

Antimelanoma Potential of *Eruca sativa* Seed Oil and its Bioactive Principles

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Bansal, *et al.*: Antimelanoma Potential of *Eruca sativa* Seed Oil

The present communication reports the comparison of *in vivo* antioxidant, antimelanoma and antimutagenic activities of *Eruca sativa* seed oil and its bio principles (allyl isothiocyanate, phenylethyl isothiocyanate and sulphoraphane) against B16F10 melanoma cells induced in C57BL/6 mice model. Among the various treatments considered for the study, isothiocyanates combination (allyl isothiocyanate, phenylethyl isothiocyanate and sulphoraphane; 1:1:1; 10 μ M) exhibited optimum antioxidant activity, 51.95 \pm 1.14 μ M glutathione per mg protein compared to seed oil 25.91 \pm 1.26 μ M. Lipid peroxidation value was 9.97 \pm 1.72 μ M malondialdehyde per mg wet weight for isothiocyanates combination against seed oil, 28.45 \pm 1.87 μ M and rendered significant protection against oxidative stress induced by melanoma in liver tissue. Isothiocyanates combination significantly suppressed various parameters, such as tumor growth, isothiocyanates combination by 36.36% while the seed oil by 15.23%; tumor weight, isothiocyanates combination by 45.9% and seed oil by 19.6%; tumor volume, isothiocyanates combination by 41.7% while the seed oil by 32.3%, measured for antimelanoma activity at a concentration of 10 μ M. Isothiocyanates combination has been found to be more cytotoxic bioagent against B16F10 melanoma cells induced in C57BL/6 mice compared to naturally occurring *Eruca sativa* seed oil.

Key words: Antimelanoma, *in vivo*, oxidative stress, B16F10 melanoma cell line, C57BL/6 mouse

The proven efficacies of phytochemicals have created a revolutionary interest and awareness among the scientific community^[1]. The secondary metabolites of the plants serve as a defence system against various infestations^[2]. Thus, unlike compounds synthesized in the laboratory, secondary metabolites from plants are virtually guaranteed to have biological activity^[3]. Plant-based chemopreventive research has been associated with the issue that whether whole plant extract or bioactive principle is more potent^[4]. Several biologically active compounds in a plant work together (chemical partnership) to produce greater effect than that of a single chemical and also delay the development of resistance while isolated bioactive principles add to our therapeutic armamentarium. Search for plant-derived chemicals with pharmacological efficacies either in crude state or isolated state becomes an important area of sustained research^[5].

The plant *Eruca sativa* (*cruciferae*) originated in the Mediterranean region, is widely distributed all over

the world, particularly in India^[6,7], where the seeds are used for the production of a traditional spicy (*taramira*) oil. The role of *Eruca sativa* seed oil for antioxidant, antimicrobial activity^[8] and inhibition of proliferation of melanoma^[9] has been reported by us. In continuation, the present communication reports the comparison of *in vivo* antioxidant (glutathione and lipid peroxidation assay) and antimelanoma activity (body weight, tumor weight, tumor delay time, chromosomal assay, micronucleus assay) of its isolated bioactive principles (allyl isothiocyanate, phenylethyl isothiocyanate and sulphoraphane either given alone or in combination) and *Eruca sativa* seed oil against B16F10 melanoma cells induced in C57BL/6 mice.

MATERIALS AND METHODS

C57BL/6 strain mice 7-8 weeks old, weighing 25 \pm 5 g were maintained in a ventilated animal house of Department of Research, Jawaharlal Nehru Cancer Hospital and Research Centre, Bhopal (India). All the mice were kept at controlled environmental condition

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(22±2°, 60±5% humidity) with 12 h light/dark cycle. They were provided with standard pallet diet and water *ad libitum*. B16F10 mouse melanoma cells were obtained from the National Centre for Cell Sciences, Pune. These cells were maintained in Dulbecco's Modified Essential Medium supplemented with antibiotics, L-glutamine and fetal calf serum. All the chemicals and reagents used were of AR grade. Protocols of the animal experiments were approved by the institutional animal ethics committee constituted and approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India.

Animal experimental design, treatment and monitoring:

To investigate the effects of test sample against reference anticancer drug (doxorubicin), mice were randomized and divided into 15 groups of 6 animals each. *Eruca sativa* seed oil in DMSO, allyl isothiocyanate (AITC), phenylethyl isothiocyanate (PEITC), sulphoraphane (SUL) either alone or in combination were the test compounds used with corn oil as vehicle, saline was used as control. Volume equivalent to 10 µM of test compound has been used. Two doses, 10 and 30 µM of isothiocyanates have been considered for the present study on the basis of earlier reported study^[10]. Group I was kept as normal control and Groups 2-15 were injected with B16F10 mice melanoma cells (4×10^5) subcutaneously in the dorsal flank on day zero. Mice were injected intraperitoneally with saline (group II), corn oil (group III), 2% dimethyl sulphoxide (group IV), 1 mg/kg of doxorubicin in 4 doses on the day 1, day 5, day 9 and day 13 of treatment (group V), allyl isothiocyanate 10 µM (group VI), allyl isothiocyanate 30 µM (group VII), phenylethyl isothiocyanate 10 µM (group VIII), phenylethyl isothiocyanate 30 µM (group IX), sulphoraphane 10 µM (group X), sulphoraphane 30 µM (group XI), AITC, PEITC and SUL combination 10 µM (group XII), AITC, PEITC and SUL combination 30 µM (group XIII), seed oil 10 µM (group XIV), seed oil 30 µM (group XV). The incorporation of (4×10^5) viable cells (highly proliferative and metastatic melanoma cells) in the dermis is likely to complete one mitotic cycle with in 24 h and develop significant tumor within 3-4 d. Therefore, this regimen was considered for screening of anticancer activity. Doxorubicin induces apoptosis by induction of DNA fragmentation and cell shrinkage in tumor cells and has been in use for more than 30 y

in treating a variety of malignancies^[11-13], therefore, it has been considered as a reference drug for the present study. B16F10 mice melanoma cells have been injected in subcutaneous layer of skin at the back of mice and single tumor was developed there only. Local tumor growth was determined by measuring blindly diameter with callipers every other day, starting with the day when tumor became palpable. B16F10 melanoma cells injected subcutaneously into mice when grew to average size of tumor volume 2000 mm³ in the control group. Tumor volume (mm³) was estimated by the formula, $4/3 \times \pi \times (1/2 \times \text{smaller diameter})^2 \times (1/2 \times \text{larger diameter})$ ^[14]. Tumor growth delay was determined according to the method of Corbett *et al.* 1997^[15] and was calculated as follows, tumor growth delay=T-C, where T represents median time (in days) required for the treatment group tumors to reach a volume of 100 mm³ and C represents median time (in days) required for the control group tumors to reach the same size. Body weights of all animals were measured every alternative day during treatment period to detect life threatening toxicity by test samples and reference drug. Mice in all groups were observed daily for survival and sacrificed on day 26 after the experimental schedule. The tumors were dissected, weighed and stored at -80° until analysis was completed. To examine the histopathology, tumors from each group of animals were removed and fixed in 10% formalin solution for 24 h. Tissues were then embedded into paraffin. A section (4 µm) was stained with haematoxylin and eosin and examined under a microscope^[16].

Assay for reduced glutathione:

The liver tissue was dissected, weighed and homogenized in saline (1.15% potassium chloride) to give a 10% homogenate (w/v). The crude homogenate was centrifuged at 2000 rpm for 15 min and supernatant was collected. Phosphate ethylene diamine tetraacetate buffer (0.9 ml) and 5, 5'-dithio-bis-(2-nitrobenzoic acid) solution (0.050 ml) was added to supernatant (0.050 ml) making the solution 1.0 ml. The reaction mixture was incubated at room temperature for 20 min and the optical density was measured at 410 nm. The GSH levels were monitored by the reduction of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) to 5-thio-2-nitrobenzoate (TNB)^[17,18].

Assay for lipid peroxidation:

After euthanasia of mouse, the liver tissue was homogenised in 1.15% potassium chloride solution

by homogeniser (1 g of tissue in 9 ml of 1.15% potassium chloride solution). Sodium dodecyl sulphate (8.1%) was added to 0.2 ml of sample in test tube and pH was adjusted to 3.5 with 5 N sodium hydroxide. To this, 1.5 ml of 0.8% aqueous solution of thiobarbituric acid was added and mixture made up of 4 ml with distilled water and heated at 95° for 60 min. After cooling under tap water, 1 ml of distilled water and 5 ml of mixture of n-butanol and pyridine (15:1) were added and shaken vigorously^[19]. The solution was centrifuged at 3900 rpm for 10 min. Upper organic layer was removed and absorbance was measured at 532 nm using UV/Vis spectrophotometer.

Chromosomal aberrations test:

Cytogenetic damage in the bone marrow cells was studied by chromosomal aberration analysis at the end of experiment. All the animals were injected 0.025% colchicine intraperitoneally and sacrificed 2 h later to arrest the cells in metaphase by cervical dislocation. The femurs were dissected and cleaned to remove adherent muscles. The bone marrow was flushed into centrifuge tubes through repeated aspirations with 2 ml medium using fine needle. After sampling of bone marrow from femurs of the animals, the cells were centrifuged at 1000 rpm for 10 min. The supernatant was discarded completely and pellet was suspended in hypotonic solution (5 ml, 0.56% potassium chloride). These tubes were kept in water bath at 37° for 20 min. After incubation, cells were recentrifuged at 1400 rpm for 5 min, the supernatant was discarded and the pellet was resuspended in freshly prepared chilled Cornoy's fixative solution (2 ml, methanol:glacial acetic acid mixture in 3:1 ratio) and again centrifuged at 1400 rpm for 10 min^[20]. The pellet was resuspended in fresh fixative and the process was repeated 2-3 times. Cells kept for overnight fixation (4°) were centrifuged for 1400 rpm for 10 min again and the pellet was redispersed in fresh fixative (0.7-1 ml) depending on the amount of pellet. The cells were agitated and mixed thoroughly using Pasteur pipette and dropped on to the precleaned chilled slides from a distance of 30-40 cm. The slides were left in air to dry. The slides were dipped in 5% Giemsa solution for 10 min and rinsed in double distill water (DDW) and air-dried. A total of 100 well spread metaphase plates/animal were analyzed for different types of chromosomal damage including breaks, fragments, exchanges and multiple aberrations including pulverizations at a magnification of (100X×10X) for

all treated groups. The slides prepared were used for the counting of Mitotic Index. Metaphase plates were prepared by the air drying method^[21].

Assay for micronucleus test:

The bone marrow was flushed out using minimum essential medium, centrifuged and the pellet was resuspended in few drops of fetal bovine serum. Smears were prepared on preclean glass slides^[22], stained with May-Grawwald (5 min) and followed by Giemsa stain (5% giemsa solution for 10 min), rinsed in DDW and air-dried^[23]. The number of polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) and the frequency of micronucleated PCEs and NCEs were recorded at a magnification of (100X×10X) for all treated groups. Tumor micro vessels and density evaluation has been carried out using inbuilt MOTIC software.

Statistical analysis:

All experimental data were given as mean±SD. Statistical analysis was carried out using the one-way Analysis of Variance (ANOVA). Post Dunnett test was applied between control, reference drug and test samples using Graph Pad Prism software. Probability values were found to be less than 0.05.

RESULTS AND DISCUSSIONS

In vivo antioxidant, antimelanoma and antimutagenic activities of *Eruca sativa* seed oil and its bioactive principles, allyl isothiocyanate, phenylethyl isothiocyanate and sulphoraphane either given alone or in combination given at the doses of 10 and 30 µM against B16F10 melanoma cells induced in C57BL/6 mice strain. However, no marked increase in all the parameters considered for the study was observed with increase in dose from 10 µM onwards. Therefore, 10 µM dose was considered optimum. No further increase in the dose was considered because of toxicity issues of Isothiocyanates.

In order to determine the content of bio-active isothiocyanates in seed oil, we exploited the volatility of isothiocyanates present in seed oil. The coupled Head Space sampling enriched by solid phase micro extraction (HS/SPME) was used. Among the several fibres tested, a polar polyacrylate fibre showed to afford the best sensitivity and linearity in GC-MS analysis. Identification of

isothiocyanates was accomplished by comparison with NIST 05 MS-library (f-fit>700; r-fit>650) and was confirmed using authentic standards in all cases. Good quality chromatograms were obtained and reported in our earlier publication^[8]. Various identifying parameters of isothiocyanates in *Eruca sativa* seed oil have been presented here also as follows (Table 1).

The down regulation level of reduced glutathione in liver of experimental mice was investigated to determine the antioxidative effect of test groups against the oxidative stress induced by melanoma cells. After induction of B16F10 mice melanoma cells, the weights and the level of GSH of liver tissues of experimental mice were recorded. The liver weight of isothiocyanates treated mice (1.20 g) was found almost near to the normal control group mice (1.26 g) compared to the tumor control mice (1.64 g). Decreased concentration of reduced GSH, 15.75 μM per mg protein in tumor control group has been observed compared to normal control 59.82 μM . The reduced GSH level of test samples at two concentrations were as follows: at 10 μM concentration AITC -36.82 μM ; PEITC -40.83 μM ; SUL -43.33 μM ; AITC:PEITC:SUL -51.95 μM ; SO -25.91 μM ; while at 30 μM concentration AITC -42.57 μM ; PEITC -44.12 μM ; SUL -47.95 μM ; AITC:PEITC:SUL -52.26 μM ; SO -26.70 μM against the reference drug doxorubicin 56.79 μM . The increase in the concentration from 10-30 μM of isothiocyanates treatment alone or in combination did not result any noticeable change in antioxidant activity. Therefore, 10 μM has been considered optimum dose.

The percentage change on level of GSH per mg protein as a function of seed oil and isothiocyanates alone or in combination against doxorubicin at 10 μM on d 26 has been presented in fig. 1.

TABLE 1: HS/SPME/GC-MS ANALYSIS OF VOLATILE ISOTHIOCYANATES IN ERUCA SATIVA SEED OIL

Retention time (min)	Compound	Mol.Wt.	Characteristic ions ^a (m/z)	Amount ($\mu\text{g/g}$)
6.237	Allyl isothiocyanate	99.15	41, 72, 99 (bp)	40.3
22.450	2-Phenylethyl isothiocyanate	163.24	91 (bp), 105, 163	158.5
28.373	Sulforaphane	177.29	55, 72 (bp), 85, 115, 160	743.1

^aThe base peak (100%) is indicated as bp, while the molecular ion (M^+), when visible in the spectrum, is given in the table

Although level of reduced GSH is higher in case of individual isothiocyanates, interestingly their combination at 10 μM concentration exhibited a further increase compared to seed oil. Subcutaneous induction of B16F10 melanoma showed a significant lowering of reduced glutathione in liver (characteristic of antioxidants) compared to normal and reduced the scavenging of reactive oxygen species. Among the groups studied, optimum value of reduced GSH per mg protein is found to be in the order, doxorubicin>isothiocyanates combination>isothiocyanates given alone>seed oil. Increased concentration of MDA (63.3 μM) per mg wet weight has also been observed compared to normal control 65 \pm 1.83 μM . Concentration of MDA/mg were as follows: at 10 μM concentration AITC -23.42 \pm 1.98 μM ; PEITC -22.9 \pm 1.88 μM ; SUL -23.5 \pm 1.85 μM ; AITC:PEITC:SUL -9.77 \pm 1.72 μM ; SO -28.45 \pm 1.65 μM ; at 30 μM concentration AITC -19.87 \pm 1.79 μM ; PEITC -19.95 \pm 1.94 μM ; SUL -17.7 \pm 1.81 μM ; AITC:PEITC:SUL -8.77 \pm 1.96 μM ; SO -24.36 \pm 1.69 μM . Among the groups studied, optimum value of MDA per mg wet weight is found to be in the order: doxorubicin>isothiocyanates combination>isothiocyanates given alone>seed oil. Based on our observation, 10 μM of AITC:PEITC:SUL exhibited optimum antioxidant activity and rendered significant protection against oxidative stress induced by melanoma in liver tissues.

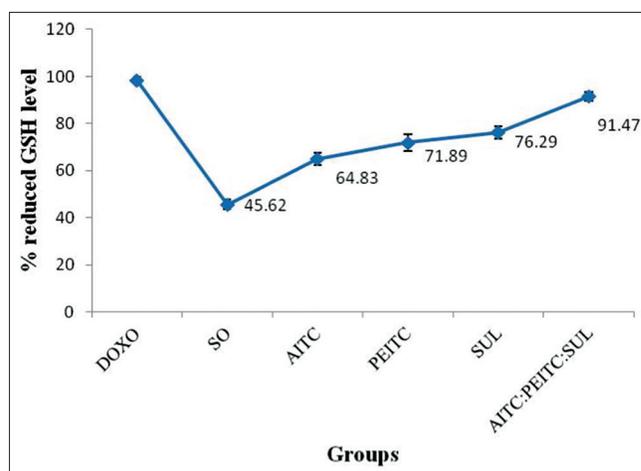


Fig. 1: Effect of seed oil, isothiocyanates on GSH/mg protein against doxorubicin at 10 μM on day 26. Percent change in GSH per mg protein after seed oil, isothiocyanates alone and in combination against doxorubicin at 10 μM on day 26. Each value is mean \pm SD (n=6). $P>0.05$ vs. tumor control, $P<0.05$ vs. normal control. AITC is allyl isothiocyanate, PEITC is phenylethyl isothiocyanate, SUL is sulphoraphane, DMSO is dimethyl sulfoxide, DOXO is doxorubicin and SO is seed oil.

Injection of B16F10 melanoma cells subcutaneously into mice was followed by 26 d observation, monitoring the mean body weight, tumor weight and tumor growth delay of all the experimental groups (Table 2). The body weights of the control and treated mice were determined periodically to assess non-specific toxicity of isothiocyanates. The average body weights of the control and isothiocyanate alone or in combination treated mice did not differ significantly by one-way ANOVA suggesting that isothiocyanates administration did not cause weight loss. The mice in isothiocyanates-treated group appeared healthy and did not show any other sign of non-specific toxicity, such as food and water withdrawal and impaired movement. Concentration of 10 μM in each case was found optimum as no significant change in body weight with the increase in concentration to 30 μM . Average tumor weight in the tumor control group and test samples treated group were depicted, and can be ordered as: doxorubicin<isothiocyanates combination<isothiocyanates given alone<seed oil<Tumor control. Similar trend was also observed when tumor delay time in all the experimental groups has been studied. Overall, significant reduction in tumor weight and tumor delay time was found in isothiocyanates combination treated mice.

TABLE 2: EFFECT OF ISOTHIOCYANATES, SEED OIL AND DOXORUBICIN ON NORMAL AND MELANOMA TUMOR BEARING MICE DAY 26

Groups	Body weight \pm SD (g)	Tumor weight \pm SD (g)	Tumor delay time (days)
Normal control	31.4 \pm 1.27	-	-
Tumor control	29.0 \pm 1.20	1.78 \pm 1.35	0
Corn oil	30.8 \pm 1.16	1.63 \pm 1.27	0
2% DMSO	32.3 \pm 1.17	1.65 \pm 1.15	0
DOXO	26.8 \pm 1.25	1.12 \pm 1.03	6
PEITC (10 μM)	27.9 \pm 1.05	1.34 \pm 1.15	3
PEITC (30 μM)	27.4 \pm 1.22	1.30 \pm 1.26	4
AITC (10 μM)	31.1 \pm 1.15	1.31 \pm 1.12	3
AITC (30 μM)	28.3 \pm 1.22	1.27 \pm 1.23	4
SUL (10 μM)	27.5 \pm 1.25	1.36 \pm 1.18	3
SUL (30 μM)	28.3 \pm 1.13	1.31 \pm 1.28	4
AITC: PEITC: SUL (10 μM)	29.4 \pm 1.07	1.22 \pm 1.06	5
AITC: PEITC: SUL (30 μM)	28.4 \pm 1.20	1.20 \pm 1.09	5
SO (10 μM)	28.6 \pm 1.22	1.52 \pm 1.38	1
SO (30 μM)	28.0 \pm 1.01	1.47 \pm 1.23	2

Each value is mean \pm SD ($n=6$). $P>0.05$ vs. tumor control, $P<0.05$ vs. normal control. NC is normal control, TC is tumor control, AITC is allyl isothiocyanate, PEITC is phenylethyl isothiocyanate, SUL is sulphoraphane, DMSO is dimethyl sulphoxide, DOXO is doxorubicin and SO is seed oil

The above findings support the view that multiple active phytochemicals result into synergism in such a way that outcome may not be additive but multiplicative^[24]. The uses of two or more bioagents have been recognized recently, providing enhanced therapeutic bioefficacy^[25,26].

The effect of the test samples on percent chromosomal aberration was measured in terms of chromatid breaks, centric rings, acrocentric association, acentric fragments, intracalary deletion, minutes and total abnormal metaphases (fig. 2). Percent of aberrant metaphase in various groups were in the range as follows: cancer control (80%), doxorubicin-treated (72%), AITC (20-34%), PEITC (36-48%), SUL (40-60%), AITC:PEITC:SUL (12-20%), seed oil (46-52%) at 10 μM -30 μM concentrations, providing the following order: isothiocyanates combination<isothiocyanates alone<seed oil<doxorubicin<cancer control group (Table 3). Isothiocyanates combination is found to exhibit maximum reduction in all the chromosomal aberrations studied in bone marrow cells compared to tumor control and standard doxorubicin.

The effect of various treatments on melanoma-induced mice was determined in terms of micronucleated polychromatic erythrocytes (MPCEs) and normochromatic erythrocytes (NCEs) per 1000 cells. Percent of MPCE and NCE in various groups were in the range as follows: cancerous control (90-97%), doxorubicin-treated (37-40%), AITC (6-22%), PEITC (10-19%), SUL (13-20%), seed oil (12-34%), AITC:PEITC:SUL (5-8%) at 10-30 μM concentrations (fig. 3). Isothiocyanates combination treated mice were significantly ($P>0.05$) reduced the micronuclei in PCEs and NCEs comparable with tumor control and doxorubicin treated group. The Isothiocyanates combination reduced the frequency of micronuclei per polychromatic (PCEs) and normochromatic erythrocytes (NCEs) compared to standard drug and tumor control group (fig. 4).

The observation finds support from Musk and Johnson^[27] who have demonstrated the role of Isothiocyanates as significant inducers of chromosomal damage protecting laboratory animals from the induction of tumors and recommended the presence of isothiocyanates in the human diet.

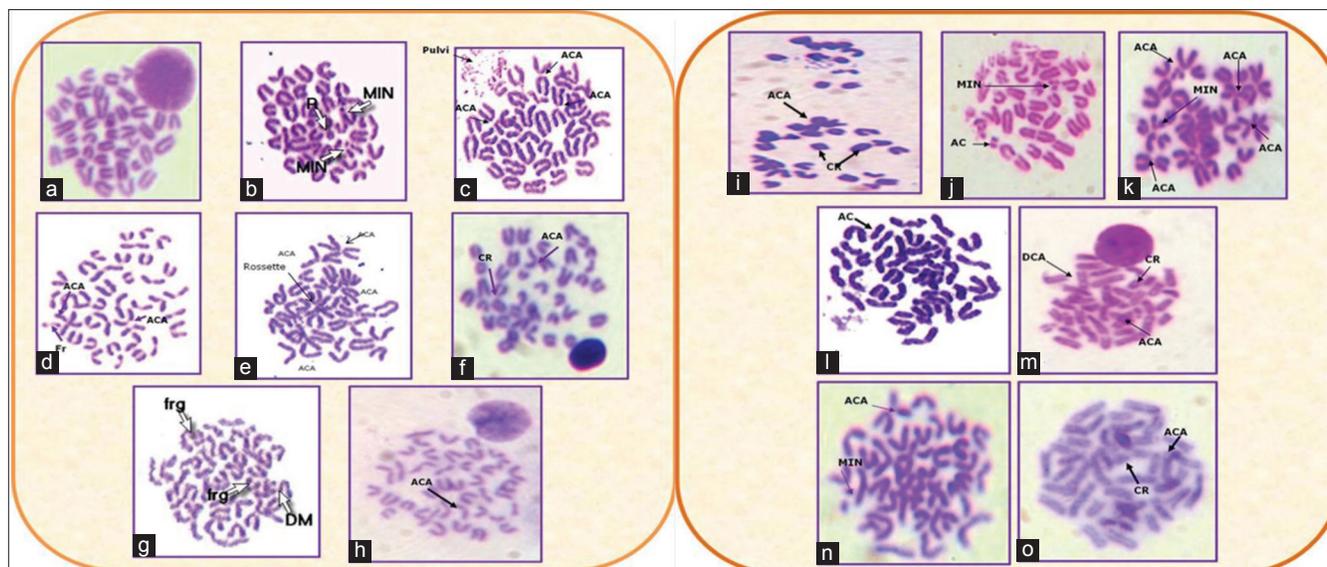


Fig. 2: Effect of treatments on chromosomal aberrations in mice bearing B16F10 melanoma. Effect of treatments on chromosomal aberrations in (a) normal control, (b) tumor control, (c) corn oil, (d) 2% DMSO (e) doxorubicin-treated, (f) AITC 10 μ M, (g) AITC 30 μ M, (h) PEITC 10 μ M, (i) PEITC 30 μ M, (j) SUL 10 μ M, (k) SUL 30 μ M, (l) AITC:PEITC:SUL 10 μ M, (m) AITC:PEITC:SUL 30 μ M, (n) SO 10 μ M, (o) SO 30 μ M treated mice after inducing B16F10 melanoma.

Accumulating evidences^[28-31] demonstrate that tumor growth and lethality are dependent on angiogenesis. An observation of histological slides (fig. 5) exhibits the decrease in tumor growth in mice by the isothiocyanates treatment which may be attributed to decreased host angiogenesis. A marked and dense microvasculature was observed in the control tumors. Tumors treated with isothiocyanates combination (31.23 \pm 6.45%) and doxorubicin (27.6 \pm 6.67%) had significantly fewer microvessels compared with the AITC (45.27 \pm 8.68%), PEITC (47.8 \pm 8.34%), SUL (42.35 \pm 8.42%) and tumor control (62.6 \pm 8.7%). Angiogenesis inhibition observed with isothiocyanates combination treatment is indicative of drug accumulation in the tumor and decreased tumor microvessel density which is further associated to the suppression of angiogenic vascularization, inhibited tumor cell proliferation and increased tumor cell apoptosis.

The mechanism of anticarcinogenic activity of isothiocyanates has not yet been fully elucidated. Isothiocyanates are reported to reduce activation of carcinogens and increase their detoxification finally exhibiting anticarcinogenic activity^[32,33]. The mechanism of the enhancement in anticancer activity of isothiocyanates combination is not easy to predict owing to their complex pharmacological actions. However, the intrinsic properties of isothiocyanates in synergism play important role towards enhancement

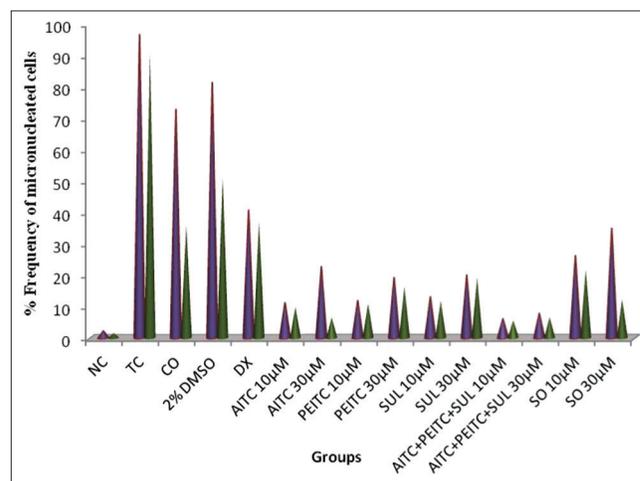


Fig. 3: Effect of treatments on MPCs and NCEs in tumor bearing mice. Effect of the isothiocyanates alone and in combination, seed oil and doxorubicin on percent micronucleated polychromatic cells (MPCs) and normochromatin erythrocytes (NCEs) in normal and melanoma tumor bearing mice on day 26. Each value is mean \pm SD (n=6). $P>0.05$ vs. tumor control, $P<0.05$ vs. normal control. NC is normal control, TC is tumor control, AITC is allyl isothiocyanate, PEITC is phenylethyl isothiocyanate, SUL is sulphoraphane, DMSO is dimethyl sulphoxide, DOXO is doxorubicin and SO is seed oil. ■ MPCs, ■ MNCE.

in target bioefficacy. Based on experimental findings and relevant available literature, a hypothesis is synthesized to explain observed synergistic action of isothiocyanates (fig. 6). Isothiocyanates act through apoptosis and exhibit anticancer activity by multiple pathways including oxidative stress^[34,35], inhibition of cell cycle progression^[36,37], angiogenesis^[38] and MAPK signalling^[39-41]. Isothiocyanates effectively disables the

TABLE 3: EFFECT OF ISOTHIOCYANATES, SEED OIL AND DOXORUBICIN ON THE BONE MARROW OF C57BL/6 MICE AFTER INDUCTION OF MELANOMA

Group	CB	CR	FR	ACA	ICD	AC	MIN
NC	12.5±1.14	0±0.0	25.0±1.13	12.5±1.02	0.0±0.0	12.5±1.05	0.0±0.0
TC	12.6±1.85	8.14±1.85	17.1±2.09	38.5±2.14	11.8±1.98	9.62±1.65	2.96±1.74
CO	17.8±2.54	10.2±1.58	9.55±1.74	27.4±1.65	8.28±1.62	11.5±1.06	3.18±1.32
2% DMSO	21.9±1.78	7.74±1.98	9.03±2.41	29.0±2.10	9.67±2.14	9.67±1.65	5.64±1.57
DOXO	25.1±1.85	7.44±1.65	8.37±1.84	31.6±1.78	9.30±0.98	10.2±1.56	5.11±1.64
AITC (10 µM)	12.6±0.94	5.26±0.68	7.36±0.84	16.8±0.98	6.31±0.75	10.5±0.72	5.26±0.86
AITC (30 µM)	14.1±1.28	5.46±1.21	7.03±1.12	19.5±1.06	8.59±1.15	10.9±1.25	4.68±1.09
PEITC (10 µM)	14.1±0.68	5.05±0.92	7.07±0.84	18.2±0.65	6.06±0.94	11.1±0.81	5.05±0.75
PEITC (30 µM)	15.3±1.75	5.64±1.56	8.06±1.34	19.4±1.32	8.87±1.45	12.1±1.64	4.83±1.68
SUL (10 µM)	14.7±1.78	3.92±1.58	7.84±1.46	19.6±1.34	7.84±1.52	12.7±1.64	4.9±1.74
SUL (30 µM)	15.3±2.45	5.83±2.34	8.02±2.25	18.2±2.12	8.75±2.32	13.1±2.41	4.37±2.48
AITC:PEITC:SUL (10 µM)	7.69±1.78	4.32±1.95	6.69±2.1	15.4±2.15	5.98±2.08	5.76±1.80	3.84±1.71
AITC:PEITC:SUL (30 µM)	16.7±1.74	5.55±2.14	11.1±2.45	19.4±3.21	5.55±2.35	11.1±2.08	5.55±1.87
SO (10 µM)	18.2±2.34	6.81±1.08	13.6±0.75	19.7±0.85	9.09±1.32	9.09±1.21	6.06±1.54
SO (30 µM)	20.0±1.24	6.66±1.48	16.7±1.09	23.3±1.36	11.7±0.95	11.7±1.75	6.66±2.48

Each value is mean±SD (n=6). $P>0.05$ vs. tumor control, $P<0.05$ vs. normal control. NC is normal control, TC is tumor control, AITC is allyl isothiocyanate, PEITC is phenylethyl isothiocyanate, SUL is sulphoraphane, DMSO is dimethyl sulphoxide, DOXO is doxorubicin and SO is seed oil. CB is chromatid breaks, CR is chromatid rings, FR is fragments, ACA is acrocentric association, ICD is intercalary deletion, AC is acentric association

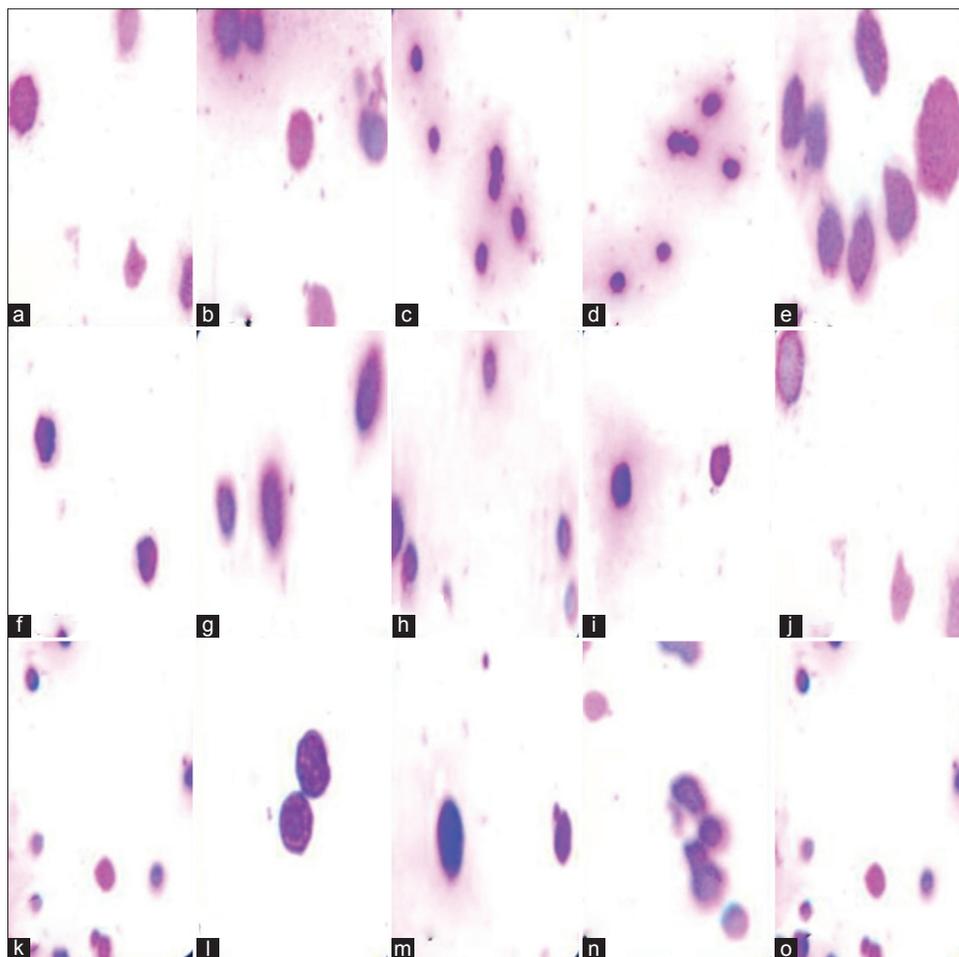


Fig. 4: Effect of treatments on micronuclei per PCEs and NCEs in tumor bearing mice. Micronuclei per polychromatic erythrocytes (PCEs) and normochromic erythrocytes (NCEs) in (a) normal control, (b) tumor control, (c) corn oil, (d) 2% DMSO (e) doxorubicin-treated, (f) AITC 10 µM, (g) AITC 30 µM, (h) PEITC 10 µM, (i) PEITC 30 µM, (j) SUL 10 µM, (k) SUL 30 µM, (l) AITC:PEITC:SUL 10 µM, (m) AITC:PEITC:SUL 30 µM, (n) SO 10 µM, (o) SO 30 µM treated mice after inducing B16F10 melanoma.

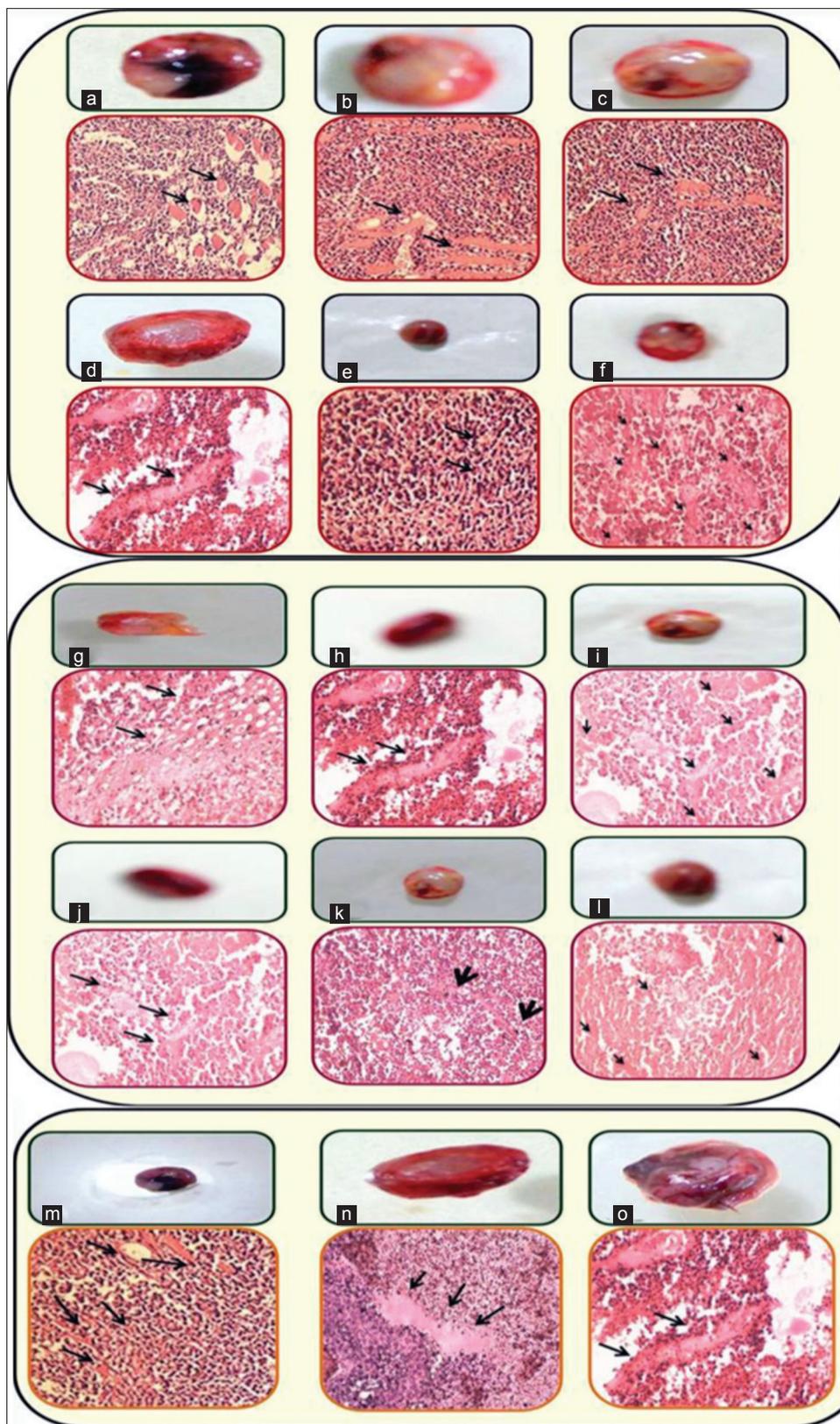


Fig. 5: Effect of treatments on micro vessels in tumor cells in mice bearing solid tumors.

Histological observation of micro vessels among the tumor cells with solid tumor: tissue sections stained by HE ($\times 100$) from (a) normal control, (b) tumor control, (c) corn oil, (d) 2% DMSO (e) doxorubicin-treated, (f) AITC 10 μM , (g) AITC 30 μM , (h) PEITC 10 μM , (i) PEITC 30 μM , (j) SUL 10 μM , (k) SUL 30 μM , (l) AITC:PEITC:SUL 10 μM , (m) AITC:PEITC:SUL 30 μM , (n) SO 10 μM , (o) SO 30 μM -treated animals. Arrows indicate micro vessels.

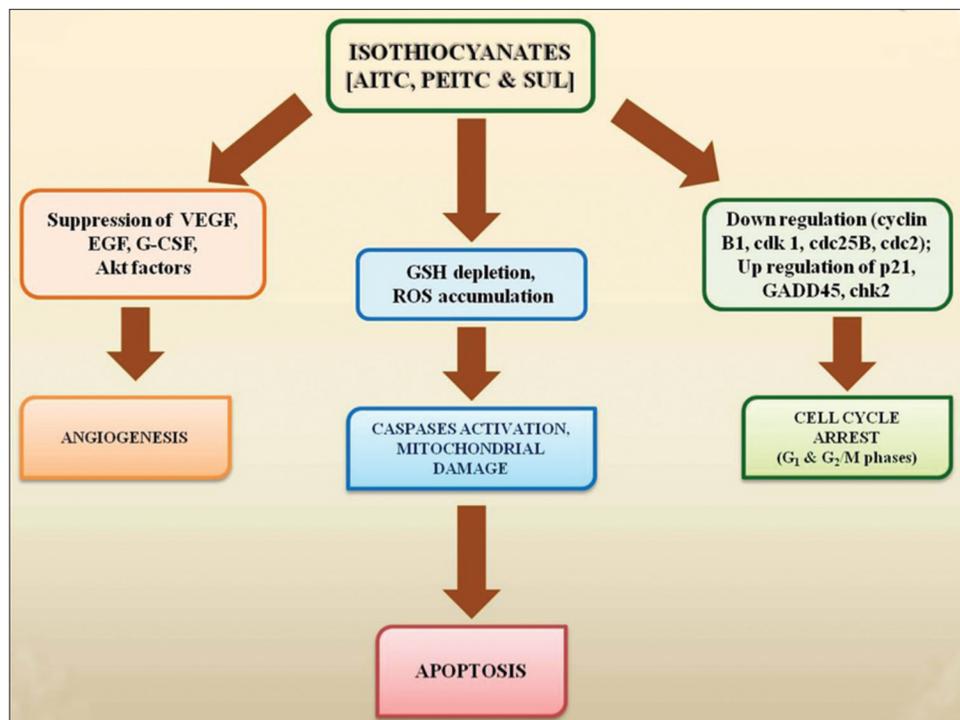


Fig. 6: Mechanistic hypothesis to explain synergistic effects of isothiocyanates in combination.

glutathione (GSH) antioxidant system and causes ROS accumulation preferentially in the transformed cells due to their active ROS output. Excessive ROS cause oxidative mitochondrial damage, cytochrome *c* release, inactivation of redox-sensitive molecules (GXP), and massive cell death. Isothiocyanates may induce cell cycle arrest (G_1 and G_2/M phases) in different phases in a cell line dependent manner. Inhibition of angiogenesis employed by ITCs to prevent cancer involves inactivation of Akt, suppression of VEGF and EGF expression, and G-CSF secretion. Isothiocyanates may also induce apoptosis through a caspase-3-dependent MAPK mechanism based on JNK and caspase-3-dependent mechanism. It induces rapid and transient induction of caspase-3 activity and stimulates proteolytic cleavage of poly-(ADP-ribose) polymerase, resulting into caspase activation and precedes DNA fragmentation.

The present piece of *in vivo* experiments highlight the effectiveness of isothiocyanates-combination among all the treatment studied and is found capable for reducing melanoma against reference drug doxorubicin. Overall, the results nicely complement each other depicting the safe and health promoting value of dietary consumption of isothiocyanates containing *Eruca sativa* seed oil. Our results also indicate that the isothiocyanates combination at

10 μM is a better cytotoxic bioagent against B16F10 melanoma cells induced in C57BL/6 mice compared to naturally occurring seed oil. It has enough potential for clinical applications and lends support to its use in traditional medicine. It is worth mentioning that neither life threatening toxicity nor a loss of body weight during the isothiocyanates combination treatment was observed compared to normal control. The finding may be considered significant in comparison with side effects (loss of body weight) normally observed in adjuvant therapy, highlighting the ability of isothiocyanates combination to inhibit melanoma growth with the view to develop new antitumor substances with low toxic potential.

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