$  until analysed.

Urine samples were filtered through Whatman No.1 filter paper and 20 ml urine was passed through a XAD-2 Amberlite column (40 mm x 10 mm)(16), to adsorb weekly anionic metabolites of the carcinogens which were eluted with 10 ml acetone. The acetone eluent was evaporated to dryness at  $60^{\circ}\text{C}$  and stored at  $-20^{\circ}\text{C}$ . The residue was reconstituted in 1.5 ml DMSO and 0.1 ml was incubated with *Salmonella* organism in the presence of activation mixture. Bacterial strain used was TA98 in the case of Benzo[a]pyrene and TA102 in the case of tobacco extracts. Experiments were repeated and an average of two experiments were taken for analysis.

*Determination of anti-mutagenic activity of Smoke Shield in human volunteers.* 45 male smokers and 10 male non-smokers were selected for the study. Age of the subjects was between 30-40 and all of them had been smoking for a minimum period of 10 years. All of them were workers in the hospital or nearby tile factory. No diet restriction was made during the study.

Experiment was conducted after obtaining permission from Institutional Human Ethics Committee and individual consents were obtained. All the subjects were instructed to continue their smoking during the study period. They (nos.30) were asked to take Smoke Shield capsule or placebo containing beeswax twice daily for one month period. Weight of the smokers and nonsmokers were monitored every one week. 24 hrs. urine samples were collected before starting the study and after one month of treatment with Smoke Shield. 200 ml of the urine was passed through XAD-2 column and mutagens were eluted in acetone. Acetone was evaporated and residue was reconstituted in DMSO and 0.1 ml was used to determine the mutagenicity with TA98 after activation with S9 fraction.

In order to find out whether Smoke Shield produced any genotoxicity, blood was collected from 15 smokers before treatment and after 1 month treatment with Smoke Shield in heparin coated and uncoated tubes and following parameters were determined: a) Haematological analysis such as white blood cells, granulocytes, RBC, platelet and hamoglobin which were analysed by Abbot Cell Dyne 800, cell counter; b) liver

function tests such as serum glutamate pyruvate transaminase, bilirubin, blood urea and serum creatinine which were done using Hitachi 902 Roche, Germany, autoanalyser.

## Results

*Antimutagenicity of Smoke Shield against direct acting mutagens in vitro.* Smoke Shield was found to significantly inhibit the mutagenicity induced by different direct-acting mutagens. Activity was dependent upon the type of *Salmonella* strain used, nature of mutagenic substance and its concentration and concentration of Smoke Shield. In all cases inhibition was found to be dose dependent (Table I). At the highest concentration used in this experiment (2 mg/plate) Smoke Shield could inhibit sodium azide induced mutagenicity by 38% (TA100) and 40% (TA 1535). Similarly, NPD induced mutagenicity was inhibited by 43% (TA98) and 80% (TA 100). MNNG-induced revertants were inhibited by 20% (TA100) and 20% (TA 1535) which was less significant compared to the inhibition produced to sodium azide and NPD induced revertant formation.

Smoke Shield was also found to inhibit the mutagenicity induced by tobacco extract (Table II). Tobacco extract, at concentrations of 50 mg/plate produced significant number of revertants to TA 102. This was inhibited by Smoke Shield. At 5 mg/plate, Smoke Shield produced an inhibition of revertant colony formation by 98.5%.

*Antimutagenic action of Smoke Shield in vitro against mutagens needing activation.* We have also tested the activity of Smoke Shield against mutagens requiring microsomal activation (Table III). Mutagens used were 2-acetamidofluorene and benzo[a]pyrene. In both cases Smoke Shield was found to produce significant inhibition of revertant colony formation. Smoke Shield produced a concentration-dependent inhibition of the mutagenicity induced by 2-acetamidofluorene. At concentration of 2 mg/plate, Smoke Shield produced an inhibition of 76% and 82% for TA100 and TA98 strain, respectively. Smoke Shield was also found to inhibit the mutagenicity induced by benzo[a]pyrene to TA 98 strain. At 5 mg/plate inhibition produced was 97%.

*Inhibition of urinary mutagenicity by Smoke Shield in vivo.* Smoke Shield was found to inhibit the mutagenicity induced by benzo[a]pyrene *in vivo* as

**Table I** - Antimutagenic activity of Smoke Shield to *S.typhimurium* strains against direct acting mutagens *in vitro*

	Sodium azide (2.5 µg/plate)		MNNG (1 µg/plate)		NPD (20 µg/plate)	
	Average number of revertants/plate					
	TA100	TA 1535	TA 100	TA 1535	TA98	TA 100
Control	768 ± 56	755 ± 73	1574 ± 251	1378 ± 251	2884 ± 165	69 ± 6
Smoke Shield 2 mg/plate	475 ± 71*** (38%)	452 ± 84** (40%)	1263 ± 252* (20%)	1103 ± 80* (20%)	1655 ± 216*** (43%)	14 ± 7*** (80%)
Smoke Shield 0.5 mg/plate	530 ± 22*** (31%)	746 ± 130 (1%)	1759 ± 32	1371 ± 66	2044 ± 107*** (29%)	47 ± 6 (32%)
Smoke Shield 0.1 mg/plate	735 ± 98*** (4%)	692 ± 60 (8%)	2022 ± 227	2451 ± 74	2418 ± 101** (16%)	53 ± 15 (23%)
SR	78 ± 14	32 ± 6	53 ± 8	43 ± 5	28 ± 8	68 ± 10

\*\*\*  $P < 0.001$ , \*\*  $P < 0.005$ , \*  $P < 0.05$

Values are average of three plates after reducing spontaneous revertants. Values in the bracket indicate percent inhibition produced by Smoke Shield.

**Table II** - Effect of Smoke Shield on tobacco induced mutagenicity *in vitro*

<i>Salmonella typhimurium</i> TA 102		
Treatment	Average Number of revertants	Percent inhibition
Tobacco extract alone (50 mg/plate)	453 ± 33	
Tobacco + Smoke Shield (5 mg/plate)	7 ± 3***	98.5
Tobacco + Smoke Shield (1 mg/plate)	91 ± 9***	79.9
Tobacco + Smoke Shield (0.2 mg/plate)	139 ± 17***	69.3
Spontaneous revertants	264 ± 15	

\*\*\*  $P < 0.001$  significantly differ from tobacco extract alone treated group. Values are average of three plates after deducting spontaneous revertants (264 ± 15).

evident from the reduction of number of revertants in the urine. Inhibition in revertant formation was 73.8% when treated with Smoke Shield (Fig.1). Smoke Shield was also found to inhibit the urinary mutagenicity induced by administration of tobacco extract in rats. (Fig.2). Smoke Shield treated rats did not produce any tobacco induced mutagenicity in the urine as seen from the revertant formation.

*Inhibition of urinary mutagenicity of smokers.* Administration of Smoke Shield was done on 45 smokers and 10 non-smokers for a period of one month. At the threshold it was found that very few study subjects had increased urinary mutagenicity, which may be related with practice of smoking local brands that contain lower tobacco content as well as dietary protection by spices and tea. Only 5 subjects showed significantly elevated levels of urinary mutagenicity. It was found that increased mutagenicity in these subjects were significantly lowered by administration of Smoke Shield (Table IV).

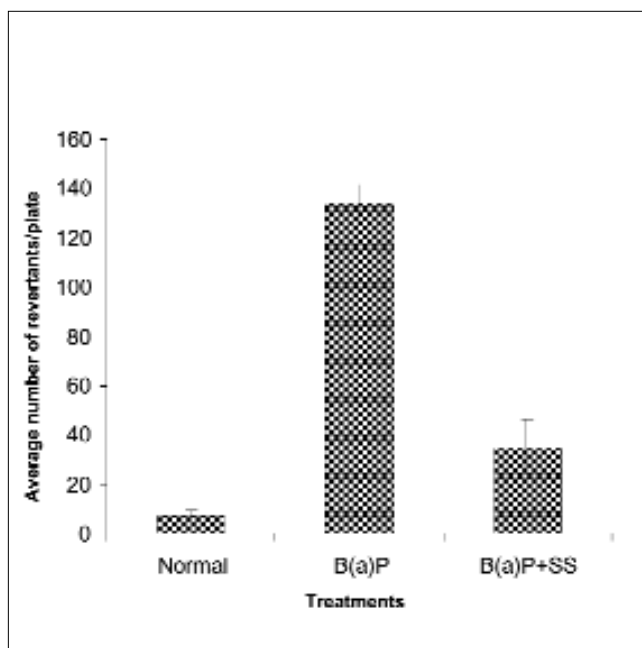
*Toxicity of Smoke Shield to human volunteers.* Smoke Shield administration at a concentration of 1 gm/kg for one month did not produce any apparent toxicity to mice (data not included). In order to deter-

**Table III** - Antimutagenic activity of Smoke Shield to *Salmonella typhimurium* against mutagens needing activation *in vitro*

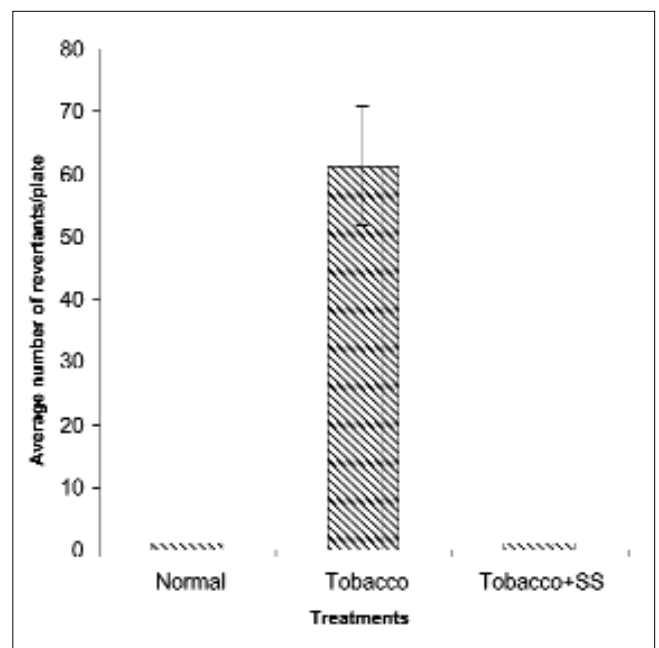
	2-Acetamidofluorene (20 µg/plate)	Average number of revertants/plate		Benzo(a)pyrene (5 µg/plate)
	TA 100	TA 98	TA 98	TA 98
Control	479 ± 26	379 ± 34		73 ± 15
2 mg Smoke Shield/plate	119 ± 22*** (76%)	69 ± 14*** (82%)		3 ± 7*** (97%)
0.5 mg Smoke Shield/plate	181 ± 19*** (62%)	129 ± 16*** (66%)		15 ± 8*** (81%)
0.1 mg Smoke Shield/plate	319 ± 18** (33%)	158 ± 16*** (58%)		25 ± 8** (66%)
SR	151 ± 17	31 ± 6		35 ± 6

\*\*\*  $P < 0.001$ , \*\*  $P < 0.005$ .

Values are average of these plates after reducing spontaneous revertants. Values in the bracket indicate percent inhibition produced by Smoke Shield.



**Fig. 1** - Inhibition of urinary mutagenicity induced by Benzo[a]pyrene in rats by Smoke Shield. Rats (nos.6/group) were treated with Smoke Shield (SS) (0.5 gm/kg, p.o) for 15 days, Benzo[a]pyrene (B[a]P) (50 gm/kg, p.o) in ground nut oil was given i.p. Animals were kept in metabolic cages and 24 h urine sample collected. Mutagenicity was determined using TA 98 strain. Values are after deducting the spontaneous revertants. \*\*\*  $P < 0.001$  significantly differ from B(a)P alone treated group.



**Fig. 2** - Inhibition of urinary mutagenicity induced by tobacco extract in rats by Smoke Shield. Rats (nos.6/group) were treated with Smoke Shield (SS) (0.5 gm/kg, p.o) for 15 days. Tobacco extract (2.5 gm/kg) was given orally. Animals were kept in metabolic cages and 24 h urine samples were collected. Mutagenicity was determined using TA 102 strain. Values are after deducting the spontaneous revertants. \*\*\* $P < 0.001$  significantly differ from tobacco alone treated group.

**Table IV** - Inhibition of urinary mutagenicity in smokers treated with Smoke Shield for one month

Smoker No. Treatment	Average number of revertants/plate (TA 98)	
	Before treatment	After
10	204 ± 6	33
16	195 ± 10	0
17	302 ± 82	39
19	52 ± 5	0
20	86 ± 11	0

*Values are after deducting spontaneous revertants (34.6 ± 4.5)*

mine whether Smoke Shield produced any toxicity to human volunteers, haematological changes, hepatic and renal function tests were done in the blood of human volunteers before and after Smoke Shield administration for one month. Administration of Smoke Shield did not produce any weight change in volunteers (average weight change was 0.1 gm ± 1.3 kg) compared to placebo 0.46 ± 3.58 kg. Administration of Smoke Shield for one month did not produce

any significant change in haematological parameters such as WBC, Granulocyte, RBC, Platelets and haemoglobin (Table V). Smoke Shield administration for one month also did not produce any significant change in liver function and renal function as compared with placebo (Table VI).

## Discussion

The results presented in this report indicate significant inhibition of mutagenicity induced by various environmental mutagens by Smoke Shield. This preparation formulated by New Chapter, USA has several spices that have been reported to be antioxidant, antimutagenic as well as anticarcinogenic. For example turmeric, a rhizome which has been used as a food additive in many Asian countries, has been proven for its effect on various types of carcinogens such as benzo[a]pyrene, aflatoxin and nitroso compounds in animals, cell culture as well as in bacteria (17-19). Curcuminoids in turmeric are confirmed active ingredients, and other polar constituents and the oil turmeron are independently and synergistically active (20). Similarly, green tea that is being used in China and Japan has several antioxidant chemopreventive agents such as epigallocatechingallate, while presence of genestein inhibits protein kinases that thereby

**Table V** - Effect of Smoke Shield on haematological parameters in smokers

	Total WBC (K/μl)	Granulocytes ((K/μl)	RBC (M/μl)	Platelets (K/μl)	Hb (g/dl)
Before	8.72 ± 2.7	4.5 ± 1.94	4.25 ± 0.92	255 ± 82	14.32
After	8.22 ± 2.02	4.15 ± 1.09	4.53 ± 0.75	248 ± 56	14.42

**Table VI** - Effect of Smoke Shield in liver function and renal function in smokers

	Serum glutamate pyruvate transaminase (U/l)	Bilirubin Total (mg/dl)	Blood urea nitrogen (mg/dl)	Serum creatinine (mg/dl)
Before treatment	24.0 ± 8.95	0.62 ± 0.13	19.8 ± 3.0	0.72 ± 0.15
After treatment	25.93 ± 14.0	0.72 ± 0.12	13.37 ± 3.0	0.83 ± 0.158

*Smokers were treated with Smoke Shield 1 bd/one month. Data is average of 15 subjects*

reduce the proliferation of tumour cells (21). Other ingredients present in the preparation are extracts of clove (22) and ginger (23), which have significant antioxidant activity as well as parsley, peppermint and rosemary. Phenolic antioxidants are present in all these spices.

Conventional turmeric extracts are typically prepared by isolating through solvent extraction one or more "standardized" constituents, specifically one or more curcuminoids. On the other hand, the turmeric extract used in the Smoke Shield is a full spectrum extract of the herb. In Smoke Shield, turmeric is first extracted using CO<sub>2</sub> gas under supercritical conditions. After extraction of the non-polar fractions, turmeric residue is subjected to a second state "post-supercritical" hydroethanolic extraction to extract the hydrophilic fraction. This dual extraction process presents a broad spectrum extract that is highly concentrated, chemical-solvent free, undamaged by heat or chemical stress, and rich with all the healing and protective turmeric oils, resins, and curcuminoids. This turmeric extract is provided with other herbs that have demonstrated profound synergy with turmeric, multiplying the power of this most important herb by several fold.

Smoke Shield was formulated primarily for reducing the smoke-induced damage in humans. Studies presented here indicate that it is highly effective in reducing the extent of mutation produced by not only smoke but many xenobiotic compounds. They not only include the direct acting mutagenic substances such as sodium azide, MNNG and NPD but also mutagens needing metabolic activation such as benzo[*a*]pyrene and 2-acetamidofluorene. In fact, Smoke Shield was found to be more active on mutagens needing activation as it may be inhibiting the activation of mutagens mediated by P450 enzymes and thereby reducing DNA-adduct formation.

Monitoring the urinary mutagenicity is a way to understand the antimutagenic potential of a preparation. Smoke Shield was found to significantly inhibit the urinary mutagenicity induced in rats by benzo[*a*]pyrene as well as by tobacco extract indicating that it is effective in reducing the mutagenicity both *in vitro* as well as *in vivo*. Polossa et al. have shown that urinary mutagenicity in cigarette smokers is considerably inhibited by one of Smoke Shield's ingredients, turmeric (24).

Preliminary studies have shown that Smoke Shield could inhibit the urinary mutagenicity of smokers who had significantly elevated urinary mutagenicity. Increased mutagenicity in cigarette smokers was

reduced to normal by Smoke Shield administration for one month.

Smoke Shield administration for one month did not produce any apparent toxicity to human volunteers. There was no weight change in these volunteers nor did they produce any adverse reactions. Haematological parameters such as WBC, RBC, haemoglobin and platelets did not alter in these volunteers after one month's administration of Smoke Shield. Both renal function test and hepatic function tests in the volunteers did not make any change after Smoke Shield treatment. Similar results were also seen in rats treated with Smoke Shield for one month.

The mechanism of Smoke Shield could be diverse. Because of the antioxidants present, Smoke Shield may be inhibiting the oxygen radicals produced, which are very important in reducing the formation DNA strand breaks (25). Smoke Shield could also inhibit the Phase I enzymes thereby reducing the activation of mutagens. *In vivo* it could also increase glutathione S-transferase, which is involved in the detoxification mechanism. It may also inhibit the protein kinases involved in the oncogene activation as well as cell proliferation. The combined effects of these beneficial processes may be responsible for the activity of these interesting properties in this preparation.

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