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Effect of *Aegle marmelos* on Biotransformation Enzyme Systems and Protection Against Free-radical-mediated Damage in Mice

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Abstract

The effect of hydroalcoholic (80% ethanol, 20% water) extract of leaves of *Aegle marmelos* was examined on carcinogen-metabolizing phase-I and phase-II enzymes, antioxidant enzymes, glutathione content, lactate dehydrogenase and lipid peroxidation, using two doses of dried extract (50 and 100 mg kg^{-1} daily for 14 days), in the liver of mice. The modulatory effect of the extract was also examined on extrahepatic organs (lung, kidney and fore-stomach) for effects on the activity of glutathione S-transferase, DT-diaphorase, superoxide dismutase and catalase.

Extract treatment significantly increased the basal levels of acid-soluble sulphydryl (−SH) content, cytochrome P450, NADPH-cytochrome P450 reductase, cytochrome b5, NADH-cytochrome b5 reductase, glutathione S-transferase, DT-diaphorase, superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase in the liver. *Aegle* acted as a bifunctional inducer since it induced both phase-I and phase-II enzyme systems. Both doses significantly decreased the activity of lactate dehydrogenase and formation of malondialdehyde in liver, suggesting a role in cytoprotection as well as protection against pro-oxidant-induced membrane damage. Butylated hydroxyanisole (positive control) induced almost all the antioxidative parameters measured in this study. The extract was effective in inducing glutathione S-transferase, DT-diaphorase, superoxide dismutase and catalase in lung, glutathione S-transferase, DT-diaphorase and superoxide dismutase in fore-stomach, and DT-diaphorase and superoxide dismutase in lung.

These significant changes in the levels of drug-metabolizing enzymes and antioxidative profiles are strongly indicative of the chemopreventive potential of this plant, especially against chemical carcinogenesis.

Aegle marmelos is a spinous tree belonging to the Rutaceae family. Its edible fruits are valued in indigenous medicine. The leaf, root, bark and seed of *Aegle* are also valued in Ayurvedic medicine in India. The root is an ingredient of dasmula (ten roots), a medicine commonly used by Ayurvedic practitioners. The leaves are bitter and used as a remedy for ophthalmia, ulcers, dropsy, cholera and beriberi associated with weakness of heart. Fresh aqueous and alcoholic leaf extracts of *Aegle* are reported to have a cardiotonic effect like digitalis and to decrease the requirement of circulatory stimulants. It contains alkaloids (aegeline, aegelenine) and essential oil. Aegeline is efficacious in asthma and other respiratory problems. The essential oil contains cineole, *p*-cymene, citronellol, citral, cuminaldehyde, D-limonene and eugenol, and has shown a broad spectrum of antibacterial and antifungal activity (Pattnaik et al 1996; Rana et al 1997). The aqueous decoction of the leaf has been shown to have a significant hypoglycaemic effect (Karunanayake et al 1984). *Aegle* leaf extract also helps in the regeneration of damaged pancreas (β-cells) in diabetic rats (Das et al 1996) and is found to be as effective as insulin in restoration of blood glucose and body weight to normal levels (Seema et al 1996).

In this study, an attempt has been made to investigate the cancer chemopreventive potential of *Aegle marmelos* by evaluating its modulatory

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effects on carcinogen-metabolizing enzymes, as well as on the antioxidative parameters involved in maintaining the reducing milieu of the cell.

Materials and Methods

Chemicals

Butylated hydroxyanisole, 1-chloro-2,4-dinitrobenzene, 5,5′-dithiobis-2-nitrobenzoic acid, reduced glutathione, oxidized glutathione, pyrogallol, 2,6-dichlorophenol-indophenol, potassium ferricyanide, triton X-100, ethylenediamine tetra-acetic acid (EDTA), bovine serum albumin (BSA), sodium pyruvate, thiobarbituric acid, reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma Chemical Co. (St Louis, MO). All other chemicals were obtained from local firms (India) and were of the highest purity grade.

Animals

Random-bred Swiss albino male mice (6–8 weeks old) were used for the study. They were maintained in our air-conditioned animal facility (Jawaharlal Nehru University, New Delhi) with a 12-h light–dark cycle, and provided (unless otherwise stated) with standard food pellets (Hindustan Lever Ltd, India) and allowed free access to tap water.

Preparation of the plant extract

The fresh leaves plucked from *Aegle marmelos* were washed thoroughly with distilled water and used for hydroalcoholic (80% ethanol and 20% distilled water) extraction in a soxhlet apparatus. The extract was lyophilized and stored at 4°C. The dried extract obtained was mixed with distilled water to obtain two dose levels (50 mg in 0·03 mL and 100 mg in 0·03 mL).

Experimental design

This experiment was designed to study the effect of *Aegle marmelos* on drug-metabolizing enzymes and antioxidant status in mice. Mice were randomly assorted into the following groups: group I ($n = 8$) mice were put on a normal diet and sham-treated with 0·03 mL distilled water through oral gavage, daily for 14 days (this group served as control); group II ($n = 8$) mice were put on a normal diet and treated with 50 mg kg^{-1} body-weight of the plant extract, suspended in 0·03 mL distilled water, through oral gavage daily for 14 days; group III ($n = 8$) mice were put on a normal diet and treated with 100 mg kg^{-1} body-weight of the plant extract, suspended in 0·03 mL distilled water, through oral gavage daily for 14 days; group IV ($n = 8$) mice were put on a diet containing 0·75% butylated hydroxyanisole for 14 days (this group served as a positive control).

Biochemical studies in liver, lung, kidney and fore-stomach

Mice were killed by cervical dislocation and the entire liver was then perfused immediately with ice-cold 0·9% NaCl and rinsed in chilled 0·15 M Tris-KCl buffer (pH 7·4). The liver was homogenized in ice-cold 0·15 M Tris-KCl buffer (pH 7·4) to yield a 10% (w/v) homogenate. A sample of this homogenate (0·5 mL) was used for assaying acid-soluble sulphydryl groups (–SH), while the remainder was centrifuged at 10 000 *g* for 20 min. The resultant supernatant was centrifuged at 105 000 *g* for 60 min. The supernatant (cytosol fraction), after discarding any floating lipid layer and appropriate dilution, was used for assaying total cytosolic glutathione S-transferase, DT-diaphorase, lactate dehydrogenase and antioxidant enzymes. The pellet representing microsome was used for assaying cytochrome P450, cytochrome b5, cytochrome P450 reductase, cytochrome b5 reductase and lipid peroxidation.

Lung, kidney and stomach were carefully removed, along with the liver, and rinsed in chilled 0·15 M Tris-KCl (pH 7·4). The fore-stomach was separated from the glandular stomach and cleaned of all its contents. These organs were then blotted dry, weighed quickly and homogenized in ice-cold 0·15 M Tris-KCl buffer (pH 7·4) to yield a 10% (w/v) homogenate. The latter was next subjected to high-speed centrifugation at 15 000 *g* for 30 min at 4°C. The resulting supernatant was used as such for assaying activity of glutathione S-transferase, DT-diaphorase, superoxide dismutase and catalase.

Estimation of cytochrome P450 and cytochrome b5

Cytochrome P450 was determined by using the carbon monoxide difference spectra. Both cytochrome P450 and cytochrome b5 contents were estimated in the microsomal suspension by the method of Omura & Sato (1964), using an absorption coefficient of 91 and 185 cm^2 $mmol^{-1}$, respectively.

Determination of NADPH-cytochrome P450 reductase and NADH-cytochrome b5 reductase

Assay of NADPH-cytochrome P450 reductase was performed according to the method of Omura & Takesue (1970) with some modifications, measuring the rate of oxidation of NADPH at 340 nm. The

enzyme activity was calculated using an extinction coefficient of $6{\cdot}22\,mM^{-1}\,cm^{-1}$. One unit of enzyme activity is defined as that causing the oxidation of 1 mole of NADPH per min.

NADH-cytochrome b5 reductase was assayed according to the method of Mihara & Sato (1972), measuring the rate of reduction of potassium ferricyanide at 420 nm by NADH. The enzyme activity was calculated using an extinction coefficient of $1{\cdot}02\,mM-1\,cm^{-1}$. One unit of enzyme activity is defined as that causing the reduction of 1 mole of ferricyanide per min.

Determination of activity of glutathione S-transferase and DT-diaphorase

The cytosolic glutathione S-transferase activity was determined according to the procedure of Habig et al (1974). The specific activity of glutathione S-transferase is expressed as μmol of reduced glutathione-1,chloro-2,4-dinitrobenzene conjugate formed per min per mg protein using an extinction coefficient of $9{\cdot}6\,mM^{-1}\,cm^{-1}$.

DT-diaphorase activity was measured as described by Ernster et al (1962). The activity was calculated using an extinction coefficient of $21\,mM^{-1}\,cm^{-1}$. One unit of enzyme activity is defined as the amount of enzyme required to reduce one μmol of 1,chloro-2,4-dinitrobenzene per min.

Estimation of acid-soluble sulphydryl group

The level of acid-soluble sulphydryl group was estimated as total non-protein sulphydryl group by the method described by Moron et al (1979). Reduced glutathione was used as a standard to calculate nmol of −SH content per gram of tissue.

Determination of activity of glutathione reductase and glutathione peroxidase

Glutathione reductase was determined by the procedure of Carlberg & Mannervik (1985). One unit of enzyme activity has been defined as nmol of NADPH consumed per min per mg of protein, based on an extinction coefficient of $6{\cdot}22\,mM^{-1}\,cm^{-1}$. Glutathione peroxidase activity was measured by the coupled assay method as described by Paglia & Valentine (1967). One unit of enzyme activity has been defined as nmol of NADPH consumed per min per mg of protein, based on an extinction coefficient of $6{\cdot}22\,mM^{-1}\,cm^{-1}$.

Determination of activity of catalase and superoxide dismutase

Catalase was estimated at 240 nm by monitoring the decomposition of H_2O_2 as described by Aebi (1984). The specific activity of catalase has been expressed as moles of H_2O_2 reduced per min per mg of protein. Superoxide dismutase was assayed by the method of Marklund & Marklund (1974). A single unit of enzyme is defined as the quantity of superoxide dismutase required to produce 50% inhibition of auto-oxidation.

Estimation of lipid peroxidation

Lipid peroxidation in microsomes was estimated by the thiobarbituric acid-reactive substances (TBARS) method, as described by Varshney & Kale (1990) and is expressed in terms of malondialdehyde formed per mg of protein.

Determination of lactate dehydrogenase

Lactate dehydrogenase was assayed by measuring the rate of oxidation of NADH, according to the method of Bergmeyer & Bernt (1971). One unit of enzyme activity is defined as that which causes the oxidation of 1 μmol of NADH per min.

Estimation of protein

Protein was determined by the method of Lowry et al (1951) using BSA as standard, at 660 nm.

Data analysis

Results are presented as mean ± s.d. Statistical analysis was performed by Mann–Whitney rank sum test. $P < 0{\cdot}05$ was considered significant.

Results

The findings of this study are shown in Tables 1–5. The mice treated with the hydroalcoholic extract of *Aegle* leaf and butylated hydroxyanisole did not show any significant alterations in body-weight or body-weight gain as compared with the control group of mice.

Hepatic studies

There was no significant change in the liver-to-body-weight ratio (L-S index) in *Aegle* and butylated-hydroxyanisole-treated mice. The protein levels in microsomal and cytosolic fractions of liver, did not show any significant change following extract treatment whereas in butylated-hydroxyanisole-treated mice the levels were increased 1·14 fold ($P < 0{\cdot}05$) and 1·21 fold ($P < 0{\cdot}01$), respectively (Table 1).

Cytochrome P450 system

Different components of the cytochrome P450 system were determined in the microsomal fraction

Table 1. Modulatory influence of two different doses of *Aegle marmelos* leaf extract and butylated hydroxyanisole on body-weight gain and toxicity-related parameters in mice.

Groups	Treatment	Body-weight (g)	
		Initial	Final
I	Control	27·30 ± 1·04 (1·00)	28·00 ± 1·16 (1·00)
II	*Aegle* (50 mg kg^{-1})	26·30 ± 0·76 (0·96)	26·80 ± 1·04 (0·96)
III	*Aegle* (100 mg kg^{-1})	26·80 ± 1·04 (0·98)	28·50 ± 0·93 (1·02)
IV	BHA (0·75% in diet)	26·30 ± 0·93 (0·96)	26·80 ± 1·02 (0·96)
Liver weight[a]	Lactate dehydrogenase[b]	Protein (mg mL^{-1})	
		Microsome	Cytosol
5·440 ± 0·523 (1·00)	3·150 ± 0·428 (1·00)	4·840 ± 0·593 (1·00)	3·570 ± 0·404 (1·00)
5·100 ± 0·476 (0·94)	2·320 ± 0·370† (0·74)	4·960 ± 0·356 (1·02)	3·630 ± 0·331 (1·02)
5·82 ± 0·42 (1·07)	2·070 ± 0·333† (0·66)	4·930 ± 0·339 (1·02)	3·410 ± 0·214 (0·96)
5·730 ± 0·550 (1·05)	1·930 ± 0·133† (0·61)	5·520 ± 0·551* (1·14)	4·300 ± 0·298** (1·21)

Values are expressed as mean ± s.d. of 6–8 animals. Values in parentheses represent relative changes in parameters assessed. [a]Expressed as percentage of final body-weight. [b]Expressed as μmol (mg protein)$^{-1}$. *$P < 0.05$, **$P < 0.01$, †$P < 0.005$, compared with control. Control group received treatment with distilled water only. BHA, butylated hydroxianisole. Treatment duration: 14 days.

of mouse liver. Haem proteins, cytochrome P450 and cytochrome b5, significantly increased at the lower dose level of *Aegle* treatment as compared with their control values. The level of induction at the lower dose was 1·15 fold ($P < 0.01$) in cytochrome and 1·12 fold ($P < 0.05$) in cytochrome b5 (Table 2).

Aegle-treated groups presented significant elevation in the specific activities of NADPH-cytochrome P450 reductase and NADH-cytochrome b5 reductase as compared with their controls. For cytochrome P450 reductase, elevation in the activity was 1·23 fold ($P < 0.005$) in group II and 1·32 fold ($P < 0.005$) in group III, showing a dose-dependent response. The corresponding increase in the activity of cytochrome b5 reductase was 1·22 ($P < 0.001$) and 1·15 fold ($P < 0.05$). Butylated hydroxyanisole induced only cytochrome b5 ($P < 0.05$) (Table 2).

Phase-II enzymes

In phase-II enzymes, glutathione S-transferase and DT-diaphorase were assayed in the cytosol of liver. Both these enzymes showed a significant dose-dependent increase in their activities in *Aegle*-treated groups of mice. At the lower dose level of treatment, specific activities of glutathione S-transferase and DT-diaphorase were elevated by 1·44 ($P < 0.005$) and 1·14 fold ($P < 0.05$) and at the higher dose, the activities were increased by 1·66 ($P < 0.005$) and 1·20 ($P < 0.01$) fold respectively.

Table 2. Modulatory influence of two different doses of *Aegle marmelos* leaf extract and butylated hydroxyanisole on mouse hepatic phase-I and phase-II drug-metabolizing enzyme levels.

Group	Treatment	CYP P450[a]	CYP b5[a]
I	Control	0·470 ± 0·015 (1·00)	0·183 ± 0·008 (1·00)
II	*Aegle* (50 mg kg^{-1})	0·543 ± 0·024** (1·15)	0·204 ± 0·016* (1·12)
III	*Aegle* (100 mg kg^{-1})	0·423 ± 0·042 (0·90)	0·171 ± 0·011 (0·93)
IV	BHA (0·75% in diet)	0·469 ± 0·025 (1·00)	0·233 ± 0·014* (1·28)
CYP P450 R[b]	CYP b5 R[c]	GST[d]	DTD
0·377 ± 0·036 (1·00)	3·120 ± 0·250 (1·00)	4·560 ± 0·157 (1·00)	0·021 ± 0·0019 (1·00)
0·463 ± 0·033† (1·23)	3·81 ± 0·214†† (1·22)	6·570 ± 0·508† (1·44)	0·0240 ± 0·0015* (1·14)
0·497 ± 0·024† (1·32)	3·600 ± 0·325* (1·15)	7·59 ± 0·438† (1·66)	0·0250 ± 0·0015** (1·20)
0·416 ± 0·033 (1·10)	2·900 ± 0·233 (0·93)	13·63 ± 0·81** (2·99)	0·0470 ± 0·0057** (2·24)

Values are expressed as mean ± s.d. of 6–8 animals. Values in parentheses represent relative change in parameters assessed. Control group received treatment with distilled water only. *$P < 0.05$, **$P < 0.01$, †$P < 0.005$, ††$P < 0.001$, compared with control. Units of activity: [a]nmol (mg protein)$^{-1}$; [b]μmol of NADPH oxidized per min per mg of protein; [c]μmol of NADH oxidized per min per mg of protein; [d]μmol of 1-chloro-2,4-dinitrobenzene-reduced glutathione conjugate formed per min per mg of protein; [e]μmol of 2,6-dichlorophenol-indophenol reduced per min per mg ofprotein. BHA, butylated hydroxyanisole; CYP P450 R, cytochrome P450 reductase; CYP b5 R, cytochrome b5 reductase; GST, glutathione S-transferase and DTD, DT-diaphorase.

Table 3. Modulatory influence of two different doses of *Aegle marmelos* leaf extract and butylated hydroxyanisole on mouse hepatic antioxidant-related parameters and lipid peroxidation.

Groups	Treatment	GSH[a]	GPX[b]
I	Control	29·50 ± 2·90 (1·00)	62·00 ± 3·18 (1·00)
II	*Aegle* (50 mg kg^{-1})	39·70 ± 4·26† (1·34)	82·00 ± 6·02** (1·32)
III	*Aegle* (100 mg kg^{-1})	41·60 ± 4·31† (1·41)	90·02 ± 5·34† (1·45)
Gr IV	BHA (0·75% in diet)	64·10 ± 5·16† (2·17)	59·63 ± 4·76 (0·96)
GR[b]	SOD[c]	CAT[d]	LPO[e]
34·20 ± 2·66 (1·00)	10·100 ± 0·626 (1·00)	47·00 ± 3·66 (1·00)	1·095 ± 0·134 (1·00)
42·40 ± 3·32** (1·24)	12·200 ± 0·986† (1·21)	61·20 ± 3·88** (1·30)	0·803 ± 0·069† (0·73)
43·70 ± 3·29† (1·27)	13·800 ± 1·242** (1·37)	63·60 ± 3·60** (1·35)	0·655 ± 0·861† (0·60)
50·00 ± 1·94† (1·46)	9·900 ± 0·605 (0·98)	49·10 ± 2·13 (1·04)	0·666 ± 0·050** (0·61)

Values are expressed as mean ± s.d. of 6–8 animals. Values in parentheses represent relative change in parameters assessed. Control group received treatment with distilled water only. $^{**}P < 0.01$, †$P < 0.005$, compared with control. Units of activity: [a]nmol of reduced glutathione per g of tissue; [b]nmol of NADPH consumed per min per mg of protein; [c]specific activity expressed as μmol (mg protein)$^{-1}$; [d]μmol of H_2O_2 consumed per min per mg of protein; [e]nmol of malondialdehyde formed per mg of protein. BHA, butylated hydroxyanisole; GSH, reduced glutathione; GPX, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase; CAT, catalase; LPO, lipid peroxidation.

Butylated hydroxyanisole significantly ($P < 0.01$) enhanced the activities of both glutathione S-transferase and DT-diaphorase (Table 2).

Antioxidative parameters

Antioxidative parameters (reduced glutathione, glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase) showed a dose-related increase in their level/activity following *Aegle* treatment. The level of reduced glutathione was increased by 1·34 and 1·41 ($P < 0.005$) fold in Group II and Group III, respectively. At the lower dose level of treatment, the specific activity of glutathione peroxidase, glutathione reductase,

Table 4. Modulatory influence of two different doses of *Aegle marmelos* leaf extract and butylated hydroxyanisole on detoxifying and antioxidant enzyme profiles in lung and kidney of mouse.

Groups	Treatment	Organ weight (%)	GST[a]
Lung			
I	Control	0·612 ± 0·053 (1·00)	0·288 ± 0·029 (1·00)
II	*Aegle* (50 mg kg^{-1})	0·608 ± 0·037 (0·99)	0·301 ± 0·036 (1·05)
III	*Aegle* (100 mg kg^{-1})	0·665 ± 0·058 (1·09)	0·305 ± 0·032 (1·06)
IV	BHA (0·75% in diet)	0·639 ± 0·045 (1·04)	0·422 ± 0·025† (1·47)
Kidney			
I	Control	1·090 ± 0·042 (1·00)	0·263 ± 0·029 (1·00)
II	*Aegle* (50 mg kg^{-1})	1·050 ± 0·075 (0·96)	0·306 ± 0·037* (1·16)
III	*Aegle* (100 mg kg^{-1})	1·100 ± 0·094 (1·01)	0·325 ± 0·024† (1·24)
IV	BHA (0·75% in diet)	1·000 ± 0·067 (0·92)	0·331 ± 0·019† (1·26)
DTD[b]	SOD[c]	CAT[d]	Protein (mg mL^{-1})
Lung			
0·0140 ± 0·0007 (1·00)	2·990 ± 0·431 (1·00)	21·70 ± 3·29 (1·00)	3·930 ± 0·400 (1·00)
0·015 ± 0·0014 (1·07)	4·200 ± 0·594** (1·41)	24·40 ± 3·17 (1·12)	3·920 ± 0·324 (1·00)
0·0160 ± 0·0008* (1·14)	4·01 ± 0·63* (1·34)	21·40 ± 3·07 (0·99)	3·590 ± 0·339 (0·91)
0·0160 ± 0·0016* (1·14)	3·16 ± 0·34 (1·06)	33·10 ± 2·29** (1·52)	3·780 ± 0·303 (0·96)
Kidney			
0·0200 ± 0·0011 (1·00)	5·950 ± 0·508 (1·00)	48·6 ± 3·8 (1·00)	5·340 ± 0·409 (1·00)
0·0210 ± 0·0011 (1·05)	7·690 ± 0·421† (1·30)	66·50 ± 6·31† (1·37)	4·92 ± 0·32* (0·92)
0·0240 ± 0·0022** (1·20)	7·410 ± 0·589† (1·25)	78·10 ± 7·08† (1·61)	5·010 ± 0·547 (0·94)
0·0310 ± 0·0008** (1·55)	6·72 ± 0·48** (1·13)	69·40 ± 3·16† (1·43)	5·220 ± 0·527 (0·98)

Values are expressed as mean ± s.d. of 6–8 animals. Values in parentheses represent relative change in parameters assessed. Control group received treatment with distilled water only. $^{*}P < 0.05$, $^{**}P < 0.01$, †$P < 0.005$, compared with control. Units of activity: [a]μmol of 1-chloro-2,4-dinitrobenzene-recuced glutathione conjugate formed per min per mg of protein; [b]μmol of 2,6-dichlorophenol-indophenol reduced per min per mg of protein; [c]specific activity expressed as μmol (mg protein)$^{-1}$; [d]μmol of H_2O_2 consumed per min per mg of protein. BHA, butylated hydroxyanisole; GST, glutathione S-transferase; DTD, DT-diaphorase; SOD, superoxide dismutase; CAT, catalase.

Table 5. Modulatory influence of two different doses of *Aegle marmelos* leaf extract and butylated hydroxyanisole on detoxifying and antioxidant enzyme profiles in fore-stomach of mouse.

Groups	Treatment	Fore-stomach weight (%)	GST[a]
I	Control	0·171 ± 0·016 (1·00)	0·451 ± 0·045 (1·00)
II	*Aegle* (50 mg kg^{-1})	0·167 ± 0·023 (0·98)	0·56 ± 0·052** (1·24)
III	*Aegle* (100 mg kg^{-1})	0·187 ± 0·018 (1·09)	0·595 ± 0·050† (1·32)
Gr IV	BHA (0·75% in diet)	0·171 ± 0·014 (1·00)	0·670 ± 0·057† (1·49)
DTD[b]	SOD[c]	CAT[d]	Protein (mg mL^{-1}
0·0140 ± 0·0014 (1·00)	3·870 ± 0·406 (1·00)	ND	5·640 ± 0·320 (1·00)
0·0150 ± 0·0014 (1·07)	6·640 ± 0·546† (1·72)	ND	4·980 ± 0·286** (0·88)
0·0160 ± 0·0016* (1·14)	5·580 ± 0·347† (1·44)		5·210 ± 0·368* (0·91)
0·0160 ± 0·0008** (1·14)	6·940 ± 0·751† (1·79)	ND	5·560 ± 0·605 (0·99)

Values are expressed as mean ± s.d. of 6–8 animals. Values in parentheses represent relative change in parameters assessed. Control group received treatment with distilled water only. *$P < 0·05$, **$P < 0·01$, and †$P < 0·005$, compared with control. Units of activity: [a]μmol of 1-chloro-2,4-dinitrobenzene-reduced glutathione conjugate formed per min per mg of protein, [b]μmol of 2,6-dichlorophenol-indophenol reduced per min per mg of protein; [c]specific activity expressed as μmol (mg protein)$^{-1}$; [d]μmol of H_2O_2 consumed per min per mg of protein. BHA, butylated hydroxyanisole; GST, glutathione S-transferase; DTD, DT-diaphorase; SOD, superoxide dismutase; CAT, catalase; ND, not detectable.

superoxide dismutase and catalase were significantly increased by 1·32, 1·24, 1·21 and 1·30 fold, respectively; at the higher dose level of treatment the increases were 1·45, 1·27, 1·37 and 1·35 fold, respectively, compared with their control values. Butylated hydroxyanisole significantly induced the basal levels of reduced glutathione and glutathione reductase (Table 3).

Lipid peroxidation and lactate dehydrogenase

Malondialdehyde formation was taken as an indicator of lipid peroxidation. Lipid peroxidation and specific activity of lactate dehydrogenase both showed a dose-dependent decrease following the administration of *Aegle*. At the lower dose level of treatment, the values were reduced by 27% and 26% ($P < 0·005$), respectively and at the higher dose level of treatment they were reduced by 40% and 34% ($P < 0·005$), respectively, as compared with their control values. Butylated hydroxyanisole significantly inhibited the levels of both lipid peroxidation and lactate dehydrogenase (Tables 1 and 3).

Extrahepatic studies

The relative weights of extrahepatic organs examined (lung, kidney and fore-stomach) remained unaffected following treatment with *Aegle* and butylated hydroxyanisole. Protein contents generally tended to decrease in *Aegle*-treated groups. The protein content was significantly reduced in the kidney in the group treated with the lower dose of *Aegle* and in the fore-stomach in both of the *Aegle*-treated groups. The protein level in the butylated-hydroxyanisole-treated group of animals remained unaltered (Tables 4–5).

The treatment with *Aegle* leaf extract showed significant, as well as dose-dependent, increases in the specific activity of glutathione S-transferase in kidney and fore-stomach relative to their control values. In the kidney and fore-stomach, glutathione S-transferase activity was elevated by 16% ($P < 0·05$) and 24% ($P < 0·01$), respectively, at the lower dose level of treatment and by 24% and 32% ($P < 0·005$), respectively, at the higher dose level of treatment. Butylated hydroxyanisole significantly increased the activity of glutathione S-transferase in all three extrahepatic organs examined (Tables 4–5).

The specific activity of DT-diaphorase increased in lung, kidney and fore-stomach only at the higher dose level of *Aegle* treatment as compared with respective control values. DT-diaphorase activity in Group III was elevated by 1·14 ($P < 0·05$), 1·20 ($P < 0·01$) and 1·14 ($P < 0·05$) fold in lung, kidney and fore-stomach, respectively. Butylated hydroxyanisole significantly increased the activity of DT-diaphorase in all three extrahepatic organs examined (Tables 4–5).

In lung, treatment with *Aegle* extract resulted in an increase in the activity of superoxide dismutase by 41% ($P < 0·01$) in Group II and by 34% ($P < 0·05$) in Group III as compared with that in control group. In the kidney there was significant enhancement, at both dose levels of *Aegle* treatment, by 1·30 and 1·25 ($P < 0·005$) fold in Group II and Group III, respectively. The specific activity of superoxide dismutase in the fore-stomach was elevated by 1·72 and 1·44 ($P < 0·005$) fold in Group II and Group III, respectively. Butylated

hydroxyanisole significantly increased the activity of superoxide dismutase in the kidney and forestomach (Tables 4–5).

Under our assay conditions, catalase activity was detectable only in lung and kidney. It showed a significant and dose-dependent increase in kidney following *Aegle* treatment. Catalase activity in kidney was enhanced by 1·37 fold ($P < 0{\cdot}005$) in Group II and 1·61 fold ($P < 0{\cdot}005$) in Group III. Butylated hydroxyanisole significantly enhanced the activity of catalase in lung and kidney (Tables 4–5).

Discussion

In recent years, a large number of naturally occurring bioactive compounds with different chemical compositions have been identified as chemopreventive agents that inhibit the process of carcinogenesis at various sites. Cancer chemoprevention is an exciting area of pharmaceutical cancer research involving the use of either natural or synthetic components to delay, inhibit or reverse the development of cancer in normal or preneoplastic conditions (Tanaka 1994; Morse & Stoner 1996; Pezzuto 1997).

The findings of this study are based on the examination of the inducibility of enzymes involved in the metabolism of xenobiotics (including carcinogens) and drugs, as this is one of the reliable markers for evaluating the chemopreventive potential of the test materials in a murine model. Butylated hydroxyanisole was used as a positive control to validate the authenticity of assay protocols since it has been proved to be a chemoprotectant in diverse animal models of site-specific carcinogenicity (Rao 1982; Hocman 1988). There were no adverse effects on the animals at the given dose levels of *Aegle* leaf extract (50 and 100 mg of the extract per kg body-weight of mice, daily for 14 days). It caused neither increase in the rate of mortality nor decrease in the body-weight of animals. The liver-somatic index remained unaltered and also, at a cellular level, there was no indication of damage as observed by the measurement of lactate dehydrogenase activity. Its activity was significantly reduced from the control value showing the possibility of protection against damage caused during normal metabolic processes. Even the higher dose (100 mg kg^{-1} daily) used had a safety margin sufficiently distant from the toxic range.

The microsomal cytochrome P450 system plays a key role in oxidative activation, inactivation and promotion of excretion of most xenobiotic compounds, and also in modulating the duration and intensity of their toxicity (Guengerich 1988; Miller 1988). Cytochrome P450 catalyses the oxidation of lipophilic chemicals by the addition of polar groups. In this study, *Aegle* increased the level of the cytochrome P450 system (cytochrome P450, cytochrome b5, NADPH-cytochrome P450 reductase and NADH-cytochrome b5 reductase). The modulation of cytochrome P450 by *Aegle* could well be an environmental determinant in addition to genetic factors for an individual to metabolize carcinogens. Indeed, it is recognized that the main function of phase-I metabolism is to prepare a compound for phase-II metabolism, which is usually the true detoxification reaction, resulting in water-soluble products that can be easily excreted.

The action of phase-II enzymes on the substrates generated by the action of phase-I enzymes on innocuous or hazardous chemicals leads to their solubilization and excretion (Gibson & Skett 1994). Glutathione S-transferase plays an important role in conjugating the metabolites resulting from the action of cytochrome P450, with endogenous ligands (reduced glutathione), favouring their elimination from the body of the organism (Hartman & Shankel 1990). It has already been reported that the induction in glutathione S-transferase is implicated in protection against various cytotoxic, mutagenic and carcinogenic chemicals (Ketterer 1988; De Flora & Ramel 1989; Reed 1990). We used 1-chloro-2,4-dinitrobenzene as a non-specific substrate in our assay for glutathione S-transferase. Thus, the specific activity of the enzyme measured was the sum of all of its isoforms. Many naturally occurring chemopreventive agents decrease potential DNA-damaging entities and convert them into excretable metabolites, facilitated through the induction of glutathione S-transferase (Coles & Ketterer 1990).

Induction of DT-diaphorase has been ascribed as a means for determining the potency of many anticarcinogenic substances (De Long et al 1986; Begleiter et al 1997). This enzyme protects against the toxicity of quinones and their metabolic precursors (polycyclic aromatic hydrocarbon, benzene, etc.) (Smart & Zannoni 1984; Karczewski et al 1999). DT-diaphorase facilitates the metabolism of quinones to hydroquinone, obliterating semiquinone radicals and subsequent oxygen radical production. The stable hydroquinone resulting from two-electron oxido-reduction of quinone by DT-diaphorase can be conjugated by glucuronide or sulphate into excretable compound and thus affords protection from reactive intermediates (De Long et al 1986; Talalay 1989).

It is evident from these findings that *Aegle* acts as a bifunctional enzyme inducer since it induced phase-I, as well as phase-II, drug-metabolizing enzyme systems. This reinforces the balance of xenobiotic metabolism towards detoxification and therefore, might be attributed to playing a major role in cytoprotection and chemoprevention. The study also reveals that *Aegle* can significantly attenuate oxidative stress by modulating cellular enzymatic and non-enzymatic antioxidant defence systems. The antioxidant potential of the plant was evaluated by examining its ability to modulate the basal level of reduced glutathione, glutathione peroxidase and glutathione reductase in the liver of mice and superoxide dismutase and catalase in liver, as well as in extrahepatic organs (lung, kidney and fore-stomach).

The elevated level of reduced glutathione protects cellular proteins against oxidation through the glutathione redox cycle and due to its nucleophilic centre and conjugating ability, it also directly detoxifies reactive oxygen species and neutralizes reactive intermediate species generated from exposure to xenobiotics, including chemical carcinogens (Ketterer 1998). The increase in glutathione reductase level plays a significant role in the reduction of oxidized glutathione to reduced glutathione at the expense of NADPH and regulates the reduced glutathione-oxidised glutathione cycle in the cell (Vanoni et al 1991). Its inhibition is likely to be deleterious to cells since it contributes in efficiently maintaining the basal level of cellular reduced glutathione (Meister 1994).

The antioxidative response of *Aegle*, as evaluated by its efficacy to modulate antioxidative parameters (reduced glutathione, glutathione reductase, glutathione peroxidase, superoxide dismutase and catalase), may be anticipated to have biological significance in eliminating reactive free radicals that are deleterious to normal cellular metabolism. These enzymes are also involved in maintaining the physiological levels of oxygen and hydrogen peroxide by hastening the dismutation of oxygen radicals and eliminating organic peroxides and hydroperoxides generated from exposure to xenobiotics. The dysfunctioning of these antioxidant enzymes has been reported in many diseases, including rheumatoid arthritis, reperfusion injury, cardiovascular diseases and immune injury, as well as in cancer (Gonzalis et al 1984; Guemouri et al 1991; Flagg et al 1993; Saydem et al 1997).

It has been suggested that a low concentration of H_2O_2 is brought about by glutathione peroxidase, whereas, catalase comes into play when the glutathione peroxidase pathway is reaching saturation with the substrate (Gaetani et al 1989). Furthermore, the significant decrease in lipid peroxidation by *Aegle* is correlated well with the induction of antioxidant enzymes. This is suggestive of the potential of the modulator in protection against membrane damage that may be imparted through modulating the various enzyme systems.

The induction of superoxide dismutase activity may also inhibit the generation of active oxygen species generated from the auto-oxidation of quinones by the action of DT-diaphorase. The augmented activity of the metalloenzyme superoxide dismutase accelerates dismutation of superoxide radicals to hydrogen peroxide, which is removed by catalase (Aebi 1984). Thus, induced superoxide, in conjunction with catalase and decrease in lipid peroxidation, by *Aegle* may antagonise any cellular injury induced by reactive oxygen species.

Administration of *Aegle* to mice in this study caused significant induction of phase-I and phase-II enzymes, –SH groups and antioxidative parameters. These overall inductive effects may presumably result in enhanced carcinogen detoxification. Blocking agents are complicated in that the microsomal mono-oxygenase system can both activate and detoxify chemical carcinogens. However, concomitant increases in the levels of phase-II enzymes and measured antioxidative parameters and inhibition of lipid peroxidation indicate enhanced detoxification reactions. Thus, the increase in cytochrome P450 level results in enhanced activation of metabolites which would further be efficiently detoxified by the elevated levels of glutathione S-transferase, DT-diaphorase, –SH groups, glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase (Wattenberg 1997). The induced levels of antioxidant enzymes can also efficiently detoxify the toxic free radicals generated during normal, as well as abnormal, cellular metabolism. Superoxide free radicals, having the capacity to damage various macromolecules, can be effectively detoxified by the superoxide dismutase catalase enzyme system.

Thus, this investigation has clearly shown the cancer chemopreventive potential of *Aegle marmelos* leaf. The next step is to experimentally substantiate the chemopreventive potential using different chemical carcinogenesis models. Our laboratory has already commenced study in this direction, employing skin cancer, stomach cancer, mammary cancer and cervical cancer model systems in appropriate animals.

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