



## ISOTHIOCYANATES: NATURALLY OCCURRING HepG2 HUMAN LIVER CARCINOMA INHIBITOR

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**Abstract:** *In vitro* anticancer screening of different isothiocyanates (ITCs) [Allyl isothiocyanate (AITC), Phenylethyl isothiocyanate (PEITC) and Sulphoraphane (SUL)], derived from naturally occurring *Eruca sativa* seed oil (SO) against HepG2 human liver carcinoma cell line has been carried out using MTT, TBE and SRB bioassays. Combination of ITCs, in ratio (1:1:1) exhibited maximum percentage inhibition of 97.12%, 96.34% and 96.56% by MTT, TBE and SRB respectively against reference drug doxorubicin at concentration 50  $\mu$ M. Percent inhibition has been found to be more pronounced in isothiocyanates combination compared to its natural source *E. sativa* seed oil.

**Keywords:** Carcinoma, *Eruca sativa*, Isothiocyanates

### INTRODUCTION

Antioxidants protect the body from destructive free radicals, which are generated when the body uses oxygen to make energy and from exposure to environmental factors. Left unchecked, free radicals cause uncontrollable chemical reactions that damage the body. Without the help of antioxidants, decay would soon destroy human bodies by damaging DNA and accelerating aging. Experimental findings indicate that antioxidants inhibit the growth of cancer cells or directly kill them (1). Research also indicates that antioxidants may work with chemotherapy to kill more cancerous cells than approach alone (2). Since it is known that antioxidants work together (3), a combination of antioxidants can be expected to have an even greater anticancer effect (4). A combination of antioxidants has been found to be more effective in reducing the growth of cancer cells than the individual antioxidant (5; 6). One major benefit of antioxidants is the fact that they do not interfere with the way that chemotherapy produces its anticancer effect. The use of antioxidants with chemotherapy showing no interference has been reported (7).

*E. sativa*, (Miller) is the most representative species of annual herb, which originated in the Mediterranean region (8), now is widely distributed all over the world, particularly in India (9), where the seeds are used for the production of a traditional spicy (taramira) oil. *E. sativa* oil is brought to the attention of the scientific community for various pharmacological properties viz. antidiabetic, antirenal-failure, antigastro-intestinal indigestion and antineurotoxic activity (10).

In continuation of our work (11; 12; 13; 14), on screening of Indian medicinal plants for various

pharmacological activities, we have recently shown that *E. sativa* is a relevant source of antimicrobial activity (15). Various isothiocyanates have been isolated from *E. sativa* seed oil (16). The combinational effects of the isothiocyanates derived from *E. sativa* seed oil have been studied on B16F10 mice melanoma cell line (17). Encouraging results on the above lines have inspired us to explore the *invitro* effect of combination of three isothiocyanates (Allyl isothiocyanate, Phenylethyl isothiocyanate and Sulphoraphane) for anticancer activity against HepG2 human liver carcinoma cell line and to compare its efficacy with *E. sativa* seed oil.

### MATERIALS AND METHOD

#### Chemicals and Reagents:

Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), glutamine, penicillin and streptomycin were purchased from Gibco BRL, USA. Trypan blue dye, MTT [3-(4, 5- dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium] dye and doxorubicin (Trade name: Adriamycin) were obtained from Sigma-Aldrich Chemical Company (UK). Allyl isothiocyanate, phenylethyl isothiocyanate and Sulphoraphane were used as test samples. Analytical standards were purchased from Fluka-Sigma-Aldrich. Double distilled water was used throughout the experiment. All other chemicals and solvents used were of highest purity commercially available (Sigma-Fluka-Aldrich) and were of analytical grade.

#### Cell culture:

HepG2 human liver carcinoma cells were purchased from National Centre for Cell Sciences, Pune, India. These cells were maintained in DMEM



medium supplemented with antibiotics, L-glutamine (2 mM) and 10% fetal calf serum. HepG2 human liver carcinoma cells were considered for *in vitro* cytotoxic studies on the basis of their metastatic nature. The cells were grown in the following conditions: CO<sub>2</sub> (5%) atmosphere in high humidity (95%) at 37°C in a CO<sub>2</sub> incubator. Each batch of cells was assessed for cell cytotoxicity by TBE, SRB and MTT assays.

#### Trypan Blue Exclusion Assay:

Cells (1×10<sup>5</sup>/plate) were incubated in poly-L-lysine precoated tissue culture petri plates with complete medium (MEM medium with fetal bovine serum) and allowed to adhere for 24 h in CO<sub>2</sub> incubator at 37 °C. The medium was replaced with incomplete medium (MEM medium without fetal bovine serum) containing serial dilution series of test samples (3 - 50 μM) separately for 24 h in CO<sub>2</sub> incubator at 37 °C. The medium was removed from the wells by aspiration and trypsinized to obtain the cell suspension. Trypan blue dye (0.4%; 0.1 ml) was mixed with cell suspension, 15 min prior to completion of incubation period (18). At the end of incubation period, the number of viable cells (unstained) was counted using a haemocytometer. Viability was expressed as a percentage of control number of cells excluding trypan blue dye.

#### Methyl Thiazole Tetrazolium Assay:

MTT assay depends on the mitochondrial enzyme reduction of tetrazolium dye to determine cell viability. Briefly, the cells were plated at a density of 1×10<sup>5</sup> cells/well into 96- well plates and allowed to adhere for 24 h in CO<sub>2</sub> incubator at 37 °C. The medium was replaced with the serum free medium containing different concentrations of test samples (3 - 50 μM) again for 24 h in CO<sub>2</sub> incubator at 37°C. MTT dye (20μl; 5 mg/ml) was added to each well 4 h prior to completion of incubation period. The medium was removed by adding 200 μl of DMSO to each well and incubated for further 10 min (19). The absorbance was read at 550 nm using ELISA reader (Synergy HT, Biotech, USA). The average values were determined from triplicate. Percent inhibition was calculated by using the formula:  $(C-T)/C \times 100$ , where C=Absorbance of control, T=Absorbance of Treatment. The IC<sub>50</sub> values of test compound were compared with standard drug (20).

#### SRB Assay:

Cells (1×10<sup>5</sup>/well) were seeded in poly-L-lysine precoated flat bottomed 96 well tissue culture plates and allowed to adhere for 48 h in CO<sub>2</sub> incubator at 37°C. The medium was replaced with the complete medium containing different concentrations of test samples (3 - 50 μM) separately again for 24 h in CO<sub>2</sub> incubator at 37°C. The plates were stained with sulphorhodamine B solution (0.4% in water) in dark for 30 min and then excess dye was washed out with acetic acid (1%) (21).The unwashed dye was eluted with tris-buffer and

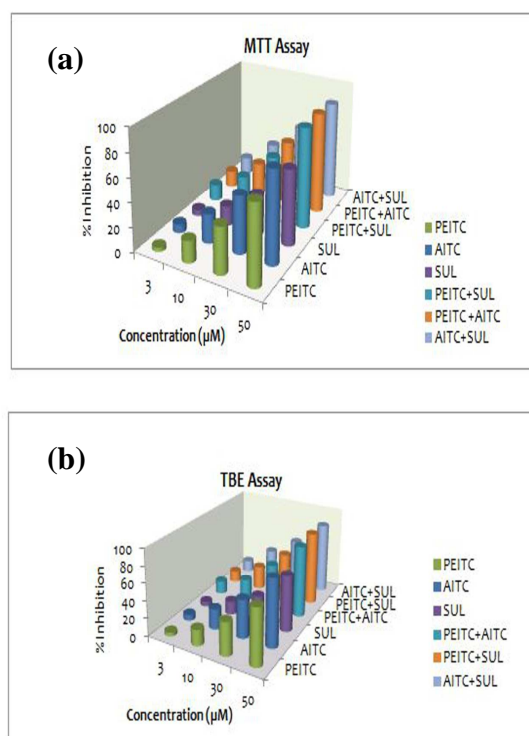
quantified at 570 nm using a microplate reader (Synergy HT, Biotech, USA).

#### Statistical Analysis:

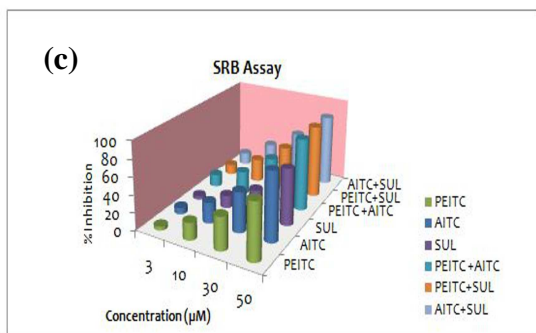
Data are expressed in percent inhibition with respect to the control. The percentages of cell inhibition were used to determine the IC<sub>50</sub> values. All experimental data were given as mean ± SD. Statistical analysis was carried out using the one-way analysis of variances (ANOVA). Post Dunnett 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 ( $p < 0.05$ ).

## RESULTS AND DISCUSSION

A combinational study of three different Isothiocyanates: Allyl isothiocyanate, Phenylethyl isothiocyanate and Sulphoraphane were screened for their *in vitro* cytotoxic activity against HepG2 human liver carcinoma cell line. Inhibitory concentration (IC<sub>50</sub>) was evaluated for all the three native phytoproducts with respect to vehicle control and standard drug (doxorubicin). Based on (IC<sub>50</sub>) values, different combinations of the three phytoproducts were prepared and monitored for their cytotoxic effect against HepG2 human liver carcinoma cell line (Fig 1).



Each value is mean ± SD (n=3).  $P < 0.01$  vs control. Control: 2% dimethyl sulphoxide, AITC: Allyl isothiocyanate, PEITC: Phenylethyl isothiocyanate, SUL: Sulphoraphane



**Fig. 1:** *In vitro* cytotoxic effect of Isothiocyanates (single and combination) on HepG2 human liver carcinoma cell line using (a) MTT (b) TBE and (c) SRB bioassays.

Cytotoxic activity of different Isothiocyanates and *E. sativa* seed oil were screened against HepG2 human liver carcinoma cell line with increasing concentrations after 48 h incubation first by the MTT, TBE followed by SRB bioassay (22). Current assays for measuring cytotoxicity are based on alterations of plasma membrane permeability and the consequent release of components into the supernatant or the uptake of dyes. In case of trypan blue exclusion assay, the dead cells uptake the dye while the viable cells are excluded (23). In MTT assay, dead cells are unable to metabolize yellow tetrazolium salt while viable cells metabolize yellow tetrazolium salt into purple formazan crystal (24) and sulphorhodamine B is an anionic aminoxanthene dye that forms an electrostatic complex with the basic amino acid residues of proteins under moderately acidic conditions, which provides a sensitive linear response (25). Three (MTT, TBE and SRB) bioassays are likely to provide data of percent inhibition of phytoproducts and hence selected for the present study.

Table.1 represents the data of percent inhibition of Doxorubicin (reference drug), AITC+PEITC+SUL (isothiocyanates combination) and SO (2mg/ml) against HepG2 human liver carcinoma cell line by the MTT, TBE and SRB bioassays. A perusal of the data highlights maximum percent inhibition of AITC-77.25%, PEITC-65.38%, SUL-63.85%, AITC+PEITC-86.19%, PEITC+SUL-85.6%, SUL+AITC-84.39%, AITC+PEITC+ SUL-97.12, SO (2mg/ml)- 68.46% for MTT assay; AITC-77.39%, PEITC-64.28%, SUL-63.49%, AITC+PEITC-84.75%, PEITC+SUL-84.39%, SUL+AITC-83.69%, AITC+PEITC+SUL-96.34, SO (2mg/ml)- 66.82% for TBE assay; AITC-77.85%, PEITC-63.42%, SUL-62.35%, AITC+PEITC-84.68%, PEITC+SUL-84.39%, SUL+AITC-83.57%, AITC+PEITC+SUL-96.56, SO (2mg/ml)- 65.92% for SRB assay at concentration of 50 µM. Percent inhibition resulting from TBE, SRB and MTT bioassays demonstrated that seed oil and

isothiocyanates exhibit significant cytotoxicity against HepG2 human liver carcinoma cell line. However, combination of isothiocyanates showed more pronounced efficacy (maximum inhibition 97.12%) at the concentration 50 µM at 24 h compared to *E sativa* seed oil against HepG2 human liver carcinoma cell line ( $p < 0.05$ ).

**Table 1:** *In vitro* cytotoxic effect of Doxorubicin, ITCs combination and Seed oil on HepG2 human liver carcinoma cell line using MTT, TBE & SRB bioassays

Samples (µM)	Bioassays (% inhibition)		
	MTT	TBE	SRB
Control	01.62±0.83	01.85±0.95	01.22±0.33
DOXO			
3	54.25±1.56	53.86±1.45	54.86±1.36
10	71.21±1.61	71.68±1.52	71.21±1.28
30	97.54±1.65	96.65±1.63	96.54±1.38
AITC+PEITC+SUL			
3	22.65±1.58	25.39±1.47	27.39±1.49
10	43.17±1.51	41.25±1.49	42.59±1.52
30	63.75±1.54	62.87±1.35	63.32±1.56
50	97.12±1.64	96.34±1.46	96.56±1.55
SO (2mg/ml)			
3	9.53±1.62	9.51±1.52	9.34±1.54
10	23.43±1.58	22.59±1.53	22.68±1.56
30	49.59±1.54	48.37±1.56	47.16±1.59
50	68.46±1.64	66.82±1.46	65.92±1.51

Each value is mean ± SD (n=3).  $p < 0.01$  vs control. Control: 2% dimethyl sulfoxide, Doxo: Doxorubicin, SO: Seed oil, AITC: Allyl isothiocyanate, PEITC: Phenylethyl isothiocyanate, SUL: Sulphoraphane.

### CONCLUSION

The present piece of work demonstrates that the combination of three isothiocyanates isolated from *E. sativa* seed oil shows more significant *in vitro* cytotoxic effect compared to its naturally occurring seed oil. The detailed *in vivo* studies are in progress. It is recommended to understand specific cellular, molecular and genetic mechanisms of combinational effects of these phytoproducts that contribute cancer growth and progression.

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