RESEARCH COMMUNICATION

Chemomodulatory Action of Brassica Compestris (Var Sarason) on Hepatic Carcinogen Metabolizing Enzymes, Antioxidant Profiles and Lipid Peroxidation

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Summary

The effect of two different doses (400 and 800 mg/kg body wt/day for 15 days) of a 95% ethanolic extract of the seeds of Brassica compestris (var sarason) was examined on carcinogen metabolizing phase-I and phase-II enzymes, antioxidant enzymes and glutathione content and lipid peroxidation in the liver of Swiss albino mice. Positive control mice were treated with butylated hydroxyanisole (BHA). Significant elevation in the levels of cytochrome p450 ($p<0.05$), cytochrome b_5 ($p < 0.05$) glutathione s-transferase ($p<0.01$), DT-diaphorase ($p<0.05$), superoxide dismutase ($p<0.01$), catalase ($p < 0.001$) and reduced glutathione ($p<0.001$) was noted in the group treated with 800 mg/kg body wt. of Brassica extract in comparison with the negative control group. Brassica compestris acted as a bifunctional inducer since it induced both phase - I and phase - II enzyme systems. Since phase-I and phase-II enzymes are considered to be reliable markers for evaluating the chemoprevention efficacy of particular test materials, these findings are suggestive of potential chemopreventive roles for Brassica seed extract.

Key Words: Chemoprevention - carcinogenesis - mustard seed (Brassica compestris var sarason) - carcinogen metabolizing enzymes - antioxidant profile

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Introduction

Laboratory studies and epidemiological evidence lead credence to chemoprevention strategy in attenuating the risk of developing cancer in human beings (Bertram et al., 1987; Boone et al., 1990). Many nutrient and non-nutrient dietary constituents of plant origin have evidence of chemoprevention by inhibiting and/or suppressing or reversing cancer incidence trend evoked by multitude of factors including environmental chemical carcinogens.

Most of the chemopreventive agents intervene either at initiation, promotion and/or progression stages conceptually associated with ontogeny of carcinogenesis, although the mechanistic detail differs depending on stage at which the chemopreventor acts effectively (Wattenberg, 1985; Morse and Stoner, 1991; Tanaka, 1994).

The hepatic drug metabolizing system comprising of phase-I and phase-II enzymes are capable of modifying the end result of carcinogenesis at "Initiation stage". Brassica vegetables including broccoli, cabbage, cauliflower and brussels sprout have been shown beneficial in preventing carcinogenesis (Beecher, 1994). Their cancer chemopreventive activity has been attributed to the presence of indole glucosinolates, aromatic isothiocyanates, dithiolthiones and phenols (Nugon-Baudon and Rabot, 1994). These compounds have been reported to exert broad based anticarcinogenic activity against variety of chemical carcinogens at multiple target sites in animal models via modulation of phase-I and phase-II drug metabolizing enzymes (Sparning and Wattenberg, 1981, Ansher et al., 1986; Zhang et al., 1994). In our laboratories, we have reported the chemopreventive action of Brassica compestris seed extract during peri-initiation and post-initiation in skin papillomagenesis of Swiss albino mice initiated with 7, 12-dimethylbenz (a) anthracene (DMBA) (Qiblawi and Kumar, 1999).

The present study was designed to further assess the anticarcinogenic activity of Brassica compestris seed extract

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and to understand the underlying mechanisms of its chemopreventive action by evaluating the levels activities of hepatic phase-I (cytochrome 450, cytochrome b5) and Phase -II (glutathione S-transferase and DT-diaphorase) enzymes. In addition hepatic antioxidant defense enzymes comprising of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and reduced glutathione have also been evaluated since inverse correlation between these antioxidant enzymes and carcinogenicity have been well documented.

Materials and Methods

Animals

Random bred male Swiss albino mice (7-8 week 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.

Test Material

The plant seeds of Brassica compestris were collected from local market only after taxonomic identity of the plant species were established. Voucher specimens of the seeds have been preserved in the herbarium, Department of Botany, University of Rajasthan, Jaipur. The seeds were powdered and soxhletted using 95% ethanolic solvent for 36 hr (12 hr x 3). Finally the extract was lyophilized, weighed and preserved at 4°C and used for treating the animals.

Chemicals

1-chloro-2,4-dinitro benzene (CDNB), 5-5 dithio-2-nitrobenzoic acid (DTNB), reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), glutathione [reduced form (GSH) and oxidized from (GSSH)], 2,6-dichlorophenol-indophenol (DCPIP), bovine serum albumin (BSA) and 3(2)-tert-butyl-4-hydroxyanisol (BHA) were obtained from Sigma Chemical Company (St.Louis, MO, USA).

Experimental Design

Animals were randomly assorted into following groups:

Group I (n=8): Animals were fed a normal diet and sham-treated with 0.05 ml double distilled water (vehicle) by oral gavage daily for 15 days; This group of animals served as a negative control.

Group II (n=8): Animals were fed a normal diet and treated with 0.05 ml of dissolved Brassica extract at 400 mg/kg body wt. by oral gavage daily for 15 days.

Group III (n=8): Animals were fed a normal diet and treated with 0.05 ml of dissolved Brassica extract at 800 mg/kg body wt. by oral gavage daily for 15 days.

Group IV (n=8): Animals were fed pulverized diet containing 0.75% BHA for 15 days; this group of animals served as a positive control.

The body weights of mice were recorded initially and at the end of the experiment.

Preparation of Homogenates, Cytosol and Microsome Fractions

Animals were killed by cervical dislocation after the termination of the experiment and the entire liver was then perfused in situ immediately with cold 0.9% NaCl and thereafter carefully removed, trimmed free extraneous tissue and rinsed in chilled 0.15M Tris KCl buffer (PH 7.4) to yield a 10% (w/v) homogenate. An aliquot 0.5 ml of this homogenate were used for assaying reduced glutathione, while the remainder part of the homogenate was centrifuged at 10,000 rpm for 20 min. The resultant supernatant was transferred into pre-cooled ultra-centrifugation tubes and centrifuged at 105,000x g for 60 min in a Beckman ultracentrifuge (model L8 70 M). The supernatant (cytosol fraction), after discarding any floating lipid layer was used for assaying glutathione S-transferase, DT-diaphorase, and antioxidant enzymes (glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase), where as the pellet representing microsome was suspended in homogenizing buffer and used for assaying cytochrome P-450, cytochrome b5 and lipid peroxidation.

Determination of Cytochrome P450

It is determined in microsomal suspension by the method of Omura and Sato (1964) by recording the difference in absorbance between 450 nm and 490 nm and using an absorption coefficient of 91 cm^2/mmol, respectively.

Determination of Cytochrome b5

This was determined in microsomal suspension by the method of Omura and Sato (1964) by recording the difference in absorbance between 424 nm and 409 nm and using an absorption coefficient of 185 cm^2/mmol respectively.

Determination of Glutathione S-transferase (GST) Activity

The cytosolic glutathione S-transferase activity was determined spectrophotometrically at 37°C according to the procedure of Habig et al. (1974). This specific activity of glutathione S-transferase is expressed at μ moles of GSH-CDNB conjugate formed/min./mg protein using an extinction coefficient of 9.6 mM^{-1} cm^{-1}.

Determination of DT-diaphorase Activity

The DT-diaphorase activity was measured as described by Ernster et al. (1962) with NADPH as the electron donor and 2,6-dichlorophenol-indophenol (DCPIP) as the electron acceptor at 600 nm. The DTD activity was calculated by reading the absorbance after 3 min. at 600 nm with an extinction coefficient value of 21 mM^{-1} cm^{-1}. One unit of enzyme activity has been defined as amount of enzyme required to reduce one μ mole of DCPIP per min.

Determination of Glutathione Reductase (GR) activity

Glutathione reductase was determined by the procedure as described by Carlberg and Mannervick (1975). The extinction coefficient of 6.22 mM^{-1} cm^{-1} was used to

determine the specific activity of glutathione reductase enzyme as n moles of NADPH consumed/min/mg protein.

Determination of Glutathione Peroxidase (GPX) Activity

The cytosolic glutathione peroxidase (GPX) activity was determined spectrophotometrically as described by Paglia and Valentine (1967). The extinction coefficient of 6.22 mM^{-1} cM^{-1} was used to determine the specific activity of glutathione peroxidase enzyme as n moles of NADPH consumed /min /mg protein.

Determination of Catalase (CAT) Activity

Catalase was estimated at 240 nm by monitoring the disappearance of H_2O_2 described by Aebi (1984). Catalase enzyme specific activity has been expressed as µ mole of H_2O_2 decomposed/min/mg protein.

Determination of Superoxide Dismutase (SOD) Activity

Superoxide dismutase was assayed using the method of Marklund and Marklund (1974), which involves inhibition of pyrogallol autooxidation at pH 8.0. A single unit of enzyme was defined as the quantity of superoxide dismutase required to produce 50% inhibition of autoxidation.

Determination of Reduced Glutathione (GSH) Content

Hepatic level of reduced glutathione was determined by the method as described by Moron et al. (1979). Reduced glutathione was used as a standard to calculate µ mole GSH/ 100 gm tissue.

Estimation of Lipid Peroxidation (LPO)

The lipid peroxidation level in the microsomal suspension was estimated spectrophotometrically by thiobarbituric acid reactive substances (TBARS) method, as described by Pryor (1976) and is expressed in terms of malondialdehyde (MDA) formed per mg protein.

Estimation of Protein

The protein content was measured according to the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard.

Statistical Analysis of the Data

Statistical significance of difference between control and experimental groups was determined by student's t-test.

Results

The results of the present investigation are depicted in Tables1-2.

Cytochrome P450 and Cytochrome b5

Treatment with Brassica seed extract at 800 mg/kg b.wt. for 15 days (group III, table 1) significantly elevated ($P < 0.05$) the cytochrome P-450 and b5 levels in the liver. Furthermore, feeding the BHA containing diet for 15 days also elevated the cytochrome P-450 ($P<0.001$) and cytochrome b5 ($P<0.01$) levels in the liver (group I versus group IV, Table 1).

Glutathione S-tranferase

Mice that were treated with the higher dose of 800 mg/ kg b.wt. of Brassica seed extract for 15 days could effectively induce glutathione S-transferase specific enzyme activity ($P<0.01$) (group I versus group III, table 2). In positive control group i.e. BHA treated animals glutathione S-transferase enzyme activity was found significantly induced relative to control group ($P<0.001$) (group I versus group IV, Table 2).

DT-diaphorase

Compared to control group of mice, DT-diaphorase specific enzyme activity was found elevated ($P<0.05$) by high dose of extract treatment at 800 mg/kg b.wt. (Group III, table-2). However with low dose treatment of the seed extract at 400 mg/kg b.wt, the enzyme specific activity remained comparable to the negative control group (group I versus group II, table 2). Furthermore, the enzyme activity was observed significantly induced in the BHA fed group ($P < 0.001$) (group I versus group IV, Table 2).

Glutathione Peroxidase and Glutathione Reductase

Treatment with Brassica seed extract at 400 mg/kg b.

Table 1. Modulatory Influence of Two Different doses of Brassica Compestris Seed Extract on Mouse Hepatic Phase-I Carcinogen Metabolizing Enzyme Levels and Lipid Peroxidation

Group (GR)	Treatment	Cyt. P 450 (nmole/mg protein)	Cyt. b5 (nmole/mg protein)	LPO (nmole malondialdehyde formed/mg protein)
Gr - I	Control (only vehicle- double distilled water 0.05 ml)	0.40 ± 0.03	0.21± 0.03	7.80 ± 10.56
Gr - II	Brasica (400 mg/kg b.wt.)	0.43 ± 0.04	0.23 ± 0.014	6.52 ± 0.93
Gr - III	Brassica (800 mg/kg b.wt.)	0.62 ± 0.04a	0.30 ± 0.02[a]	5.42 ± 0.93
Gr - IV	Positive Control (0.75% BHA in diet)	0.58 ± 0.03c	0.30 ± 0.01[b]	5.6 ± 0.65

Values are expressed as mean ± S.E. of 6-8 animals. Treated duration: 15 days.
[a]($p < 0.05$), b($p<0.01$) and c($p < 0.001$) represent significant changes against control.
Abbreviation: Cyt p 450 - cytochrome p 450; Cytb 5-cytochrome b5; LPO: Lipid peroxidation .

wt. for 15 days revealed no effect on glutathione peroxidase and glutathione reductase enzyme levels. On the contrary, Brassica seed extract at 800 mg/kg b. wt and BHA diet was effective in inducing glutathione peroxidase (P<0.1) and glutathione reductase (P<0.001) specific enzyme activities (group I versus group IV, Table 2).

Superoxide Dismutase and Catalase

The animals treated orally with Brassica seed at 800 mg/kg b.wt. showed significant increase in the SOD activity (P<0.01) and CAT activity (P<0.001) compared with the control group (group I versus group III, table 2). On the contrary low dose treatment was in effective in inducing significant elevation in liver SOD and CAT enzyme activities (group II, Table 2). Mice were fed with BHA in the diet also showed an increase in the hepatic SOD and CAT activity compared with the control group (P<0.001).

Reduced Glutathione

Mice that were treated with Brassica seed extract at 800 mg/kg b.wt. as well as those fed the BHA containing diet for 15 days revealed significant elevation in GSH content (P < 0.001) (group I versus group III & IV, Table 2).

Lipid Peroxidation

There was no statistically significant variation in hepatic LPO in either the Brassica seed extract treated groups of both dose levels or the BHA treated group compared with the control group (Table 1).

Discussion

Cancer chemoprevention can be defined as prevention by administration of chemical entities, either as individual drugs or as naturally occurring constituents of the diet. Chemoprevention has earned serious consideration as a mean of controlling cancer incidence, as it is no longer merely a theoretical strategy, but an approach yielding more impressive experimental and clinical results (Morse & Stoner, 1993).

One of the most effective ways to screen compounds that might be of use in inhibiting the chemically induced carcinogenesis is to look for its ability in inducing the phase-I and phase-II metabolizing enzymes. Diet augmented with cruciferous vegetables have been found to be very significantly alter the xenobiotic metabolizing enzymes both in liver and intestine, accelerating the disposal of chemical carcinogens thereby destroying their ability to damage DNA (Whitty and Bjeldanes , 1987) . Further it has been shown that phenethyl isothiocyanate and allyl isothiocynate induces apoptosis, DNA fragmentation and cell death of human leukemia HL 60 cells in vitro (Thornalley, 2002)

Results obtained form the present study testify Brassica administration on mice affects liver enzyme activities correlated with attenuating the risk of chemical carcinogenesis. There were no adverse effects on the animals at the given dose levels (400 and 800 mg/kg body wt.)/day for 15 days. BHA was used as positive control to validate the authenticity of assay protocols and response to modulator in our animal model system (Hocman, 1988; Iverson, 1999). Ninety five percent ethanolic seed extract of Brassica compestris produced a substantial elevation in the hepatic microsomal cytochrome P-450 and cytochrome b5 levels in the group of animals that was treated for 15 days at high dose level. Induction of phase I enzymes such as cytochrome P-450 catalyses ùactivational mechanism" of polycyclic aromatic hydrocarbons to proximate and/or ultimate carcinogens (Wattenberg, 1985). As seen with most

Table 2. Modulatory Influence of Two Different doses of Brassica Compestris Seed Extract on the Hepatic Phase II Carcinogen Metabolizing and Antioxidant Enzymes Levels and Reduced Glutathione Level

Group (GR)	Treatment	GST (µmole CDNB-GSH conjugate formed min/mg protein)	GSH (µmole GSH/100 gm tissue)	GPX (nmole of NADPH consumed/ min/mg/ protein)	GR (nmole of NADPH consumed/ min/mg/ protein)	SOD (Specific activity expressed as µmole/mg protein)	CAT (µmole H_2O_2 consumed/ min/mg/ protein)	DTD (µmole of DCPIP reduced/ min/mg protein)
Gr - I	Control (only vehicle- double distilled water 0.05 ml)	2.00 ± 0.27	4.06 ± 0.14	18.74 ±1.28	48.07 ± 2.06	2.38 ± 0.60	73.59 ± 2.15	0.075 ± 0.001
Gr - II	Brasica (400 mg/kg b.wt.)	2.41± 0.19	4.80 ± 0.21	17.08 ± 0.98	45.59 ± 0.81	3.26 ± 0.49	70.07 ± 2.60	0.072 ± 0.003
Gr - III	Brassica (800 mg/kg b.wt.)	3.68 ± 0.18[b]	6.44 ± 0.34[c]	22.71 ± 1.03	57.97 ± 0.85	4.71 ± 0.44[b]	96.77 ± 2.68[c]	0.096 ± 0.004[a]
Gr - IV	Positive Control (0.75% BHA in diet)	3.54 ± 0.48c	5.75 ± 0.62[c]	21.76 ± 0.98[a]	54.01 ± 2.76[c]	4.35 ± 0.41	84.12 ± 1.33[c]	0.092 ± 0.004[c]

Values are expressed as mean ± S.E. of 6-8 animals. Treated duration : 15 days. [a](p < 0.1), [b](p<0.01) and [c](p < 0.001) represent significant changes against control. Abbreviation: BHA: Butylated Hydoxyanisole,GST: Glutathione-s-transferase; GSH-reduced glutathione; GPX-glutathione peroxidase; GR-glutathione reductase; SOD-superoxide dismutase; CAT-catalase;DTD :DT-diaphorase activity.

chemopreventive agents, this evokes a generalized "electrophilic counter attack" response characterized by the induction of phase-II enzymes and an increase in tissue glutathione levels (Prestera et al., 1993). The enhanced level of cytochrome b5 augments the functional activity of cytochrome P-450. Because hepatic metabolism of xenobiotic chemicals often quantitively predominate over organ-specific metabolism (Wall et al., 1991), induction of the levels of cyt. b5 and cyt. P-450 may thus lead towards over all protection from the potent carcinogenic forms in conjunction with phase-II enzymes.

Glutathione S-transferase exists in many isozymic forms common to several mammalian species (Habig et al., 1974). In the present study, where in CDNB was used as a non-specific substrate for assaying total cytosolic GST, it was observed that oral treatment of Brassica seed extract for 15 days significantly enhanced the GST activity in the liver of mice. The induction of conjugative enzymes such as glutathione S-transferase is an important detoxification mechanism for withstanding the neoplastic effects of various chemicals (Wattenberg, 1980). Further, it may be speculated that increase of GST activity will detoxify hydrophobic electrophiles by binding them covalently or by establishing their conjugation with GSH (Chasseaud, 1979) since electrophiles represent the ultimate reactive carcinogenic forms of carcinogens.

Enhancement in the DT-diaphorase activity was observed in the liver of mice treated with high dose level of Brassica seed extract. DT-diaphorase induction facilitates bio-reductive activation metabolism of quinones by two electron reduction to hydroquinone, obliterating semi-quinone and oxygen radical formation. Hydroquinone formed via two electron reduction by DT-diaphorase can be conjugated by glucuronide or sulfate and excreted (Benson et al., 1980; Lind et al., 1982). It may thus be inferred that induction of DT-diaphorase in the liver by the Brassica extract, provides in vivo protection by favouring xenobiotic detoxification, alleviating the binding of metabolically activated indirectly acting carcinogens, such as polycyclic aromatic hydrocarbons (PAH) to the genomic DNA of host.

Reference to antioxidant enzymes status in liver of Brassica seed extract treated animals, only catalase and superoxide dismutase specific activities were found elevated over the control basal values. Glutathione peroxidase and glutathione reductase were however comparable to control animals. SOD and CAT play an important role in the detoxification of reactive oxygen species such as O_2^-, OH^-, and H_2O_2 which are involved in genotoxicity and various stages of chemical carcinogenesis (Troll et al., 1984; Cerutti, 1985; Oberley and Oberley, 1986). Thus, the induction of SOD and CAT activities will augment in maintaining the physiological level of oxygen and hydrogen peroxide by dismutation of oxygen radicals and decomposition of hydrogen peroxide (Gonzales et al., 1984) hence offering a protective role against the free radicals.

The level of lipid peroxidation in the liver showed no significant difference in the Brassica treated groups compared with the control.

The present study also revealed that Brassica seed extract, could elevate the hepatic content of GSH. Glutathione represents an important defense mechanism in protecting cells against oxygen-derived free radicals and also from cellular lethality after exposure to anticancer drugs or ionizing radiations (Orrneius and Moldeus, 1984; Biaglow et al., 1987). The depletion of hepatic glutathione in vivo enhances lipid peroxidation (Younes and Siegers, 1980). On the contrary the presence of higher level of glutathione, scavenges the electrophilic moieties involved in cancer initiation by conjugation to less-toxic reduction products (De flora and Ramel, 1988; Ketterer, 1988).

Thus, the present findings suggest that feeding of Brassica seed extract has the potential to block or suppress the events associated with chemical carcinogenesis through it is modulatory influence on carcinogen metabolizing enzymes and antioxidant defense system in Swiss albino mice. In addition to our earlier report of Brassica compesris extract protection against DMBA induced skin carcinogenesis (Qiblawi and Kumar, 1999), the study of chemopreventive potential of Brassica compestris using different chemical carcinogenesis models is in progress in our laboratory.

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