Anticarcinogenic activity of meso-zeaxanthin (MZ), a xanthophyll carotenoid with profound antioxidant activity, was evaluated against 3-methylcholanthrene (3-MC)-induced sarcoma in mice. Oral administration of MZ at different doses significantly increased tumor latency period. In 3-MC control group, animals started developing sarcoma on 6th week. However animals (treated with 3-MC and MZ (50 and 200 mg/kg b.wt) started developing sarcoma on 10th and 18th week, respectively. Survival of tumor-bearing mice was significantly increased by MZ treatment. Animals in 3-MC control group started dying due to tumor burden from 8th week. All animals treated with MZ (50 and 200 mg/kg b.wt) along with 3-MC were found to be alive even after 16 and 20 wk, respectively. Oral administration of MZ inhibited different CYP450 isozymes like CYP1A1 (FROD), CYP1A2 (MROD), and CYP2B12 (EROD), which are involved in carcinogen metabolism in a dose-dependent manner. Moreover, levels of phase II enzymes like UDP-glucuronosyltransferase and glutathione-S-transferase, which are involved in detoxification of carcinogens, were significantly increased by MZ treatment. Results indicated that mode of action of MZ may be through inhibition of carcinogen activation coupled with enhancement of detoxification process. MZ may also inhibit promotion phases of carcinogenesis by its antioxidant activity.

INTRODUCTION

Carotenoids are one of the phytonutrients belonging to the category of tetraterpenoids that impart red, yellow, and orange colors to fruits and vegetables. Structurally they are in the form of a polyene chain with and without aromatic substituted rings.
Preparation of MZ

Fresh marigold flowers were dried and were extracted with hexane to obtain oleoresin. The oleoresin was then subjected to saponification to obtain linolein. The saponified oleoresin containing linolein was then mixed with a solvent such as phenyl carboline and an alkali such as potassium hydroxide in a vessel fitted with a water condenser. The mixture was then heated under stirring for a period of 3 to 36 h at around 80–200°C. Sufficient alkali was used for accelerating the saponification. The product obtained was purified by HPLC for the identification of MZ.

For the present study we purchased MZ, which was prepared according to the above mentioned procedure, from Omni Active Health Technologies Pvt. Ltd. (Mumbai, India).

Stability and Storage of MZ

Stability data indicated that 200 mg/ml stock formulation of MZ was stable for up to 14 days when maintained at either 3–5°C or at 25°C (5). In the present study, a 5% suspension of MZ was prepared in sunflower oil and was stored in dark bottles at 4°C for 14 days to prevent oxidation.

HPLC Analysis of MZ

HPLC analysis of MZ was done using HPLC system-Waters 2489 UV/Vis detector. Column used was YMC pack SIL 250×4.6 mm 5 LD (YMC, Inc., Aflatenan, PA). Mobile phase used was hexane:ethyl acetate (75:25, v/v) and detector wavelength was 252 nm. Flow rate was 2 ml per min, total run time was 45 min, and detector sensitivity was 0.5 AUFS.

Anticarcinogenic Activity of MZ on 3-MC Induced Sarcoma

Sixty male Swiss albino mice were randomly divided into 4 groups (15 animals/group). Hair from the dorsal side of the animals was removed 24 h before the experiment. A single dose of 3-MC (200 μg/animal/dose) in 0.1 ml of dimethyl sulphoxide was administered subcutaneously (s.c.) on the dorsal surface of each animal to induce sarcoma (11). Animals in Group I were treated with 3-MC alone (3-MC control) and animals in Group II were treated with sunflower oil along with 3-MC (vehicle control). Animals in Groups III and IV received MZ 50 and 250 mg/kg b.wt respectively along with 3-MC. The treatment with MZ was started (oral gavage) 24 h after the injection of 3-MC and was continued for 6 days a wk for 20 consecutive wks. The animals were observed for 30 wk for the onset of sarcoma as well as their survival.

Effect of MZ on Phase I Carcinogen Metabolising Enzymes

Thirty rats were randomly divided into 5 groups (6 animals/group). Animals in Group I was kept as untreated (normal). Animals in Group II received phenobarbitone, which can induce microsomal P450 enzymes (PB control). Animals in Group III received sunflower oil along with phenobarbitone (vehicle control); VC. Animals in Groups IV and V received MZ 50 and 250 mg/kg b.wt respectively along with phenobarbitone. MZ.
was administered orally once daily for 15 days. Administration of phenobarbital (60 mg/kg b.w; i.e., peritoneally, once daily) was started on 12th day and continued for 4 days. On the 16th day 1 h after MZ administration the animals were sacrificed and the liver was excised. Liver homogenate (25%) was prepared in ice cold Tris buffer (pH 7.4, 0.1 M). The liver homogenate was initially centrifuged at 14,000 g for 20 min in a cold centrifuge (Remi). The supernatant was further centrifuged at 10,500 g for 1 h at an ultracentrifuge (Servall) and liver microsomes were washed and resuspended in cold phosphate buffer (pH 7.4, 0.1 M). The effect of MZ on the dealkylation of methoxy resorufin by 7-methoxyresorufin-O-demethylase (MROD)-CYP1A2, pentoxifylline by 7-pentoxylresorufin-O-depentylase (PRED)-CYP2B1/2 and ethoxy resorufin by 7-ethoxyresorufin-O-deethylase (EROD)-CYP1A1 were studied (12,13). Assay mixture contained sodium phosphate buffer (0.1 M, pH 7.4), 6.25 mM MgCl₂, 60 μM EDTA, 5 μM 7-methoxy resorufin, 7-pentoxyl resorufin, and 7-ethoxy resorufin, 100 μg microsomal protein (from MZ pre-treated and untreated animals) and 100 μM NADPH in a final volume of 1 ml. The reaction time was 5 min with a preincubation period of 5 min without the addition of NADPH at 37°C. The reaction was stopped by the addition of 2 ml of chilled methanol. The precipitated protein was centrifuged and supernatant was used for the estimation of enzyme activity using a fluorescent spectrophotometer (Hitachi F-2500) at the excitation wavelength of 355 nm and the emission wavelength of 453 nm. Calibration curves were constructed by determining the fluorescence of known amounts of the authentic resorufins. Concentration of proteins in each sample is measured by the method of Lowry et al. (14). Results were expressed as nano moles of resorufin formed/min/mg protein.

Effect of MZ on Phase II Carcinogen Metabolising Enzymes

Estimation of Glaucarboxylase-5-Transferease (GST) Activity

Twenty-four rats were randomly divided into 4 groups (6 animals/group). Animals in Group I was kept as untreated (normal), Animals in Group II received sunflower oil only (VCO). Animals in Groups III and IV received MZ 50 and 250 mg/kg b.w, respectively. MZ was administered orally once daily for 15 days. On the 16th day, 1 h after the carcinogen administration the animals were sacrificed and liver was excised. Liver homogenate (25%) was prepared in ice cold Tris buffer (pH 7.4, 0.1 M). GST activity was estimated by the method of Habig et al. (15). Briefly, the reaction mixture contained 0.1 M phosphate buffer (pH 6.5), 1 mM CDNB in ethanol, and 1 mM GSH. Reaction was started by the addition of liver homogenate (1 mg of protein) from MZ pre-treated and untreated animals. The initial reading was taken at 340 nm against a reference cuvette containing the complete assay mixture without tissue homogenate and the reading was continued for 5 min with a 1-min interval. The activity of GST was calculated and expressed as nano moles of CDNB-GSH conjugate formed/min/mg protein.

Estimation of UDP-Glucuronyl Transferase Activity

UDP-glucuronyl transferase (UDPGT) activity was estimated by the method of Issakabcher et al. (16) modified by Hollman and Touster (17). Incubation mixture contained 0.5 ml Tris HCl buffer (1M, pH 7.4), 0.2 ml Triton X-100, 0.05 ml MgCl₂ (50 mM), 0.05 ml p-nitro phenol, 0.1 ml water, and 0.1 ml of liver homogenate (1 mg of protein) from MZ pre-treated and untreated animals of the above experiment were incubated at 37°C for 2 min. After incubation, the reaction was started by the addition of 0.1 ml of UDP-glucuronic acid (30 mM). The reaction was arrested at different time points (0, 10, and 15 min) by adding 2 ml of ice cold trichloroacetic acid (5%, v/v) into tubes containing 100 μl of reaction mixture. After brief centrifugation, 1 ml of supernatant solution was made alkaline with 0.25 ml of 2M NaOH and the absorbance was read at 450 nm using spectrophotometer. The activity of UDPGT was expressed as nano moles/min/mg protein.

Statistical Analysis

Values were expressed as mean ± SD. The mean values were statistically analyzed by Kruskal–Wallis test. The statistical software package SPSS (version 18) was used for the analysis. Significant levels of control groups were determined by comparing with normal group, whereas significant levels of MZ-treated and vehicle control groups were determined by comparing with control group. P value < 0.05 was considered to be statistically significant.

Statistical analysis of survival of tumor-bearing animals treated with and without MZ was done by Wilcoxon (log rank) test.

RESULTS

HPLC Pattern

Chromatogram of HPLC analysis of MZ is given in Fig. 2. Purity of MZ was 91.75% and impurities present were translin (0.36%) and other carcinoids (1%).

![HPLC Chromatogram](image)

FIG. 2. HPLC of meco-anthrarin.
Effect of MZ Administration on 3-MC Induced Sarcoma

Tumor Incidence

The effect of MZ on tumor incidence (i.e., on development of 3-MC-induced sarcoma) is shown in Fig. 3. In 3-MC alone treated control group, animals started developing sarcoma on 6th wk after the carcinogen administration and within 20 wk all the mice were found to develop sarcoma. Animals treated with the lowest dose of MZ (50 mg/kg b.wt) started developing sarcoma only on 15th wk after 3-MC treatment. In 250 mg/kg b.wt MZ-treated group, only 1 animal developed sarcoma on 18th wk indicating very low incidence of sarcoma development at higher dose of MZ. The size of the tumor in MZ-treated animals were found to be comparatively small when compared to those of 3-MC control animals (Fig. 4).

Survival of Animals

The effect of MZ on survival of animals treated with 3-MC is given in Fig. 5. There was a significant reduction ($P < 0.001$) in the mortality rate of animals due to tumor burden in MZ-treated groups when compared to that of 3-MC control group. Death of animals due to tumor burden in 3-MC alone treated control group commenced from 8th wk. But all the animals treated with both 3-MC and MZ (50 and 250 mg/kg b.wt) were alive even after 16 and 20 wk, respectively. In 250 mg/kg b.wt MZ-treated group, 9 animals were found to be alive with no tumor when the experiment was completed (i.e., on 30th week). These results indicated that MZ significantly decreased tumor development and increased survival of animals.

Inhibition of Different Isomers of Microsomal Cytochrome P450 Enzymes by MZ

In normal animals, the activities of PROD, MROD, and EROD were 4.4 ± 2.1, 6.1 ± 3, and 7.1 ± 1 nano moles of resorufin formed/min/mg protein, respectively. In phenobarbital alone treated control animals, the activities of those 3 CYP450 isoenzymes were significantly increased ($P = 0.004$) to 24 ± 5.1, 18.4 ± 6, and 16 ± 3.2 nano moles of resorufin formed/min/mg protein, respectively. The increase in the

FIG. 3. Effect of *mazza-zeaxanthin* (MZ) on 3-methylcholanthrene (3-MC) induced tumor incidence in mice. 3-MC was administered as a dose of 200 μg/kg intraperitoneally (i.p.). Treatment with MZ was started 24 h after the injection of 3-MC and was continued for 6 days in a week for 20 consecutive wk. The animals ($n = 15$) were observed for 30 wk for the onset of sarcoma. a: Number of animals developed sarcoma; b: percentage of animals developed sarcoma.

FIG. 4. Effect of *mazza-zeaxanthin* (MZ) on 3-methylcholanthrene (3-MC) induced sarcoma formation in mice. A: 3-MC alone treated control animal with well-developed sarcoma; B: 3-MC + sunflower oil-treated vehicle control animal with well-developed sarcoma; C: 3-MC + 250 mg/kg b.wt MZ-treated animal with reduced sarcoma formation.
activities of CYP1A1 (PROD) was sixfold, CYP1A2 (MROD) was threefold, and CYP2B11 (EROD) was twofold. Oral ad-
ministration of MZ significantly decreased ($P = 0.003$ for EROD; $P = 0.004$ for MROD and PROD) phenobarbitone-
induced increase in the activities of CYP450 isoenzymes in a
dose-dependent manner. In 50 mg/kg b.wt MZ-treated group,
the activities of PROD, MROD, and EROD were $7.8 \pm 3.2$, $6.9 \pm 2.8$, and $10 \pm 1.4$ nmoles of retinol formed/min/g pro-
tein respectively, and in $250$ mg/kg b.wt MZ-treated group,
the activities of all these isoenzymes were reduced to $3.9 \pm 2.5$, $4 \pm 2.2$, and $6.8 \pm 0.98$ nmoles of retinol formed/min/g pro-
tein, respectively, which were almost close to those of normal
animals (Fig. 6). Effect of MZ Administration on Phase II Enzymes
UDPGT activity of normal animals was $26.79 \pm 0.9$ nmoles
mole/min/g protein and this was significantly increased ($P =
0.004$) by MZ pretreatment ($50$ and $250$ mg/kg b.wt) to $34.93$
$0.7$ and $55.6 \pm 1.5$ nmoles/min/g protein respectively.
GST activity was also significantly increased ($P = 0.004$) to
$75.1 \pm 11.6$ and $130.8 \pm 33.1$ nmoles/min/g protein by
MZ pretreatment ($50$ and $250$ mg/kg b.wt) respectively when
compared to that of normal animals, which was $37.6 \pm 0.04$
nano moles/min/g protein (Fig. 7). DISCUSSION
In the present study, anticarcinogenicity of carcinoid MZ
was studied against 3-MC induced sarcoma model. 3-MC was
found to be metabolized in liver by microsomal CYP450 en-
zymes to several oxygenated metabolites mainly dihydrodiols
and epoxides. These active electrophilic carcinoenic species
interact with critical cellular target molecules and thereby cause
genetic damage (18). Results of this study revealed that MZ at
doses 50 and 250 mg/kg b.wt markedly inhibited 3-MC-induced
sarcoma development in mice. The reliable criterion for judging
the value of any anticancer drug is the prolongation of life span of
the animal and reduction of tumor incidence. The present study
demonstrated reduction in sarcoma development and increase
in life span of tumor-bearing mice treated with MZ in a dose-
dependent manner. The reduced life span of 3-MC control mice
was evidently due to excessive tumor burden. It can therefore
be inferred that MZ increased the life span of sarcoma-bearing
mice by preventing tumor progression.
Chemoprevention is an effective means of cancer control.
Chemopreventive agents are either blocking agents or suppress-
ing agents. These agents can be further subdivided into bifunc-
tional inducers (induce both phase I and phase II enzymes),
monofunctional inducers (induce phase II enzymes only), and
dual-acting agents (inhibit phase I enzymes and induce phase II
enzymes) (19). In order to understand the possible anticarcino-
genetic activity of MZ, its effect on phase I carcinogen metaboli-
tizing enzymes was studied. The result indicated that MZ could
significantly inhibit different CYP450 isoenzymes (CYP1A1,
CYP1A2, and CYP2B1/2), which are involved in the activation of
many known chemical carcinogens. This inhibitory effect
could be one of the mechanisms of action of MZ against chem-
ical carcinogenesis.
Another major mechanism of protection against chemical
carcinogenesis is mediated by the induction of enzymes in-
volved in the detoxification of chemical carcinogens. Phase II
enzymes such as GST and UDPGT are the major enzymes in-
volved in the detoxification process. Transcriptional control

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FIG. 6. Inhibitory effect of meso-zeaxanthin (MZ) on phenobarbital induced CYMO activity. (a) Effect of MZ on the deactivation of tertiary research by 7-ethoxyresorufin-O-deethylase (EROD-CYP1A1). When mean value of normal animals was compared with that of phenobarbital (PB) control animals, there was a significant increase in EROD activity. P = 0.004 (**P < 0.01). When mean value of PB control animals was compared with that of vehicle control (VC) group animals, there was no significant decrease in EROD activity. P = 0.402 (P > 0.05). When mean value of PB control animals was compared with those of 30 and 250 mg/kg MZ treated animals, there was a significant decrease in EROD activity. P = 0.003 (**P < 0.01). (b) Effect of MZ on the deactivation of tertiary research by 7-methoxyresorufin-O-deethylase (MROD-CYP1A1). When mean value of normal animals was compared with that of PB control animals, there was a significant increase in MROD activity. P = 0.004 (**P < 0.01). When mean value of PB control animals was compared with that of VC group animals, there was no significant decrease in MROD activity. P = 0.057 (P > 0.05). When mean value of PB control animals was compared with those of 30 and 250 mg/kg MZ treated animals, there was a significant decrease in MROD activity. P = 0.004 (**P < 0.01). (c) Effect of MZ on the deactivation of primary research by phenacetin-O-deethylase (P450-2E1). When mean value of normal animals was compared with that of PB control animals, there was a significant increase in P450-2E1 activity. P = 0.004 (**P < 0.01). When mean value of PB control animals was compared with those of 30 and 250 mg/kg MZ treated animals, there was a significant decrease in P450-2E1 activity. P = 0.004 (**P < 0.01). The values were expressed as mean ± SD. The mean values were statistically analyzed by Kruskal-Wallis test. Results were expressed as same units of resorufin-forming protein.

FIG. 7. Effect of meso-zeaxanthin (MZ) administration on phase II enzymes. (a) UDPGT activity. When mean value of normal animals was compared with that of vehicle control (VC) group animals, there was a significant increase in UDPGT activity. P = 0.037 (P < 0.05). When mean value of normal animals was compared with that of PB group animals, there was a significant decrease in UDPGT activity. P = 0.006 (**P < 0.01). (b) Glutathione S-transferase (GST) activity. When mean value of normal animals was compared with that of PB group animals, there was no significant decrease in GST activity. P = 0.390 (P > 0.05). When mean value of normal animals was compared with those of 30 and 250 mg/kg and MZ treated animals, there was a significant increase in GST activity. P = 0.004 (**P < 0.01). The values were expressed as mean ± SD. The mean values were statistically analyzed by Kruskal-Wallis test. Results were expressed as same units of activity/mg protein.

The expression of phase II enzymes is mediated through the antioxidant response element (ARE) found in the regulatory regions of their genes. The binding of transcription factor Nrf2 to ARE in response to treatment with certain phytochemicals appears to be essential for the induction of prototypical phase II enzymes (20). The present study revealed that MZ treatment significantly elevated activities of different phase II enzymes like GST and UDPGT in a dose dependent manner. This ability of MZ is possibly due to the induced activation of Nrf2 and the expression of antioxidant enzymes. L and Z are the other macular carotenoids present in macula lutea of primates retina along with MZ. Previous studies in
our laboratory showed that L (20), Z, and MZ could scavenge superoxide, hydroxyl, nitric oxide, DPHH, and ABTS radicals and inhibit tissue lipid peroxidation in vitro in a concentration-dependent manner (3). The free radicals scavenging activities of MZ were found to be similar to those of Z. However, the IC50 values of MZ for scavenging superoxide radical, DPHH radical, and singlet oxygen were found to be low when compared to those of Z. Oral administration of MZ and L for 1 mo was found to increase the levels of antioxidant enzymes like catalase, superoxide dismutase, and glutathione peroxidase as well as GSH levels in mice (3,11). MZ and L were found to have hepatoprotective (8,22) and antiinflammatory properties. Moreover, MZ was found to inhibit effectively the carcinogenicity induced by nitrosodiethyl amine (NDEA) in rats (10). Epidemiological studies indicated that high intake of LZZ could reduce the risk of variety of cancers including lung and colon cancer (23). Because carotenoid MZ has substantial antioxidant activity, the anticarcinogenic activity of this carotenoid can also be due to the queching of oxygen radicals produced during promotion stage of carcinogenesis.

In conclusion, we can say that MZ is a "dual-acting agent" as it inhibited specific CYP450 isoenzymes and at the same time augmented the deoxification process through induction of phase II detoxification enzymes. A limitation of this study is that we have used high concentrations of MZ (50 and 250 mg/kg b.wt) to prevent 3-MC-induced carcinogenesis. Recommended daily supplement of MZ is only 0.5 mg/kg/day. Here we have used an acute dosage of 3-MC (200 μg/animal/dose) so higher concentrations of MZ may be needed to counteract the genotoxic effect of the carcinogen. More data on bioavailability of MZ is not available. Hence detailed studies on bioavailability as well as molecular mechanism of MZ are required. MZ can be considered as a chemopreventive agent by virtue of its protective effects like antioxidant effect, antiinflammatory effect, singlet oxygen quenching effect, inhibitory effect on specific CYP450 isoenzymes, potential to induce phase II enzymes, chemoprotective effect as well as anticarcinogenic effect. MZ is nonotoxic and generally regarded as safe. This carotenoid will be highly useful in the chemopreventive strategy against cancer using dietary supplements.

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