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## Potential of enhancing tumor toxicity by crude ethanolic extracts of fruits of *Helicteres isora* (L.)

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**Abstract**

**Objective:** The present study was aimed to evaluate therapeutic potential of *Helicteres isora* (L.) which holds the potential of an anticancer drug.

**Methods:** We have examined the anticancer properties of ethanolic extract of fruits of *H. isora* on two cancer cell lines from different origins with different p53 status namely, MCF7 and HT29. Different *in vitro* cell assays were conducted to evaluate the cell death caused by the ethanolic extract of the fruits.

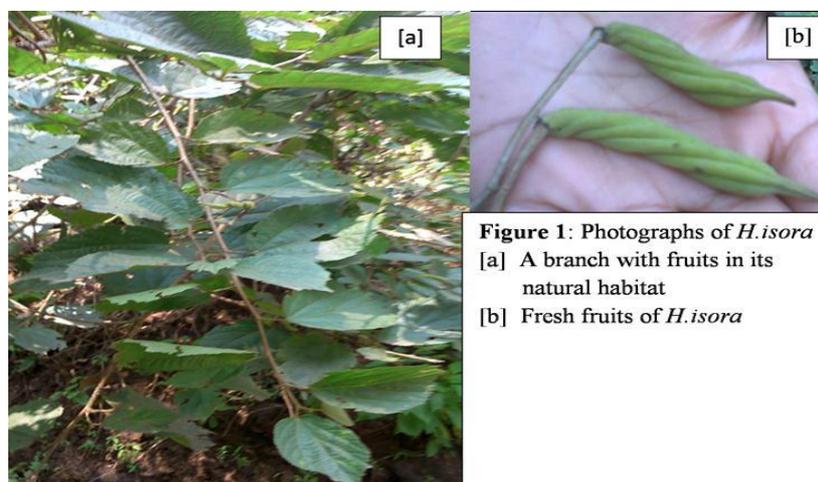
**Results:** Results showed that MCF7 cells with wild type p53 were more sensitive towards the fruit extract than HT29 cells with mutated p53. On the other hand, the extract exhibited insignificant or low toxicity to normal cell lines (MCF10). The cytotoxic effect of the fruit extract was found dependent on dose, time and type of the cell lines.

**Conclusion:** It is concluded that the fruit extract induced selective toxicity in cancer cells but normal cells showed low sensitivity. It is suggested that differential response of tumor and normal cells was associated with their differences in cytosolic redox status acted upon by antioxidants in the extract under study. Our results have opened new vistas for understanding the mechanism of action of the fruit extract of *H. isora*.

**Keywords:** *H. isora*, bioactive compounds, anticancer drug, tumor toxicity, antioxidants

**1. Introduction**

Cancer is a disease of growing health concern around the world. Together with conventional treatment modalities, additional target and mechanism-based preventive approaches as secondary line of defense are urgently required for prevention and management of cancer. Epidemiological evidence together with laboratory studies lead credence to plant-based chemoprevention strategy in attenuating the risk of developing cancer in human beings. Plants are naturally gifted with several defense machinery including bioactive compounds to protect themselves from the radiation induced injuries and oxidative stress. The prospects and scope of research on herbals and their bioactive compounds induced toxicity on cancer cells with considerable relevance in chemopreventive approach motivated us to study the therapeutic efficacy of a native medicinal plant as anticancer drug candidate, namely, *H. isora* which is a tropical south-east Asian shrub available throughout India. In Hindi, it is popularly acknowledged as Marorphali where “maror” refers to twists and “phali” means fruit. It is called Avartani or Mrigshringa in Sanskrit where “mrig” stands for deer and “shringa” means horn, since twisted shape of the fruit resembles that of a deer’s horn (Figure1, Images are from authors own collection).



**Figure 1:** Photographs of *H. isora*  
[a] A branch with fruits in its natural habitat  
[b] Fresh fruits of *H. isora*

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The fruit was chosen because of its medicinal importance partly based on Indian System of Medicine (ISM) and old medicinal literatures. Different parts of the plant are traditionally used in ISM to cure various ailments. There is scantily known information about chemotherapeutic and chemopreventive effects of various parts of *H. isora*. Although evidence exists for the use of fruits of *H. isora* in local medical practices, but reliable and valid research is lacking regarding their uses either alone or in combination with other therapeutic agents. The presence of wide range of phytochemical constituents such as polyphenols, tannins and alkaloids that exhibit therapeutic effects<sup>[1, 2]</sup>, indicates that plant could serve as pilot for the development of novel agents for various pathological disorders including cancer. However, little information is available regarding chemical constituents and bioactivity of this ethno-medicinally important species. Though, the literature surveyed does give us an indication that antioxidant activity in the fruits exists<sup>[1, 3, 4]</sup> and this enables evaluation of its role in cancer treatment. Fruits of *H. isora* may be ideal protector of ROS mediated cellular damage as they contain variety of compounds with known pharmacological action. This may lead to an important breakthrough in cancer prevention and treatment. This prompted us to investigate chemotherapeutic effect of fruits of *H. isora*. Our major aim was to identify herbal extract which selectively and efficiently kills cancer cells but protects the integrity of healthy/normal cells.

## 2. Methods

### 2.1. Collection and authentication

Fresh fruits of *H. isora* (HI) were collected from the vicinity of Vindhyachal range of Chitrakoot district, M.P., India. The plant materials were further identified and authenticated by Central Regional Circle, Botanical Survey of India (BSI), Allahabad, U.P., India. A voucher specimen (NGBU/RC/1301) was deposited at the Herbarium of the BSI and at Research Center (NGBU/RC/1303), Nehru Gram Bharati University, Allahabad, UP. for further references.

### 2.2. Chemicals

Analytical grade, Merck (Darmstadt, Germany), Sigma Chemicals Co (St, Louis, MO, USA) and SRL (India) brand chemicals and reagents were used for the entire experimentation; unless stated otherwise.

### 2.3. Preparation of extract for treatment

The freshly collected fruits of *H. isora* were subjected to thorough washing with tap water followed by shade drying at room temperature. The shade dried fruits were coarsely powdered separately and stored in air tight containers before use. The dry powder was subjected to soxhlet extraction. Ethanol (90%) was used as solvent medium. After soxhlation, the extract was filtered through Whatman No. 1 filter paper, collected, air dried and stored in the refrigerator at 4 °C. The crude/ethanolic extract of *H. isora* fruits has been termed HIF. The extract was mixed and diluted to 100 ml with DMSO before any experimental analysis.

### 2.4. Cell Culture

MCF7 and MCF10 cells were purchased from National Centre for Cell Science, Pune, India. HT29 cells were provided by Micelles Life Sciences Pvt. Ltd., Lucknow, India. All the procured human cell lines viz. MCF7 (breast cancer cell line, p53 wild type, estrogen receptor positive), HT29

(colon adenocarcinoma cell line, mutant p53), MCF10 (normal epithelial cell lines) were cultured in cell culture flasks maintained by using Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% Penicillin-Streptomycin at 37 °C in 5% CO<sub>2</sub> incubator and sub-cultured every 2-3 days to maintain exponential growth. In order to evaluate the effect of HIF, confluent cells were incubated with varying concentrations of the test compound for different time intervals. Different *in vitro* cell assays were conducted to evaluate the cell death caused by the HIF. Cytotoxicity experiments were conducted with cells in exponential growth phase. Confluent cells were treated with different test compounds and incubated at 37 °C (5% CO<sub>2</sub> and 98% humidity).

### 2.5. Determination of Cytotoxicity by Trypan Blue Assay

The trypan blue dye exclusion assay was performed according to method described by Masters (2011)<sup>[5]</sup>. In brief, MCF7, HT29 and MCF10 cells, (3x10<sup>5</sup> cells/ml) suspended in DMEM supplemented with FBS (10%) were incubated with increasing concentrations of HIF (200-1000 µg/ml) at 37 °C (+ 5% CO<sub>2</sub>) for 24 and 48 hrs. After treatment with different dilutions of HIF, trypan blue dye (final concentration of dye: tenth volume of 0.4% dye in Phosphate Buffer Saline) was added to it (100 µl) and mixed well. The percent viability/cytotoxicity of cells was determined by cell counting i.e. number of unstained (live) cells with total number of cells<sup>[5, 6]</sup>:

$$\% \text{ viability} = (\text{live cell count} / \text{total cell count}) \times 100$$

### 2.6. Studies on cell proliferation

For assessment of cell proliferation, MTT assay was performed as described previously with slight modifications<sup>[7, 8]</sup>. Exponentially growing MCF7, HT29 and MCF10 cells were harvested from tissue culture flasks and a stock cell suspension (10<sup>5</sup> cells/ml) was prepared with media. 96-well tissue culture plates were seeded with 1x10<sup>5</sup> cells/well in 0.1 ml of DMEM supplemented with 10% FBS. Cells were incubated for different time periods. HIF aliquots were prepared just prior to the experiment in 0.1% DMSO and serially diluted with suitable medium to get different concentrations (200-1000 µg/ml). After 24 hrs of incubation, cells were treated with 100 µl of HIF from respective concentrations and the plates are again incubated for 24 and 48 hrs. The cells in the control group received only the medium containing the 0.1% DMSO (vehicle). Each treatment was performed in triplicates. After the treatment, HIF containing media was removed and washed with 200 µl of PBS. The formazan crystals formed were solubilized by incubating the cells with 10% SDS overnight. To each well of the 96 well plate, 20 µl of MTT reagent (Stock: 5 mg/ml in PBS) was added and incubated for 4 hrs at 37 °C (5% CO<sub>2</sub>). After incubation, spent medium was discarded and plate was inverted on tissue paper to remove the MTT reagent. DMSO on reacting with formazan gives purplish color. The absorbance or optical density (OD) of purple colored solution developed was read using a conventional ELISA plate reader at 570 nm (Bio-Tek instruments, USA).

$$\% \text{ cytotoxicity} = \frac{(A_{\text{Control}} - A_{\text{Blank}}) - (A_{\text{Sample}} - A_{\text{Blank}})}{(A_{\text{Control}} - A_{\text{Blank}})} \times 100$$

### 2.7. Assessment of DNA damage

DNA fragmentation was assayed using the Apoptotic DNA Ladder kit (1kb) from Sigma. The tumor cells ( $2 \times 10^6$  cells) were treated with different concentrations of HIF (200-1000  $\mu\text{g/ml}$ ) alone, along with their controls, for 24 and 48 hrs in a  $\text{CO}_2$  incubator at  $37^\circ\text{C}$ . The DNA was extracted following the protocol provided with the kit and run on a 1% agarose gel stained with 5  $\mu\text{l}$  of ethidium bromide. The gel was photographed using the gel and blot imaging system from SynGene.

### 2.8. Estimation of intracellular reactive species (RS)

Reactive species play an important role in maintaining redox balance of cells. However, overproduction of RS results in oxidative stress mediated cell damage. This assay reflects the generation RS in cells which is an indicator of oxidative stress.

#### (a) Assessment of intracellular ROS by DCHFDA Assay

The generation of intracellular ROS was measured using DCHFDA (2, 7-dichlorodihydrofluorescein diacetate) as the fluorescence probe as described previously, with slight modifications [7]. Briefly, MCF7 cells ( $3 \times 10^5$  cells/ml) were incubated with 200-400  $\mu\text{g/ml}$  of HIF with respective controls in PBS for 30 min at  $37^\circ\text{C}$ , followed by labeling with the fluorescence probe DCHFDA. Aliquots of 200  $\mu\text{l}$  obtained after different dose treatments were diluted to 3 ml with PBS, followed by measurement of fluorescence intensity (lexZ490 and 520 nm) in quartz cuvette using Fluorescence Spectrometer (LS50B, Perkin Elmer, USA).

**(b) Assessment of RNS by Nitrate/Nitrite assay:** Nitrate is converted to nitrite using nitrate reductase and total nitrite is measured. Instead of discarding the cell culture media containing FBS, it is transferred to a fresh 96 well plate. To estimate RNS production, Griese reagent was added to superfluous media of cells (100  $\mu\text{l}$ ) where RNS are present as byproducts of cellular metabolism. Reading was taken for evaluation of nitrate and nitrite production. To obtain the nitrate conc., endogenous nitrite was subtracted from the total nitrite value.

### 2.9. Statistical Analysis

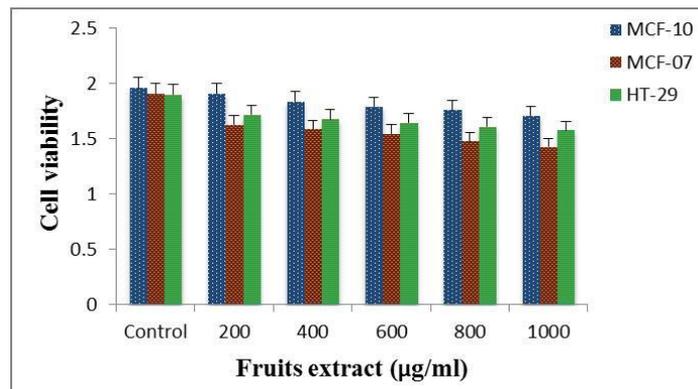
Wherever necessary, the data were analyzed using analysis of variance (ANOVA) test and expressed as Mean  $\pm$  SD using Microsoft excel and Graph pad prism software version 5.03. A probability value of  $P \leq 0.05$  was considered to denote a statistically significant difference. Each sample was analyzed individually in triplicates.

## 3. Results

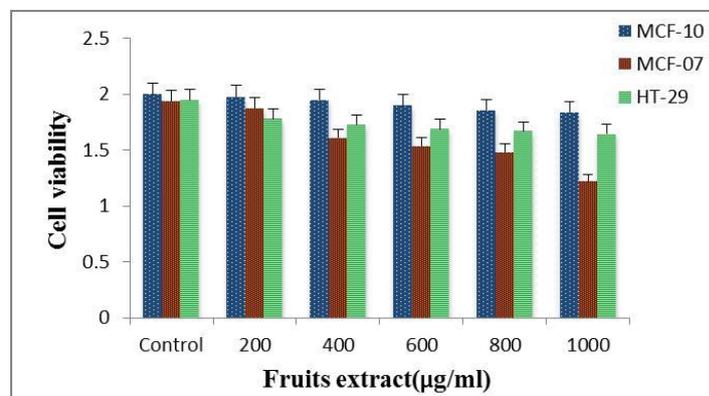
### 3.1. Effect of HIF on cell viability

The cytotoxic effect of HIF on cells from different sources of origin (normal and carcinoma) was studied using trypan blue dye exclusion method. The total cell count of carcinoma cells decreased with much higher percentage as compared to normal MCF10 cells [Figure 2(a) and 2(b)]. The values are

expressed as mean  $\pm$  SD of triplicates (ANOVA).  $P \leq 0.05$  was considered significantly different.



**Fig 2 (a):** Viability of normal and carcinoma cell lines after 24 hrs of treatment with HIF. Compared to cell viability of control (2), viability for MCF7 cells varied from 1.88-1.22. While viability of HT29 cells ranged from 1.78 to 1.65. MCF10 cells were least responsive towards HIF; viability remained between 1.98-1.84.

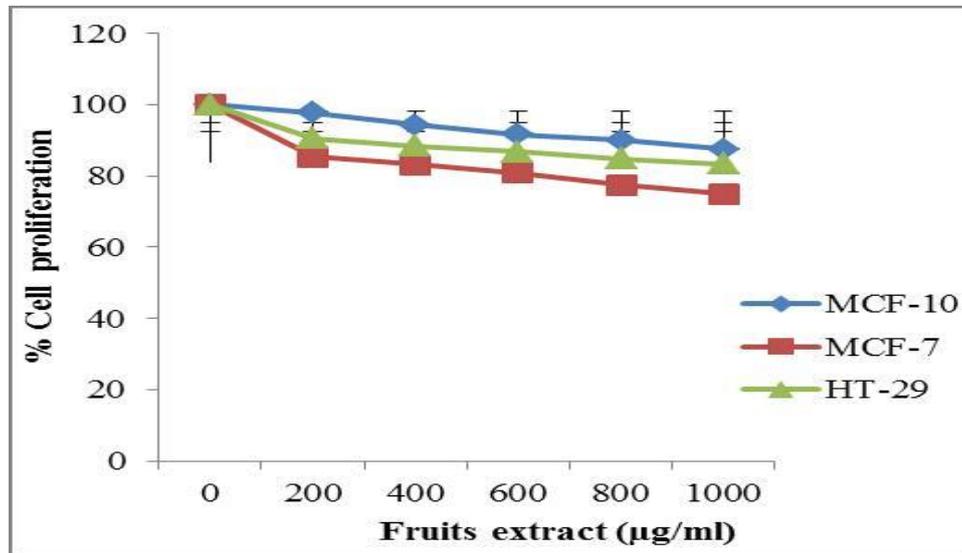


**Fig 2(b):** Viability of normal and carcinoma cell lines after 48 hrs of treatment with HIF. Cell viability for MCF7 cells varied from 1.63-1.43 as compared to control (1.91). Viability of HT29 cells ranged from 1.72 to 1.58. MCF10 cells were least responsive towards HIF; there viability remained between 1.91-1.71.

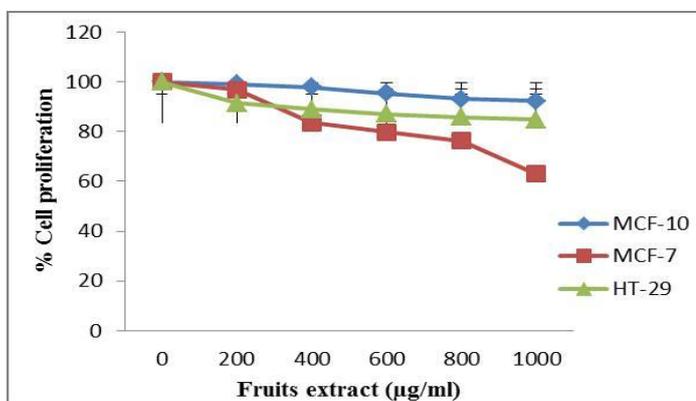
Typically, viability of cancer cells progressively decreased with increase in dose concentrations and incubation times. MCF7 cells were more sensitive than HT29 carcinoma cells. Normal cells (MCF10) were least responsive towards varying concentrations of HIF.

### 3.2. Effect of HIF on proliferation of cells

Cytotoxic effect of HIF was estimated by MTT assay. It can be seen [figure 3(a) and 3(b)] that the treatment exhibited noteworthy antiproliferative effects on the cancer cell lines. Cytotoxic activities of HIF on the cell lines were determined, which revealed the dose dependent activity.



**Fig 3(a):** Cytotoxicity from HIF after 24 hrs of treatment. For doses from 200-1000 µg/ml, percent cell proliferation of MCF7 cells was observed to vary from 85.3-74.95, while cell proliferation for HT29 cells was between 90.5-83.3%. Percent cell proliferation for MCF10 cells remained between 97.6-87.4.

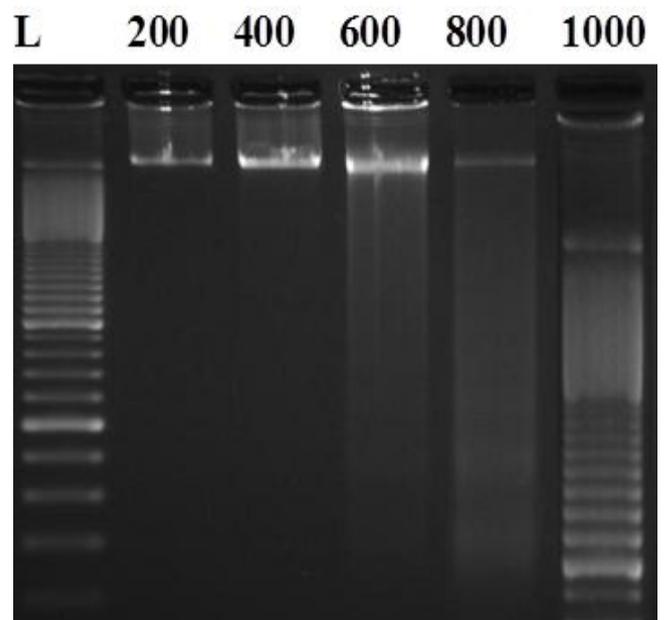


**Fig 3(b):** Cytotoxicity from HIF after 48 hrs of treatment. For doses from 200-1000 µg/ml, percent cell proliferation of MCF7 cells was observed to vary from 96.9-62.89, while cell proliferation for HT29 cells was between 91.4-84.8%. Percent cell proliferation for MCF10 cells remained between 99.1-92.3.

Proliferation of treated cells decreased with the increasing concentration of HIF, which was also found dependent on the period of incubation. The values are expressed as mean  $\pm$  SD of triplicates (ANOVA).

### 3.3. Effect of HIF on DNA damage

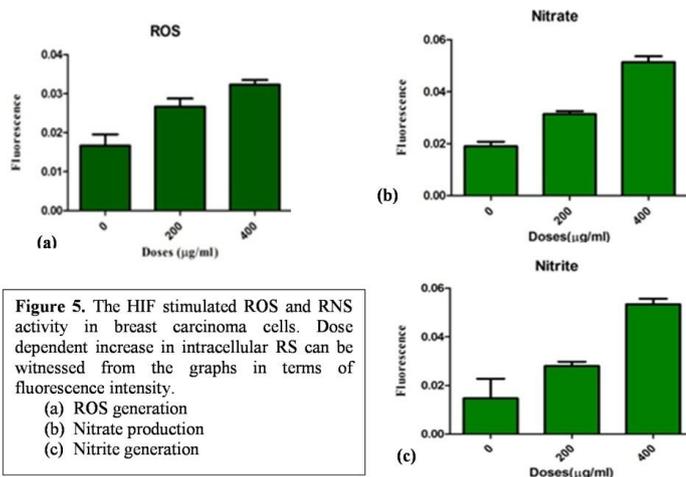
DNA Fragmentation was performed basically on MCF7 cell lines as it showed more stability in results (in terms of sensitivity towards HIF). 1 kb ladder (L) from Sigma was used for the assay. DNA isolated from treated cells was subjected to agarose gel electrophoresis, a DNA ladder characteristic of apoptotic DNA was observed. A significant increase in inter-nucleosomal DNA fragmentation of MCF7 cells was observed at 24 hrs. The whole DNA was ruptured in less than 30 hrs, repeatedly; therefore this assay was not taken for further incubation with HIF. Figure 4 shows the image after 24 hrs incubation, where, L=Ladder and 200,400,600,800, 1000 are used test dilutions in µg/ml of the solvent.



**Fig 4:** Photograph of DNA fragmentation in breast carcinoma cells (MCF7) from treatment with HIF. Lane 1 has DNA from control (Ladder), Lane 2-Treated with 200 µg/ml of HIF, Lane 3-Treated with 400 µg/ml HIF, Lane 4-600 µg/ml HIF, Lane 5-800 µg/ml HIF and Lane 6- 1000 µg/ml of HIF. Cells showed more sensitivity (in terms of DNA damage) towards low doses of HIF.

### 3.4. Measurement of HIF induced free radicals

ROS was measured by using DCHFDA as the probe at 200-400 µg/ml dose concentrations of HIF. Among all cell lines used, MCF7 cells displayed most sensitivity towards HIF treatment, henceforth were chosen for further investigations. Figure 5 shows the HIF induced free radical generation in breast cancer cell lines.



**Figure 5.** The HIF stimulated ROS and RNS activity in breast carcinoma cells. Dose dependent increase in intracellular RS can be witnessed from the graphs in terms of fluorescence intensity.  
 (a) ROS generation  
 (b) Nitrate production  
 (c) Nitrite generation

**Fig 5:** The fruit extract stimulated ROS and RNS activity in breast carcinoma cells. Data is expressed as mean + SD of three samples (ANOVA). Dose dependent increase in intracellular RS can be witnessed from the graphs in terms of fluorescence intensity.

The cytotoxicity induced by HIF may have association with pro-oxidant behavior of the extract in MCF7 cells. Recent therapeutic trends suggest that inducing ROS-mediated oxidative stress in tumor cells by exogenous sources might lead to selective killing of tumor cells that are already under stress [7, 9]. HIF showed a better efficiency in terms of inducing ROS and RNS in Estrogen Receptor(ER)-positive breast cancer cells. Reports have shown that excess ROS may drive tumor cells towards apoptosis by increasing their sensitivity [7, 9, 10]. Normal cells may be spared because low level of intrinsic ROS are inadequate to trigger apoptosis.

#### 4. Discussion

Increasing research findings including reports from our research group suggest that many bioactive compounds isolated from plants possess anticancer properties with negligible side effects; partly it may be due to presence of antioxidants and/or their free radical scavenging activity or ability to induce ROS-mediated apoptosis in cancer cells [7, 11, 12]. We aimed to explore therapeutic potential of one such indigenous herbal as anticancer drug candidate which can efficiently kill cancer cells but spare the healthy ones. The present study was aimed to investigate cytotoxic effect of HIF on cancer cells differing in their p53 status. MCF7 cells with wild type p53 and HT29 cells with mutant p53 were chosen as standard cell models.

This study reports for the first time the cytotoxic activity of HIF on breast cancer cell lines (MCF7), colon cancer (HT29) and normal human cell line (MCF10). From *in vitro* cell assays it was found that HIF induced substantial toxicity to MCF7 cells with wild type p53 (also ER-positive). A high correlation between the viable cell number and the formazan product was observed during MTT assay. However, HT29 cells (with mutant p53) were less sensitive to HIF. This may be partly ascribed to the absence of p53 dependent signaling process in these types of cells. Studies have revealed that cells which do not express p53, are resistant to cytotoxic effect of many chemotherapeutic drugs [13, 14]. Furthermore, a dose dependent increase in ROS and RNS, and subsequent DNA damage in MCF7 cells indicates towards pro-oxidant mode of action of HIF in selected cancer cell lines. It is known from earlier studies that bioactive compounds such as polyphenols

and alkaloids have cancer preventing properties and may prove potential chemopreventive drug candidates [7, 15, 16]. They can influence important cellular and molecular mechanisms associated with carcinogenesis such as expression of signal transduction pathways (MAPKs), transcription factor NF- $\kappa$ B, modulation of cell cycle regulation and apoptosis. HIF showed noteworthy antioxidant activities and was found to contain substantial amount of polyphenols [1, 2] and alkaloid content (authors results unpublished). Recent studies demonstrating selective killing of cancer cells through ROS-mediated mechanism by herbals (including reports from our research group) supports our findings [9, 10, 17, 18]. These studies suggest that in cancer, it is possible to take advantage of increased stress level to selectively kill cancer cells. In addition, differences on cytotoxic effect on cancer cell types over time have been observed, which also suggests a specific and differential manner of gene expression regulation by these extracts. It should be noted that previous *in vitro* investigation by Pradhan and co-workers (2008), showed cytoprotective role of alcoholic extract (50%) of *H. isora* fruits [19]. They showed that the extract displayed significant antitumor activity in melanoma cell, on the contrary protected normal human blood lymphocytes [19]. Henceforth, the results of our study along with contemporary investigations from other labs, allow us to conclude that HIF possess anticancer activity probably because of its pro-oxidant mode of action and/or p53-dependence. Mitochondrial toxicity or interactions with specific cell receptors such as in the case of ER-positive cells can be another mode of cell killing. Henceforth, it is worth mentioning that the anticancer effect of HIF seems to be cell or target specific, as cancer cells demonstrate higher sensitivity than normal cells when incubated with HIF. However, appropriate dose levels that can be applied to humans for anticancer effects, still needs to be identified in upcoming investigations. Therefore, more studies are needed to clarify the molecular mechanisms of fruit extracts as initiators of anti-carcinogenic effects and to evaluate their potential as anticancer agents. Further research must focus towards targeting anti-metastasis or anti-angiogenesis and understanding of signaling pathways involved in modulating cellular behavior of cells.

It looks promising to achieve ideal goal of increased tumor cell killing and protection to normal cells. The differential effect of HIF on different types of cell lines are in similar line with the previously reported mechanisms from our laboratory which showed that crude extract of herbals were non-toxic to normal cells. For instance, the herbal formulation Triphala and extract of *Nigella sativa* increased tumor toxicity by up-regulation of ROS level and spared normal cells [5, 15, 20]. Furthermore, it was demonstrated that p53(+) and p53(-) cells exhibited differential sensitivity towards Triphala. Consequently, the crucial involvement of p53 in apoptotic response is well accepted [21]. However, more elaborate work is required to characterize the exact active principles of HIF responsible for its dynamics, to elucidate its mechanisms of action.

It is obligatory to be cautious when attempting to extrapolate these observations to *in vivo* (animal tumor models) and most importantly, to humans, since none of these experimental features (detailed molecular mechanism) have been proved to occur among humans yet. Moreover, most of the mechanistic data have been obtained *in vitro* and may not necessarily be physiologically relevant. Altogether, experimental conditions

(dose, cell type, culture conditions and treatment time) should be necessarily taken into account, as they determine the biological outcome which shows the unpredictability of the treatment outcome and the need to understand the molecular mechanism of action of these herbal extracts in each particular context. Because of the complexity and inter-relationship of signaling pathways, more information on the primary targets within cells for each extract is required such as nuclear signals, potential interaction with receptors to initiate the signaling cascade and the entry into the cell.

It might be possible to determine whether pure bioactive compounds from HIF are acting through common mechanisms, to achieve the same final cytotoxic effect. Further, investigations in combination therapy such as along with radiotherapy, chemotherapy may become object of extensive research in future. Vast potential exists to identify the lead molecules from various extracts of *H. isora* and determine their mechanisms of action on cellular targets in diverse pathological conditions. Additionally, more extensive, well-controlled clinical trials are needed to fully evaluate the potential of bioactive compounds in terms of optimal dose, route of administration, cancer targets and potential interactions (synergistic effect) with other chemotherapeutic drugs such as paclitaxel, fulvestrant, silibinin and radiotherapy.

## 5. Conclusion

Our results have shown substantial toxicity to various tumor cell lines as evaluated by appropriate assays. *In vitro* studies showed that cancer cells with wild type p53 were more sensitive towards dose treatments than cells with mutant p53. Dose dependent increase in reactive species and DNA damage in MCF7 cells suggest a role of ROS mediated apoptosis in the tumor cells. It was interesting to find that the extract under study produced low or insignificant toxicity in normal cells. The mechanisms of differential cytotoxic effects of extracts from fruits remain to be investigated in further studies. These results open new thrust areas to search, identify and isolate pure compounds from *H. isora* fruit, with a goal to effectively treat ER-positive breast cancer with wild type p53 gene as in case of MCF7 cell lines. It seems interesting to extend these studies for further research on various other cell lines with wild type and mutant p53, receptor specific cell lines and many such parameters, to evaluate the target or cytosolic dependent response of fruit extracts. The endogenous level of antioxidant should also be studied at animal model followed by clinical evaluation to establish significant ROS dependent-independent therapeutic applications.

In summary, bioactive compounds derived from fruits of *H. isora* represent good sources of natural chemo-preventive drug candidates. However, further investigation on the molecular mechanism of action of these bioactive compounds is essential for the validation of their potential as chemopreventive agents.

## 6. Acknowledgement

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