Multi drug resistance and inhibition:
Is it a reality with plant based drug combinations?
Effect of *Mentha piperita* and *Brassica compestris* singularly and in combination
on the xenobiotic metabolism enzymes/antioxidant status and lipid peroxidation in mice.

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Summary

The present study reports the modulatory influence of ethanolic extracts of *Mentha piperita* and *Brassica compestris*, used in single and in combined doses, on drug metabolizing phase I and phase II enzymes, antioxidant enzymes, glutathione content (GSH) and lipid peroxidation(LPO) in the liver of 8-9 weeks old Swiss albino mice. Oral treatment with the leaf extract of *Mentha* at 400 and 800 mg/kg body weight, for 15 days significantly elevated the levels of Cytochrome P_{450} (Cyt P_{450}) and enzymatic activities of cytochrome P_{450} reductase (Cyt P_{450}R), cytochrome b_5 reductase (Cyt b_5R), Cu-Zn Superoxide dismutase (Cu-ZnSOD) and glutathione reductase(GR). The activities of glutathione-s-transferase (GST), glutathione peroxidase (GPX) and catalase (CAT) were significantly increased and malondialdehyde (MDA) formation was reduced markedly at higher dose level. Animals treated orally with the seed extract of *Brassica* at 400 and 800 mg/kg body wt. for 15 days showed significant increase in the levels of Cyt P_{450}, GSH and the enzymatic activities of Cyt P_{450} R, GR, GST, Cu-ZnSOD and manganese superoxide dismutase (MnSOD). GPX and CAT activities showed significant increase at higher single doses of *Mentha* and *Brassica*. Both high and low individual dose levels of *Mentha* and *Brassica* showed significant decrease in MDA formation in liver. Animals treated with the combined dose of *Mentha* and *Brassica* (200+200 mg/kg body wt.) for 15 days showed elevated levels of Cyt P_{450} and no significant changes in any enzymatic activities observed. The high levels of thiobarbituric acid reactive substances (TBARS) indicated enhanced lipid peroxidation in liver. Oral treatment with the high combined dose of *Mentha* and *Brassica* (400+400 mg/kg body wt.) for 15 days showed increased levels of Cyt P_{450}, Cyt b_5 and enhancement in the activities of Cyt P_{450}R, GST, GR, Cu-ZnSOD, MnSOD, while there was significant inhibition in the activities of GPX and CAT. LPO showed significant increase and the levels of GSH were depleted.

Butylated hydroxyanisole (BHA) , a pure antioxidant compound, was used as a positive control. This group showed significant increase in the hepatic levels of GSH content, Cyt b_5, GPX, GR and CAT whereas MDA formation was inhibited significantly. The treatment with either *Mentha* or *Brassica* extract is suggestive of their chemopreventive efficacy against chemotoxicity including carcinogenicity on account of enhanced GSH level and enzyme activities involved in xenobiotic metabolism and maintaining antioxidant status of cells. However, no significant chemopreventive activity was observed when *Mentha* and *Brassica* were used in combination. This warrants further investigation of active principle(s) present in the extract responsible for the observed effects employing various carcinogenesis model systems.

Keywords : *Mentha*, *Brassica,* Chemoprevention, Carcinogen/ drug metabolizing enzymes, Antioxidant enzymes, Lipid peroxidation.

Several epidemiological and experimental studies have provided initial lead for the identification of a variety of potential cancer preventing substances in the dietary components such as cereals, fruits and vegetables, that are known to inhibit various cancers, particularly skin tumors (Block et al.,1992; Wattenberg,1992; Wargovich,1999;Martin et al. 2001 and Demierre & Nathanson,2003). Plant derived glutathione-dependent antioxidants plays a fundamental role in cellular defense against reactive free radicals and other oxidant species (Sen, 1997; Gul et al., 2000), by decreasing ROS production and the rate of accumulation of oxidized DNA and protein and by enhancing antioxidant defense system (Ames et al., 1993 and Birt et al.,1995).

Mentha piperita (Linn.) of family Labaitae is a perennial, glaborous and strong scented herb. It is carminative, antispasmodic, decongestant, a cholagogue and has anaesthetic activity (Ody,1993; Mcintyre, 1995; Murray, 1995 and Mabey, 1998). *Mentha* is shown to have antioxidant and antiperoxidant properties due to the presence of eugenol, ferulic acid, p-coumaric acid, caffeic acid, rosmarinic acid and ∝-tocopherol (Rastogi & Mehrotra, 1991; Krishnaswamy & Raghuramulu, 1998; Al-Sereiti et al., 1999). *Brassica compestris* (Linn) var Sarason of family Cruciferae, is a perennial herb with pungent watery sap and yellow flowers. It is known to contain indole-3-carbinol (I3C), sulforaphane (glucosinolates), aromatic isothiocyanates, dithiolthiones and phenols (74-76) and is a source of monofunctional phase II inducers (Frankel et al., 1993). *Brassica* is also known to induce enzyme detoxification (Ziegler et al., 1992 and Hecht, 1999) by inducing phase II enzyme system (Prochaska et al., 1992 and Zhang et al., 1992). Dietary manipulations by cruciferous vegetables are known to act at molecular level and prevent the development of cancer (Bonnensen et al.,2001).

Multi drug resistance and inhibition (MDRI) in plant based drugs is associated with the failure of chemopreventive action of several drugs differing in chemical composition and mechanism of action. The putative sites of drug action include : (i) Uptake of the drug by the target cells; (ii) Activation or changes in the metabolism of the drug in the cell; (iii) Interaction with cellular targets; (iv) subsequent signaling events triggering induction/inhibition of antioxidant profile. Thus in chemopreventive studies, MDRI may arise due to : (i) drug interaction resulting in inactivation of their antioxidative capacity, either due to decreased absorption or mutual suppression; (ii) drug interaction resulting in the formation of new oxidative/peroxidative compound, either prior to or after lipophilic

to hydrophilic transformation by phaseI enzymes; (iii) increased excretion of drug, resulting in reduced level of drug in circulation and its decreased diffusion to the target cells (Jain, 2001 and Pluen et al., 2001).
Several plant based drug combinations have been found not only to be ineffective but having serious side effects and in extreme cases, the drug interactions resulting in diminished efficacy (Werneke, 2004).
Our present knowledge on chemoprevention of cancer has revealed the presence of a diverse array of naturally occurring bioactive compounds that inhibit the multistep process of carcinogenesis (Tanaka, 1994; Morse & Stoner, 1996; Pezzuto, 1997). The present investigation has been designed to assess the chemopreventive potential and modulatory influence and multi drug resistance/inhibition of *Mentha piperita* and *Brassica compestris* by evaluating the levels / activities of biochemical markers, namely phase I and phase II carcinogen/drug metabolizing enzymes and antioxidative parameters as well as lipid peroxidation in mice.

Materials and Methods

Chemicals

Bovine serum albumen (BSA), 1- chloro-2,4- dinitrobenzene (CDNB), ethylenediamine tetraacetic acid (EDTA), disodium salt and sulfosalicylic acid were procured from Amersco, USA. Dithio-bis-2-nitrobenzoic acid (DTNB), Folin's reagent, reduced glutathione (GSH), glutathione reductase (GR), nicotinamide adenine dinucleotide phosphate reduced (NADPH), ∝-naphthylamine, Xanthine, sulfanilic acid and xanthine oxidase were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Orthophosphoric acid (OPA) was procured from BDH chemicals. 2-Thiobarbituric acid (TBA) and 3(2)- tert-butyl-4-hydroxyanisole (BHA) were procured from Himedia (Mumbai).The rest of the chemicals were obtained from the local firms and were of highest purity grade.

Animals

Random-bred Swiss albino female mice (8-9 weeks old; 22-25g body wt.) were maintained in our animal facility (University of Rajasthan, Jaipur) with a 12 hr light/dark cycles and provided with standard food pellets (Hindustan Lever Ltd., India) and tap water *ad libitum*.

Preparation of ethanolic plant extracts

The plant materials were collected only after taxonomic identity of the plant species were established. Voucher specimens of the plants have been preserved in the herbarium of our institute. Fresh *Mentha* leaves were collected, shed dried and powdered subsequently. It was then extracted with 90% ethanol for 36 hours at 38-40°C. *Brassica* seeds were collected, powdered and extracted with 70% ethanol for 36 hours at 42-45°C. Both extracts were vacuum evaporated to obtain them in lyophilized state. The extracts were redissolved in double distilled water (ddw) , prior to oral administration.

Experimental Design
Animals were assorted into 8 groups.

Group I (n=8) : Animals were fed a normal diet and sham treated with 0.1 ml of double distil water (vehicle) through oral gavage, daily for 15 days. This group of animals served as control.
Group II (n=8) : Animals were fed a normal diet and treated daily with 400 mg/kg body wt. of *Mentha* leaf extract in 0.1 ml of double distil water (vehicle) through oral gavage for 15 days.
Group III (n=8) : Animals were fed a normal diet and treated daily with 400 mg/kg body wt. of *Brassica* seed extract dissolved in 0.1 ml of double distil water (vehicle) through oral gavage, for 15 days.
Group IV (n=8) : Animals were fed a normal diet and treated daily with the combined dose of 200 mg/kg body wt. of *Mentha* extract and 200 mg/kg body wt. of *Brassica* seed extract dissolved in 0.1 ml of double distil water (vehicle) through oral gavage, for 15 days.
Group V (n=8) : Animals were fed a normal diet and treated daily with 800 mg/kg body wt. of *Mentha* extract dissolved in 0.1 ml of double distil water (vehicle) through oral gavage, daily for 15 days.
Group VI (n=8) : Animals were fed a normal diet and treated daily with 800 mg/kg body wt. of *Brassica* seed extract dissolved in 0.1 ml of double distil water (vehicle) through oral gavage, daily for 15 days.
Group VII (n=8) : Animals were fed a normal diet and treated daily with the combined dose of400 mg/kg body wt. of *Mentha* extract and 400 mg/kg body wt. of *Brassica* seed extract dissolved in 0.1 ml of double distil water (vehicle) through oral gavage, daily for 15 days.
Group VIII (n=8) : Animals were fed a diet containing 0.75% of butylated hydroxyanisole for 15 days. This group served as a positive control for phase II enzymes and antioxidative parameters.

Body weights of mice were recorded initially, at weekly intervals and at the end of the experiment. Animals were starved overnight prior to the day of termination of the experiment.

Preparation of homogenate, cytosol and microsomal fractions

Animals were sacrificed by cervical dislocation after 15 days of treatment. The liver was perfused in situ immediately with ice cold 0.9% NaCl (Saline), removed and rinsed in chilled 0.15M tris (hydroxymethyl) aminomethane (Tris)- KCl (pH 7.4). The liver was then blotted dry, weighed and homogenized in ice cold 0.15M Tris- KCl buffer (pH 7.4) to yield a 10% (w/v) homogenate. An aliquot of this homogenate (0.5 ml) was used for assaying reduced glutathione (GSH), while the remainder was centrifuged at 10,000 rpm for 20 min. The resultant supernatant was transferred into precooled ultracentrifugation tubes and centrifuged at 1,05,000 g for 60 min. in a Beckman ultracentrifuge (model L870M).

The resulting supernatant (cytosolic fraction), after the floating lipid layer, if any, was discarded and appropriately diluted, was used for assaying total cytosolic glutathione-s-transferase (GST) and antioxidant enzymes. The pellet (microsome) was suspended in homogenizing buffer and used for assaying cytochrome P_{450}, cyt b_5,Cyt P_{450} reductase, cyt b_5 reductase and lipid peroxidation.
The kidney and stomach were removed, blotted dry and rinsed in chilled 0.15M Tris-KCl (pH 7.4). The stomach was slit open longitudinally, cleaned and flushed with buffer 6-8 times. The kidney and stomach were then blotted dry, weighed and homogenized in ice cold 0.15M Tris-KCl buffer (pH 7.4) to yield a 10% (w/v) homogenate. The resultant supernatant was subjected to centrifugation at 15,000 rpm for 30 min. at 4°C. The supernatant obtained was diluted appropriately and used for assaying GST, SOD and CAT.

Determination of Cytochrome P450 and Cytochrome b_5

Cyt P_{450} was determined using carbon monoxide difference spectra.Both Cyt P_{450} and Cyt b_5 contents were estimated in microsomal suspension by the method of Omura and Sato (1964), by recording the difference in absorbance between 450 & 490 nm and 420 & 490 nm respectively. The absorption coefficient of 91 cm^2/nmol was used for Cyt P_{450} and 185 cm^2/nmol for Cyt b_5.

Determination of NADPH-Cytochrome P_{450} reductase (NADPH-Cyt P_{450}R) and NADH-Cytochrome b_5 reductase(NADH-Cyt b_5 R) activity

NADPH-Cyt P_{450} R was assayed according to the method of Omura and Takesue (1970) with some modifications, measuring the rate of oxidation of NADPH at 340nm. The enzyme activity was calculated using the extinction coefficient 6.22mM/cm. 1 mol of NADH / min. was oxidized by one unit of enzyme activity.
NADH-Cyt b_5 R was assayed by the method of Mihara and Sato (1972). The enzyme activity was calculated using the extinction coefficient of 1.02mM/cm. 1 mol of ferricyanide was reduced /min. by one unit of enzyme activity.

Determination of Glutathione-s-transferase (GST) activity

GST activity was determined by the method of Habig et al., (1974) with some modifications as proposed by Ahmad et al. (2000). The reaction mixture consisted of 0.1M phosphate buffer (pH 7.4), 1mM GSH, 1mM 1-chloro-2- dinitrobenzene (CDNB) and 10% post mitochondrial supernatant (PMS) in a total volume of 2 ml. The change in absorbance at 25°C was recorded at 340nm and the enzyme activity was calculated as nmol CDNB conjugate formed/ min/ mg protein using a molar extinction coefficient of $9.6* 10^3$ $M^{-1}cm^{-1}$.

Estimation of Reduced glutathione (GSH) and determination of Glutathione reductase (GR) and Glutathione peroxidase (GPX) activity

The level of GSH was determined by the method of Jollow et al. (1974). The optical density of reaction product was read at 412 nm on a spectrophotometer and results were expressed as nmol GSH/g of tissue.

GR activity was measured according to the method of Carlberg and Mannervick (1975). The activity was calculated as nmol NADPH oxidized $min^{-1}mg^{-1}$ protein using molar extinction coefficient of $6.22*10^3$ $M^{-1}cm^{-1}$.
The method of Mohandas et al. (1984) was employed for assaying GPX activity with some modifications (Ahmad et al., 2000). The enzyme activity was calculated as nmol NADPH oxidized/min/mg of protein, using a molar extinction coefficient of $6.22*10^3$ $M^{-1}cm^{-1}$.

Determination of Cu-Zn Superoxide dismutase (Cu-ZnSOD), Mn Superoxide dismutase (MnSOD) and Catalase (CAT) activity

Sod activity was measured according to the method of Elstner and Heupel (1976). The procedure was based on the SOD mediated inhibition of nitrite formation from hydroxylammonium in the presence of O□ ⁻ generators. SOD activity was determined by incubating the homogenate with xanthine and hydroxylamine chloride. The reaction was initiated by the addition of xanthine oxidase. An aliquot of the incubation mixture was added to a mixture of sulfanilic acid and ∝- naphthylamine and the absorbance was read at 529 nm (Ultraspec III spectrophotometer, Pharmacia LKB Biochrom, Cambridge, England).
To differentiate between mitochondrial (MnSOD) and cytosolic (Cu/ZnSOD) superoxide dismutase activity in the tissue homogenate, MnSOD was determined by the addition of potassium cyanide (5mM) to the incubationmedium for 20min. at room temperature.The difference between the total and potassium cyanide-inhibited enzyme activity was defined as MnSOD activity. The activity of SOD in the sample was determined from a calibration curve of the percentage of inhibition of nitrite formation versus SOD activity, whichwas constructed using known amounts of purified SOD containing3500U/mg of protein. One unit of enzyme activity was defined as the amount of SOD required to reduce cytochrome C by 50% in the coupled system with xanthine and xanthine oxidase at pH 7.8 at 25□Cin a 3ml reaction volume. SOD activity is expressed as U/mg protein.
CAT activity was assayed using the method of Clairborne (1985) with some modifications as described by Ahmad et al.,(2000). The assay mixture consisted of 0.1M phosphate buffer (pH 7.4), 0.019M hydrogen peroxide and 10% PMS in a final volume of 3ml. Catalase activity was calculated in terms of nmol of H_2O_2 consumed/min/mg protein.

Estimation of Lipid peroxidation (LPO)

LPO was estimated by the procedure of Utley et al.,(1967) with some modifications as adopted by Fatima et al., (2000). The assay mixture contained 0.67% thiobarbituric acid (TBA), 10% chilled trichloroacetic acid (TCA) and homogenate (10%) in a total volume of 3ml. The rate of LPO is expressed as nanomoles of thiobarbituric acid reactive substances (TBARS) formed /h/gm of tissue using molar extinction coefficient of $1.56*10mcM^{-1}cm^{-1}$.

Estimation of Protein

Protein contents in various samples were estimated by the method of Lowry et al., (1951) using folin's reagent and bovine serum albumin (BSA), at 660nm.

Statistical Analysis

The values were presented as mean±s.e.m. The significance of the differences between the data pairs was evaluated by Rank Sum test (SigmaStat 2.0 software from Jandel Scientific). The level of significance was set at $p<0.05$.

Results

The findings of this study supports in vivo antioxidant potential of *Mentha and Brassica*, when used in individual doses. However, the combined doses of *Mentha and Brassica* are found to be ineffective as well as inhibitory, as described in the following sections. The body weight gain profile of mice did not show any adverse effects after treating individually with *Mentha or Brassica* and BHA.

Hepatic Studies

The relative liver weights were increased significantly in mice treated with BHA ($p<0.05$) and in animals treated with *Mentha* ($p<0.05$) at higher dose level. The microsomal protein content was elevated significantly with BHA ($p<0.001$) and with high individual doses of *Mentha* ($p<0.001$) and *Brassica* ($p<0.001$). Oral treatment with the combined dose of *Mentha* and *Brassica* did not show any increase in liver wt. or microsomal protein.

LPO was found to be decreased significantly in animals treated with BHA ($p<0.005$) and in animals treated with either *Mentha* ($p<0.005$) or *Brassica* ($p<0.005$) at higher dose level. LPO showed significant enhancement with the higher combined dose of *Mentha* and *Brassica* ($p<0.001$).

Table I

Effect of BHA and single and combined doses of *Mentha* and *Brassica* on body wt. gain, liver wt and LPO (toxicity related parameter).

Group	**Treatment**	**Duration (Days)**	**Body Weight (g)**		**Liver wt.×100/ Final body wt.**	**LPO (nmol of MDA/h/gm tissue**	**Microsomal protein (mg/ml)**
			Initial	**Final**			
I	Control (Vehicle only ie ddw)	15	25.2±0.93(1.00)	27.4±0.24(1.00)	5.77±0.89(1.00)	4.69±0.14 (1.00)	7.18±0.19 (1.00)
II	*Mentha* (400mg/kg bd wt)	15	27.5±1.01(1.01)	28.1±0.35(1.02)	5.98±0.11(1.02)	4.82±0.16(1.03)	7.32±0.21(1.01)
III	*Brassica* (400mg/kg bd wt.)	15	25.8±0.86(0.97)	26.7±0.77(0.98)	5.99±0.12(1.04)	4.05±0.06(0.95)	7.09±0.42(0.98)
IV	*Mentha &Brassica* (200+200mg/kg bd wt)	15	27.4±0.78(1.02)	27.6±0.33(1.00)	5.57±0.22(0.98)	5.01±0.10(1.13)	7.06±0.14(0.98)
V	*Mentha* (800mg/kg bd wt.)	15	26.7±0.44(1.00)	27.5±0.18(1.00)	6.82±0.37(1.38)*	2.28±0.23(0.46)#	8.31±0.22(1.18)**
VI	*Brassica* (800mg/kg bd wt.)	15	25.6±1.03(0.96)	26.5±1.11(0.97)	6.91±0.41(1.54)#	3.18±0.09(0.69)#	8.17±0.24(1.14)**
VII	*Mentha & Brassica* (400+400 mg/kg bd wt)	15	27.2±0.88(1.01)	26.7±0.48(0.94)	5.65±0.23(0.99)	6.02±0.46(1.46)#	7.01±0.18(0.97)
VIII	BHA (0.75% in diet)	15	27.9±0.21(1.03)	28.8±0.32(1.03)	6.79±0.38(1.34)*	2.85±0.42(0.49)#	8.55±0.40(1.21)**

Values are expressed as mean±s.e.m. of 6–8 animals. Values in parentheses represent relative changes in parameters assessed. $^{*}(p<0.05)$, $^{\#}(p<0.005)$ and $^{**}(p<0.001)$ represent significant changes relative to control. BHA: butylated hydroxyanisole, dd.w.: double distilled water and LPO: lipid peroxidation.

Table 2

Modulatory influence of *Mentha* extract, *Brassica* extract and BHA on mouse hepatic phase I drug metabolizing enzyme.

Group	**Treatment**	**Duration (Days)**	**Cyt P_{450} (nmol/mgprotein)**	**Cyt b_5 (nmol/mgprotein)**	**Cyt P_{450}R (µmol NADPH oxidized/min/mg protein)**	**Cyt b_5R (µmol NADH oxidized/min/mg protein)**
I	Control (Vehicle only ie ddw)	15	0.422±0.71(1.00)	0.373±0.28(1.00)	0.217±0.12(1.00)	3.95±0.17 (1.00)
II	*Mentha* (400mg/kg bd wt)	15	0.428±0.22(1.08)	0.380±0.11(1.00)	0.346±0.11(1.83)*	3.82±0.26(0.99)
III	*Brassica* (400mg/kg bd wt.)	15	0.538±0.84(1.43)*	0.377±0.28(1.00)	0.222±0.33(1.01)	3.99±0.32(1.00)
IV	*Mentha &Brassica* (200+200mg/kg bd wt)	15	0.576±0.23(1.51)*	0.401±0.16(1.07)	0.228±0.46(1.02)	4.03±0.33(1.01)
V	*Mentha* (800mg/kg bd wt.)	15	0.536±0.14(1.42)#	0.503±0.87(1.88)**	0.368±0.48(1.88)**	4.81±0.38(1.66)#
VI	*Brassica* (800mg/kg bd wt.)	15	0.592±0.71(1.79)#	0.365±0.42(0.99)	0.359±0.28(1.85)*	5.08±0.45(01.88)#
VII	*Mentha & Brassica* (400+400 mg/kg bd wt)	15	0.612±0.66(1.88)*	0.371±0.27(1.00)	0.220±0.35(1.00)	5.27±0.18(1.92)*
VIII	BHA (0.75% in diet)	15	0.420±0.17(1.00)	0.478±0.33(1.45)**	0.225±0.32(1.01)	4.10±0.25(1.04)

Values are expressed as mean±s.e.m. of 6–8 animals. Values in parentheses represent relative changes in parameters assessed. $^{*}(p<0.05)$, $^{\#}(p<0.01)$ and $^{**}(p<0.001)$ represent significant changes relative to control. Cyt P_{450}: Cytochrome P_{450}, Cyt b_5 : Cytochrome b_5,Cyt P_{450} R : Cytochrome P_{450} reductase, Cyt b5 : Cytochrome b_5 reductase.

Cytochrome P_{450}, Cytochrome b_5, Cyt P_{450} R and Cyt b_5 R

The hemeprotein Cyt P_{450} showed a dose dependent induction relative to control (Gr I), in animals treated with either *Mentha* or *Brassica*. In animals treated with higher doses of *Mentha* or *Brassica* (Gr V&VI), Cyt P_{450} level was elevated 1.42 ($p<0.01$) and 1.79 ($p<0.01$) folds. However in BHA fed mice there was no significant change in Cyt P_{450} level but the levels of Cyt b_5 increased significantly ($p<0.001$). Cyt b_5 was also significantly induced at the higher dose of *Mentha* ($p<0.001$).

The activity of Cyt P_{450} R was increased significantly at the higher doses of both *Mentha* ($p<0.001$) and *Brassica* ($p<0.05$), when administered alone. The individual higher doses of both *Mentha and Brassica* (Gr V&VI) showed 1.66($p<0.01$) and 1.88($p<0.01$) fold increase in the activity of Cyt b□R. The combined higher dose of *Mentha and Brassica* (GR VII) also showed 1.92 ($p<0.05$) fold elevation in the activity of Cyt b_5R.

Glutathione-s-transferase (GST)

The activity of hepatic GST increased in a dose dependent manner at low and high doses of *Brassica* by 1.39 ($p<0.005$) and 1.84 ($p<0.001$) fold respectively. *Mentha* at higher dose (Gr V) showed 1.39 ($p<0.005$) fold increase in the activity of GST. BHA fed animals (Gr VIII) showed 1.82 ($p<0.005$) fold elevation in GST specific activity.

Reduced glutathione (GSH), Glutathione peroxidase (GPX) and Glutathione reductase (GR)

Animals treated with the higher dose of *Mentha* (Gr V) and *Brassica* (Gr VI) showed 1.59 ($p<0.001$) and 1.78 ($p<0.05$) fold increase in the level of GSH. However, the higher combined dose of *Mentha* and *Brassica* (Gr VII) indicated significant reduction in the level of GSH ($p<0.001$). GPX activity was significantly induced at higher doses of *Mentha* (Gr V) and *Brassica* (Gr VI) by 1.71 ($p<0.05$) and 1.75 ($p<0.001$) fold respectively. The higher combined dose of *Mentha* and *Brassica* ($p<0.001$) significantly inhibited GPX activity. GR activity was found to be significantly induced at higher individual doses of *Mentha* , *Brassica* and at higher combined dose of *Mentha* and *Brassica* by 1.67 ($p<0.005$), 1.76 ($p<0.005$) and 1.72 ($p<0.005$) respectively.

Table 3

Modulatory influence of *Mentha* extract, *Brassica* extract and BHA on mouse hepatic phase II drug metabolizing enzyme and Glutathione based antioxidant/ detoxification enzymes.

Group	**Treatment**	**Duration (Days)**	**GSH (nmol GSH/ gm tissue)**	**GST (nmol CDNB conjugates/min/mg protein**	**GPX (nmol NADPH oxidized/min/mg protein)**	**GR (nmol NADH consumed/min/mg protein)**	**Protein (mg/ml)**
I	Control (Vehicle only ie ddw)	15	49.82±6.01(1.00)	160.54±3.54(1.00)	184.42±5.57(1.00)	10.31±0.52(1.00)	8.01±0.54 (1.00)
II	*Mentha* (400mg/kg bd wt)	15	51.72±5.45(1.05)	164.13±8.21(1.08)	187.01±10.63(1.07)	11.15±1.12(1.08)	9.32±0.39(1.11)
III	*Brassica* (400mg/kg bd wt.)	15	52.94±8.73(1.08)	176.06±5.34(1.39)**	186.06±5.34(1.06)	12.64±0.83(1.14)	10.75±0.57(1.23)*
IV	*Mentha &Brassica* (200+200mg/kg bd wt)	15	52.85±7.52(1.06)	162.03±4.18(1.05)	178.30±8.70(0.98)	13.37±0.11(1.19)	9.13±0.11(1.07)
V	*Mentha* (800mg/kg bd wt.)	15	64.12±4.18(1.59)#	175.06±2.93(1.39)**	201.31±4.09(1.71)*	21.43±0.19(1.67)**	10.92±0.36(1.26)#
VI	*Brassica* (800mg/kg bd wt.)	15	73.44±8.73(1.78)*	188.24±3.15(1.84)#	206.59±3.72(1.75)#	27.22±0.22(1.76)**	10.49±0.27(1.21)#
VII	*Mentha & Brassica* (400+400 mg/kg bd wt)	15	34.85±7.52(0.48)#	180.18±5.02(1.80)#	166.58±7.12(0.76)#	25.66±0.43(1.72)**	8.68±0.66(1.03)
VIII	BHA (0.75% in diet)	15	53.18±4.33(1.13)	184.06±6.28(1.82)**	188.23±6.54(1.09)	15.17±0.75(1.22)	9.13±0.23(1.07)

Values are expressed as mean±s.e.m. of 6–8 animals. Values in parentheses represent relative changes in parameters assessed. *(p<0.05), $^{\#}$(p<0.001) and **(p<0.005) represent significant changes relative to control. GSH : Glutahione; GST : Glutathione-s-transferase; GPX : Glutathione peroxidase; GR : Glutathione reductase

Table 4

Modulatory influence of *Mentha* extract, *Brassica* extract and BHA on mouse hepatic antioxidant/ detoxification enzymes.

Group	**Treatment**	**Duration (Days)**	**Cu-ZnSOD (U/min/mg protein)**	**MnSOD (U/min/mg protein)**	**CAT (nmol H O consumed/min/mg protein)**
I	Control (Vehicle only ie ddw)	15	6.11±0.37(1.00)	0.93±0.07(1.00)	74.32±3.62(1.00)
II	*Mentha* (400mg/kg bd wt)	15	10.82±3.52(1.38)*	0.99±0.02(1.01)	76.14±4.08(1.02)
III	*Brassica* (400mg/kg bd wt.)	15	13.20±0.89(1.49)#	1.81±0.17(1.59)#	78.65±3.74(1.09)
IV	*Mentha &Brassica* (200+200mg/kg bd wt)	15	8.87±0.58(1.07)	0.95±0.82(1.00)	71.50±4.10(0.99)
V	*Mentha* (800mg/kg bd wt.)	15	16.73±1.08(1.72)*	1.89±0.06(1.63)#	78.50±4.17(1.09)
VI	*Brassica* (800mg/kg bd wt.)	15	19.22±0.82(1.89)*	1.05±0.27(1.02)	87.46±3.32(1.59)#
VII	*Mentha & Brassica* (400+400 mg/kg bd wt)	15	15.16±1.11(1.65)#	1.94±0.22(1.72)**	62.70±2.87(0.77)*
VIII	BHA (0.75% in diet)	15	16.81±0.73(1.73)#	2.02±0.15(1.80)**	89.16±3.18(1.66)*

Values are expressed as mean±s.e.m. of 6–8 animals. Values in parentheses represent relative changes in parameters assessed. $^{*}(p<0.05)$, $^{\#}(p<0.01)$ and $^{**}(p<0.005)$ represent significant changes relative to control. Cu-ZnSOD : Cu-Zn Superoxide dismutase; MnSOD : Mn Superoxide dismutase; CAT : Catalase

Cu-Zn Superoxide dismutase (Cu-ZnSOD), Mn Superoxide dismutase (MnSOD) and Catalase (CAT)

The specific activity of Cu-ZnSOD was increased significantly by 1.38 ($p<0.05$) and 1.49 ($p<0.01$) fold at lower doses of *Mentha* (Gr II) and *Brassica* (Gr III). The higher doses of *Mentha* (Gr V) and *Brassica* (Gr VI) showed increase in Cu-ZnSOD activity in a dose responsive manner. Animals treated with the combined higher dose of *Mentha* and *Brassica* (Gr VII) and BHA fed animals showed significant elevation of Cu-ZnSOD activity by 1.65 ($p<0.01$) and 1.73 ($p<0.01$) fold respectively. MnSOD activity showed significant elevation at low dose of *Brassica* by1.59 ($p<0.01$) fold and at high dose of *Mentha* by 1.63 ($p<0.005$) fold.The basal constitutive level of CAT was increased significantly at higher dose of *Brassica* by 1.59 ($p<0.01$) fold and by 1.66 ($p<0.05$) in BHA fed group. The higher combined dose of *Mentha* and *Brassica* (Gr VII) inhibited CAT activity significantly by 0.77 ($p<0.05$) fold.

Effect of *Mentha, Brassica* (single and combined doses) and BHA on GST, Cu-ZnSOD, MnSOD and Catalase activity in extrahepatic organs (Kidney and Stomach)

Glutathione-s-transferase (GST)

GST-specific activity was induced significantly in the stomach of mice in all the groups in a dose dependent manner. Low and high dose of *Mentha* (Gr II & V) showed 1.45 ($p<0.01$) and 1.64 ($p<0.01$) fold increase in GST activity. 1.37 ($p<0.05$) and 1.66 ($p<0.05$) fold increase in GST activity was noted at low (Gr III) and high (Gr VI) doses of *Brassica*. BHA fed animals also showed significant increase in GST specific activity by 1.58 ($p<0.05$) fold. In kidney the GST specific activity was induced significantly by 1.59 ($p<0.01$) fold in BHA fed group (Gr VIII).

Cu-Zn Superoxide dismutase (Cu-ZnSOD) and Mn Superoxide dismutase (MnSOD)

In stomach Cu-ZnSOD specific activity showed significant increase at high individual doses of *Mentha, Brassica* and dose responsive elevation with the combined doses of *Mentha and Brassica*. The magnitude of induction was 1.38 ($p<0.005$) fold in Gr IV, 1.74 fold ($p<0.01$) in Gr V, 1.83 ($p<0.01$) fold in Gr VI and 1.82 ($p<0.01$) fold in Gr VII. The maximum induction was noted in BHA fed animals (Gr VIII) at 1.96 ($p<0.005$) fold. In kidney, the significant increase in Cu-ZnSOD specific activity was observed in Gr V, Gr VII and Gr VIII at 1.52 ($p<0.05$), 1.58($p<0.05$) and 1.77 ($p<0.001$) respectively.

In stomach MnSOD specific activity showed increase by *Mentha* in dose responsive manner, with Gr II showing 1.37 ($p<0.05$) and Gr V showing 1.68 ($p<0.01$)fold increase respectively. The combined dose of *Mentha* and *Brassica* also showed significant elevation of MnSOD activity at low and high dose by 1.42 ($p<0.05$) and 1.76 ($p<0.05$) fold respectively. The higher dose of *Brassica* showed 1.70 ($p<0.01$) fold increase and BHA fed group showed maximum induction of MnSOD activity at 1.88 ($p<0.05$) fold.

In kidney, MnSOD specific activity was significantly increased at low combined dose of *Mentha and Brassica* (Gr IV) by 1.53 ($p<0.001$) fold and in Gr VIII by 1.64 ($p<0.01$) fold.

Catalase (CAT)

In stomach, CAT specific activity was significantly increased at low dose of *Brassica* by 1.38 ($p<0.005$) fold and at low combined dose of *Mentha* and *Brassica* by 1.43 ($p<0.005$) fold. The higher dose of *Mentha* (Gr V) showed 1.50 ($p<0.01$) fold increase and BHA fed group showed maximum induction at 1.62 ($p<0.01$) fold in the catalase activity.
In kidney, the CAT induction was significantly high at low (Gr III) and high (Gr VI) doses of *Brassica* in a dose responsive manner at 1.36 ($p<0.05$) and 1.47($p<0.05$) fold respectively. Gr VIII showed maximum induction at 1.74 ($p<0.01$) fold.

Discussion

The cancer morbidity is greatly influenced by the variation in dietary composition, which consist of naturally occurring dietary and non-dietary carcinogens and anticarcinogenic modulators in the form of phytochemicals, which exerts their influence by way of activation or suppression of biotransformation and/or antioxidant enzymes that are involved in detoxification of xenobiotic compounds, including chemical carcinogens (Greenwald, 2002; Hursting et al., 2001; Goodman, 1997; Craig, 1996; Talalay et al., 1995, Lin,1998; Gaylor and Kadlubar, 1991). The findings of the present investigation demonstrate that individual administration of *Mentha* extract or *Brassica* extract had no adverse effect on normal health of the animal at the given dose levels, as there was no apparent decrease or increase in body wt. Lipid peroxidation measured as an index of cell damage also supported this observation. Thus, even the higher dose used had a safety margin well clear of the toxic range. This information is crucial, since chronic treatment with any agent for achieving chemopreventive prophylaxis should be free from any undesirable side effect. An increase in the levels of microsomal protein at individual high doses of *Mentha* and *Brassica,* is indicative of induced protein synthesis and possibly that associated with endoplasmic reticulum, which could be responsible for the increase in mouse liver weight. BHA, a known antioxidant compound, was used to verify our protocols and response in the animal model system. It has already been shown to be effective in cancer chemoprevention (Manohar and Rao, 1984; Hocman, 1988; Williams et al.,1999).
The levels of microsomal Cyt P_{450} were found to be elevated at both dose levels of individual and combined doses of *Mentha* and *Brassica*. Cyt P_{450}, a mixed function oxidase enzyme, plays a key role in oxidative activation, inactivation and in promoting the excretion of most xenobiotics including carcinogens as well as in modulating the duration and intensity of their toxicity (Guengerich and Shimada, 1991; Ingelmann,2001). During oxidative metabolism in the microsomal microenvironment involving the cytochrome P_{450} system, the electron flows from NADPH or NADH through a flavoprotein P_{450} reductase or cytochrome b_5 reductase to different isomorphic forms of cytochrome P_{450} and cytochrome b_5 (Gibson and Skett, 1994). As no inhibition in the activity of these reductases or b_5 content by *Mentha* extract and *Brassica* extract was noted, it can be assumed that no inhibition in microsomal electron transfer critical for Cyt P_{450} functional capability is operational.

Table 5

Modulatory influence of *Mentha* extract, *Brassica* extract (single and combined doses) and BHA on antioxidant/ detoxification Enzymes profile in mouse kidney.

Group	**Treatment**	**Duration (Days)**	**Relative wt of kidney (%)**	**GST (nmol CDNB conjugates/min/ mg protein)**	**Cu-ZnSOD (U/min/mg protein)**	**MnSOD (U/min/mg protein)**	**CAT (nmol H_2O_2 consumed/min/mg protein)**
I	Control (Vehicle only ie ddw)	15	1.20±0.14(1.00)	0.36±0.02(1.00)	5.54±0.12(1.00)	0.75±0.02(1.00)	58.12±1.17(1.00)
II	*Mentha* (400mg/kg bd wt)	15	1.22±0.17(1.01)	0.35±0.02(0.99)	5.59±0.18(1.05)	0.77±0.01(1.12)	61.28±1.35(1.09)
III	*Brassica* (400mg/kg bd wt)	15	1.23±0.22(1.02)	0.38±0.04(1.08)	5.83±0.19(1.21)	0.73±0.02(0.96)	72.43±2.12(1.36)*
IV	*Mentha &Brassica* (200+200mg/kg bd wt)	15	1.19±0.25(0.99)	0.34±0.05(0.98)	5.63±0.19(1.12)	0.95±0.05(1.53)**	59.73±1.72(1.04)
V	*Mentha* (800mg/kg bd wt.)	15	1.23±0.16(1.02)	0.35±0.05(0.99)	6.28±0.15(1.52)*	0.76±0.03(1.07)	57.85±1.25(1.02)
VI	*Brassica* (800mg/kg bd wt.)	15	1.24±0.21(1.03)	0.37±0.01(1.07)	5.85±0.12(1.24)	0.75±0.01(1.00)	79.16±1.65(1.47)*
VII	*Mentha & Brassica* (400+400 mg/kg bd wt)	15	1.22±0.25(1.01)	0.38±0.03(1.08)	6.57±0.23(1.58)*	0.74±0.04(0.98)	55.32±2.15(0.95)
VIII	BHA (0.75% in diet)	15	1.21±0.19(1.00)	0.52±0.04(1.59)#	7.21±0.21(1.77)**	0.97±0.08(1.64)#	81.14±1.35(1.74)#

Values are expressed as mean±s.e.m. of 6–8 animals. Values in parentheses represent relative changes in parameters assessed. $^{*}(p<0.05)$, $^{\#}(p<0.01)$ and $^{**}(p<0.001)$ represent significant changes relative to control. Cu-ZnSOD : Cu-Zn Superoxide dismutase; MnSOD : Mn Superoxide dismutase; CAT : Catalase; GST : Glutathione-s-transferase.

Table 6
Modulatory influence of *Mentha* extract, *Brassica* extract (single and combined doses) and BHA on antioxidant/ detoxification Enzymes profile in mouse stomach.

Group	**Treatment**	**Duration (Days)**	**Relative wt of stomach (%)**	**GST (nmol CDNB conjugates/min/ mg protein)**	**Cu-ZnSOD (U/min/mg protein)**	**MnSOD (U/min/mg protein)**	**CAT (nmol H_2O_2 consumed/min/mg protein)**
I	Control (Vehicle only ie ddw)	15	0.91±0.20(1.00)	0.22±0.05(1.00)	5.10±0.15(1.00)	0.90±0.01(1.00)	69.14±2.18(1.00)
II	*Mentha* (400mg/kg bd wt)	15	0.84±0.03(0.05)	0.31±0.05(1.45)#	5.22±0.14(1.04)	1.27±0.02(1.37)*	73.42±3.12(1.05)
III	*Brassica* (400mg/kg bd wt)	15	0.90±0.03(0.99)	0.29±0.01(1.37)*	5.38±0.23(1.08)	1.04±0.02(1.08)	82.46±2.34(1.38)**
IV	*Mentha &Brassica* (200+200mg/kg bd wt)	15	0.89±0.04(0.98)	0.35±0.02(1.51)#	6.30±0.18(1.38)**	1.32±0.05(1.42)*	84.18±3.42(1.43)**
V	*Mentha* (800mg/kg bd wt.)	15	0.93±0.04(1.02)	0.39±0.02(1.64)#	7.57±0.30(1.74)#	1.64±0.04(1.68)#	85.23±4.18(1.50)#
VI	*Brassica* (800mg/kg bd wt.)	15	0.95±0.02(1.03)	0.40±0.05(1.66)*	7.95±0.35(1.83)#	1.69±0.04(1.70)#	74.37±3.27(1.08)
VII	*Mentha & Brassica* (400+400 mg/kg bd wt)	15	0.86±0.02(0.96)	0.42±0.04(1.72)*	7.88±0.44(1.82)#	1.73±0.02(1.76)*	72.57±3.14(1.03)
VIII	BHA (0.75% in diet)	15	0.92±0.05(1.01)	0.37±0.04(1.58)*	8.14±0.22(1.96)**	1.85±0.02(1.88)*	89.27±4.27(1.62)#

Values are expressed as mean±s.e.m. of 6–8 animals. Values in parentheses represent relative changes in parameters assessed. $^{*}(p<0.05)$, $^{\#}(p<0.01)$ and $^{**}(p<0.005)$ represent significant changes relative to control. Cu-ZnSOD : Cu-Zn Superoxide dismutase; MnSOD : Mn Superoxide dismutase; CAT : Catalase; GST : Glutathione-s-transferase.

Under the present experimental conditions, the levels of GSH were enhanced at high individual doses of *Mentha* and *Brassica*. However, the high combined dose of *Mentha* and *Brassica* showed depletion in GSH level. GSH enhances metabolic clearance of peroxidized lipids and protects cells from the lethal effects of toxic and carcinogenic compounds (Kowalski et al.,1990; Arrick and Nathan, 1984). Therefore GSH depletion is often associated with cytotoxicity and there are some indications that conjugation reactions can be more detrimental to the cell than redox cycles (Stenius et al., 1989; Kappus, 1986). Thus, it seems possible that GSH depletion may promote tumor development through a mechanism that involves cytotoxicity (Murray et al., 1987). The enhanced LPO ,as indicated by High levels of TBARS, with the combined high dose of *Mentha* and *Brassica* is also indicative of the cytotoxic effect. It may be due to the interaction between the components of *Mentha* and *Brassica,* leading to the formation of oxidant based product resulting in cytotoxicity (Girotti, 1990). *Mentha* and *Brassica* extract treatment at individual and combined doses significantly elevated hepatic GST activity. GST is a critical detoxification enzyme that functions primarily in conjugating 'functionalized P_{450} metabolites' with endogenous ligand (GSH), favoring their elimination from the organism (Hartman and Shankel, 1990).

There are persuasive evidences to support induction of GST and protection against a wide spectrum of cytotoxic, mutagenic and carcinogenic chemicals (Ketterer, 1988; Reed, 1990). The specific activity was the sum of all its isoforms, as we used CDNB as a nonspecific substrate for GST. The protective effects of many naturally occurring chemopreventive agents against carcinogenesis have been ascribed to decreased bioavailability of potential DNA-damaging entities and their destruction into excretable metabolites, facilitated through induction of GST (Coles and Ketterer, 1990). The elevated GST activity and depleted levels of GSH at high combined dose of *Mentha* and *Brassica* might be attributed to increased electrophilic metabolites production leading to glutathione conjugates formation by elevated GST activity and the availability of GSH might not be able to keep pace with GST activity. Furthermore, certain xenobiotics are toxicologically activated by this conjugation route, either as such or as a result of further processing of glutathione conjugate. This might be the case with the high combined dose of *Mentha* and *Brassica.*

The attenuated GR level plays a significant role in the reduction of GSSG to GSH at the expense of NADPH and regulates the GSH-GSSG cycle in the cell, allowing a constant redox balance (Meister, 1994; Vanoni et al., 1991). Its high activity with the single and combined doses of *Mentha* and *Brassica* indicates increased availability of GSH though with the combined dose the rapid consumption of GSH far exceeds its formation resulting in disturbed redox balance. Both GPX and CAT, which plays an important role in the detoxification of peroxides and hydroperoxides, were induced at individual high doses of *Mentha* and *Brassica* and were found to be inhibited at combined high dose of *Mentha* and *Brassica.* The low GPX activity is lethal under the conditions of oxidative stress (Remacle et al., 1992), which is evident in combined dose of *Mentha* and *Brassica,* underlining the toxic interaction between the components of both extracts. Reduced GPX activity could be due : 1) to reduced transcription of GPX, 2) to 'using up' of the antioxidative capacity of GPX or, 3) to suppression of GPX activity by hydroperoxide products of lipid peroxidation (Remacle et al.,1992). The glutathione redox cycle, controlled by GPX, is a major source of protection against low levels of oxidative stress, whereas CAT becomes more significant in protecting against severe oxidative stress (Yan and Harding, 1997). Both GPX and CAT can neutralize peroxides but GPX has been reported to be more important than CAT to neutralize lipid peroxides (Fernandes et al., 1996) as indicated by lower affinity of CAT for H□O□ than GPX (Izawa et al., 1996).

The constitutive levels of another important antioxidant enzymes viz. Cu-ZnSOD and MnSOD were found to be augmented by individual as well as combined doses of *Mentha* and *Brassica*, accelerating dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen. Induction of Cu-ZnSOD with individualdoses of *Mentha* and *Brassica* is in conjunction with CAT induction, protecting cells from any free radical-induced injury. However, with the combined dose of *Mentha* and *Brassica*, the overproduction of H_2O_2 by $O_2\cdot^-$ dismutation might be responsible for SOD toxicity (Mates et al., 1999). Alternatively, the overscavenging of hydroperoxy radical HO2- (the conjugate acid of $O_2\cdot^-$) by excess SOD may reduce radical chain termination and result in increased lipid peroxidation (Mates et al., 1999).

Thus, the results of the current study demonstrate that the individual doses of *Mentha* and *Brassica* increases the activities of phase I and II enzymes, and enzymes involved in alleviating oxidative stress. Treatment with *Mentha* and *Brassica* resulted in striking induction of specific activities of detoxifying enzymes in the liver as well as in stomach and kidney, which strongly suggest the possibility of cancer chemopreventive potential of *Mentha* and *Brassica. Mentha* may be effective due to the presence of eugenol and ferulic acid which are known to induce detoxifying enzymes, viz. UDP-glucoronyl transferase and glutathione-s-transferase which conjugates many ultimate carcinogens (Yokota et al., 1988). The effect of *Brassica* may be due to its various components, chiefly sulforaphane (Maheo et al.,1997) and Indole-3-carbinol (Bradfield and Bjeldanes, 1984 and 1987). The combined dose not only failed to induce antioxidant enzymes but showed enhanced toxicity by inhibiting antioxidant enzymes and increasing LPO. Presumably, the antagonistic interaction between the phytochemicals of *Mentha* and *Brassica* have resulted in inactivaton of chemopreventive components, such as the breakdown of carbon chain length of isothiocyanates moiety in *Brassica* , which is associated with tumor preventive activity, as a longer isothiocyanate chain is apparently more suitable for insertion into cells due to increased lipophilicity (Morse et al., 1989). The inhibitory effect of *Mentha* and *Brassica* combination may be either due to the formation of an unknown inhibitory compound as a result of interaction of *Mentha* and *Brassica* components or due to the mutual structural steric hinderance between the phytochemicals as both *Mentha* and *Brassica* contains structurally large and long chain compounds.

The results of the present studies were further confirmed by the DMBA induced skin papillomagenesis study in Swiss albino mice, where the high combined dose of *Mentha* and *Brassica* showed significantly high tumor burden and tumor incidence along with the significant decrease in the average latent period. The individual high doses of *Mentha* and *Brassica* exhibited significant reduction in cumulative number of papillomas and tumor burden along with the enhanced duration of the average latent period (Sharma and Kumar, 2005).

However, further studies are warranted to : 1) understand the underlying mechanism of inactivation/inhibition by the combined dose of *Mentha* and *Brassica*; 2) To judge the chemopreventive efficacy of *Mentha* and *Brassica* by using different site-specific animal models of carcinogenesis and to identify the active principle(s) present in the extract responsible for the observed efficacy.

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