



Original / *Cáncer*

# Anti-proliferative action of silibinin on human colon adenomatous cancer HT-29 cells

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## Abstract

**Background:** Silibinin a flavonoid from milk thistle (*Silybum marianum*) exhibit a variety of pharmacological actions, including anti-proliferative and apoptotic activities against various types of cancers in intact animals and cancer cell lines. In the present study, the effect of silibinin on human colon cancer HT-29 cells was studied.

**Method:** Incubations of cells with different silibinin concentrations (0.783-1,600 ug/ml) for 24, 48 or 72 h showed a progressive decline in cell viability.

**Results:** Loss of cell viability was time dependent and optimum inhibition of cell growth (78%) was observed at 72 h. Under inverted microscope, the dead cells were seen as cell aggregates. IC<sub>50</sub> (silibinin concentration killing 50% cells) values were 180, 110 and 40ug/ml at 24, 48 and 72 h respectively.

**Conclusion:** These findings re-enforce the anticancer potential of silibinin, as reported earlier for various other cancer cell lines (Ramasamy and Agarwal (2008), Cancer Letters, 269: 352-62).

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Key words: Colon cancer. HT-29. Proliferation. Silibinin.

## ACCIÓN ANTI-PROLIFERATIVA DE SILIBININA SOBRE EL CÁNCER ADENOMATOSO DE COLON HUMANO LAS CÉLULAS HT-29

### Resumen

**Antecedentes:** Silibinina un flavonoide a partir de la leche de cardo mariano (*Silybum marianum*) exhiben una variedad de acciones farmacológicas, incluyendo actividades anti-proliferativas y apoptóticas contra varios tipos de cánceres en animales intactos y líneas celulares de cáncer. En el presente estudio, se estudió el efecto de silibinina en células humanas de cáncer de colon HT-29.

**Método:** Las incubaciones de las células con diferentes concentraciones silibinin (0,783-1.600 ug/ml) para 24, 48 o 72 horas mostró un descenso progresivo de la viabilidad celular.

**Resultados:** La pérdida de la viabilidad celular fue de tiempo de inhibición dependiente y óptima de crecimiento de las células (78%) se observó a las 72 horas. Bajo microscopio invertido, las células muertas fueron vistos como los agregados de células. IC<sub>50</sub> (concentración de silibinina matar a las células 50%) los valores fueron 180, 110 y 40 ug/ml a las 24, 48 y 72 horas, respectivamente.

**Conclusión:** Estos resultados volver a hacer cumplir la potenciales contra el cáncer de silibinina, como se informó anteriormente para varias otras líneas celulares de cáncer (Ramasamy y Agarwal (2008), Cancer Letters, 269: 352-62).

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Palabras clave: Cáncer de colon. HT-29. Proliferación. Silibinin.

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## Abbreviations

DMSO: Dimethyl Sulphoxide.  
ELISA: Enzyme linked immune sorbant assay.  
FACS: Fluorescence-activated cell sorting.  
IC50: Inhibitory Concentration 50.  
MTT: 5,(4,5-dimethylthiazole-2-yl)-2, 5 dimethyl tetrazolium bromide.  
µg: Microgram.

## Objectives

Analysis of anti proliferative activity of Silibinin on HT-29 cell line at different time periods

## Introduction

Silibinin is a flavonoid, and an active ingredient of milk Thistle (*Silybum marianum*) extracts. The compound exhibits numerous pharmacological activities<sup>1</sup>. Silibinin exhibits anti-inflammatory, anti-oxidant and cytotoxic effects in number of studies<sup>2</sup>. The drug has been used in humans as anti-hepatotoxic agent in the treatment of hepatic carcinoma, Cirrhosis, and as cytotoxic agent against chemotherapeutic side effects in children with acute lymphoblastic lymphoma<sup>3-5</sup>. FACS (Flourescence activated cell sorter) studies by Agarwal et al.<sup>6</sup> have shown elevated expression of cyclin dependent kinase D activity, resulting in cell cycle arrest and apoptosis in colon cancer HT-29 cell line. Inhibition of skin cancer cell growth by silibinin has been reported<sup>7</sup>. Hogan et al.<sup>8</sup> have shown that silibinin inhibits cell proliferation and causes cell cycle arrest of a number of colon cancer cell lines; namely Fet, Geo and HCT-116. Silibinin is reported to exhibit chemo preventive and chemo therapeutic actions against various types of cancers in animals systems also<sup>9-12</sup>. Anti-angiogenic properties of silibinin have been described by Yang et al.<sup>13</sup> in LoVo colon cancer cell line. Singh et al.<sup>14</sup> have reported cancer preventive and therapeutic efficacy of silymarin in animal cancer cell line cultures also.

HT-29 is adenomatous cancer cell line derived human colon. Compared to other cell lines, these cells are more malignant and are frequently used as an experimental model of colon cancer in cell culture. In the present study, the effect of silibinin on the growth of HT-29 cells was investigated in cell culture. These findings suggest that silibinin is a potent inhibitor of the growth of the cells, which produced nearly 80% inhibition of cell viability after 72 h and aggregation of the dead cells *in vitro*.

## Material and Methods

All chemicals used were of analytical grade quality. Silibinin and MTT were procured from Sigma Chemical

Co., St.Louis (MO, USA). HT-29 cells were obtained from National Centre for Cell Science (Pune) India.

## HT-29 Cell Culture

Human colorectal adenocarcinoma cell line HT-29 cells were maintained in Mc Coy's 5A media (Invitrogen), supplemented with heat inactivated 10% fetal calf serum. Streptomycin and penicillin were added to the medium to protect against the bacterial contamination. After cells had reached 60-80% confluency, they were trypsinized using Trypsin-EDTA solution (Gibco) followed by sub culturing at 1:4 -1:8 dilutions. Culture medium was changed every 72 h. The cells were cultured at 5% CO<sub>2</sub>- Air mixture at 37°C in humidified incubator. The cells were treated with silibinin dissolved in Dimethylsulfoxide (DMSO) at concentrations of 0.783, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200, 400, 800, and 1,600 µg/ml and cell viability was determined at 24, 48 and 72 h by performing MTT [5,(4,5-dimethylthiazole-2-yl)-2, 5 dimethyl tetrazolium bromide] assay.

## MTT Assay

MTT cell proliferation assay was performed following the method describe by Noh et al.<sup>15</sup> This assay is based on the reduction of tetrazolium salt of MTT to form ultra purple Formozan by the viable cells. 50 µl of the HT-29 cell suspension was placed in 96 well plates at a density of 1X10<sup>6</sup> cells per well and incubated with MTT (5 mg/ml in phosphate buffered saline) in humidified incubator for 2-3 h. The cells were treated with silibinin dissolved in DMSO. The purple blue MTT formazan precipitate was dissolved in 100 ul of DMSO. Metabolically active cells were quantified by measuring the Optical Density at 580nm in STAT Fox 325+stop-type ELISA reader (Awareness Technology Inc, USA). Cell viability was tested after 24, 48 and 72 h. The values of IC50 (concentration which inhibited 50% of cells) was determined at each of the time intervals, by plotting data on cell viability vs silibinin concentration. All assays were done in triplicate at each of the time intervals. A parallel manual count of the cells was also carried out to corroborate the MTT assay results. Microphotograph of the cells was taken by an inverted microscope (Olympus, Japan) after trypan blue staining of the cells<sup>16</sup>.

## Results

Figure 1 shows the structure of silibinin molecule, which is a bioactive flavonoid and active ingredient of milk thistle (*Silybum marianum*).

As shown in figure 2A control cells and figure 2B cells incubated with DMSO appeared well separated

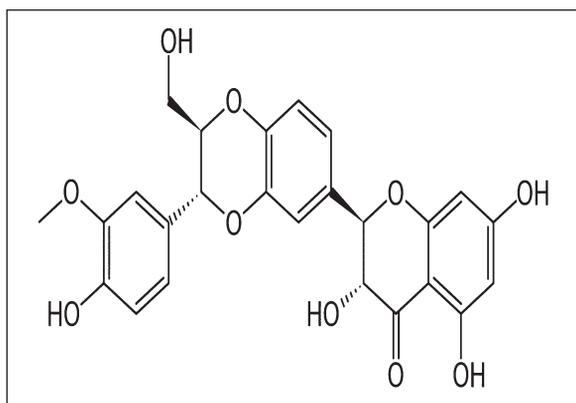


Fig. 1.—Structure of silibinin.

and normal under the growth conditions. However, the cells grown in presence of silibinin for 24 h showed an aggregated mass of dead cells, at a concentration of 1600  $\mu\text{g/ml}$  of the drug (fig. 2C) under inverted microscope. MTT assay revealed a progressive decline in cell viability with an increase in the drug concentration from 0.783 to 1600  $\mu\text{g/ml}$  (fig. 3). At the highest concentration of silibinin used, after 24 h, 71% of the cells were found dead, while only 29% viable cells were present in the culture well. Similarly, when HT-29 cells were incubated with the drug for 48 h, revealed a large mass of the dead cells, when viewed under microscope (fig. 2D). MTT assay also revealed a steep decline in cell viability, as the drug concentration was increased. At the highest silibinin concentration, 73% of the cells were killed, while 27% of viable cells were seen under these conditions (fig. 3).

HT-29 cells, when incubated in presence of the different concentrations of silibinin for 72 h showed a larger

mass of the aggregated dead cells (fig. 2E). The cell proliferation and viability as determined by MTT assay revealed a decline in cell viability as the drug concentration was increased from 0 to 1600  $\mu\text{g/ml}$  in the culture medium. At the highest concentration of silibinin used, 78% of the cells were recorded as dead, while 22% of viable cells were observed under these conditions (fig. 3). Silibinin concentration, which inhibited 50% of the cells (IC<sub>50</sub>) values were determined from the data presented in figure 3. As shown in table I, cells incubated with silibinin for 24 h showed IC<sub>50</sub> value of 180  $\mu\text{g/ml}$ , which was further reduced to 110  $\mu\text{g/ml}$  for HT-29 cells incubated with the drug for 48 h. The value of IC<sub>50</sub> for cells incubated with silibinin for 72 h was 40  $\mu\text{g/ml}$  under these conditions. These results show that efficacy of the drug in killing HT-29 cells *in vitro* was markedly enhanced with time when the cells were allowed to be in contact with silibinin up to 72 h.

## Discussion

Interest in the use of naturally occurring compounds as chemopreventive agents for carcinogenesis has been on the rise in recent years since a variety of fruits, vegetable and phytochemicals offer high anti-cancer efficacy and low toxicity to normal tissues. Silibinin is one such compound, which has strong anti-proliferative activity against various cancer cell lines<sup>1,2</sup>. In the present study, MTT assay was used to determine cell viability in the absence and presence of different concentrations of silibinin using HT-29 cells. The data, presented herein show, that viability of the cells was reduced by over 72% in presence of silibinin, after 24-72 h. These results in general are in agreement to

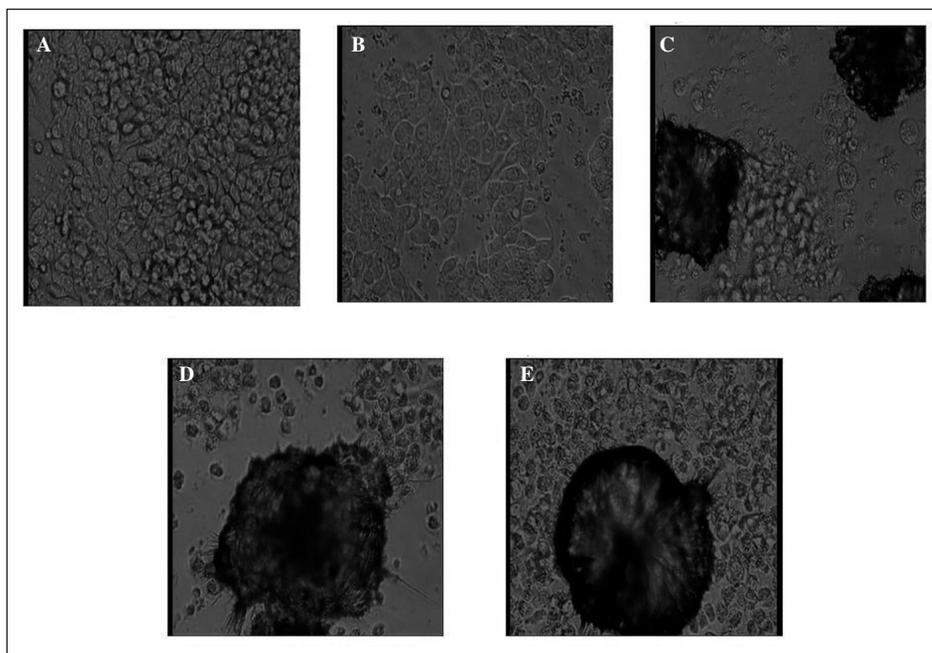


Fig. 2.—A) Control cells; B) Cells incubated with DMSO; C) Cells incubated with 1,600  $\mu\text{g/ml}$  silibinin for 24 h; D) Cells incubated with 1,600  $\mu\text{g/ml}$  silibinin for 48 h; E) Cells incubated with 1,600  $\mu\text{g/ml}$  Silibinin for 72 h.

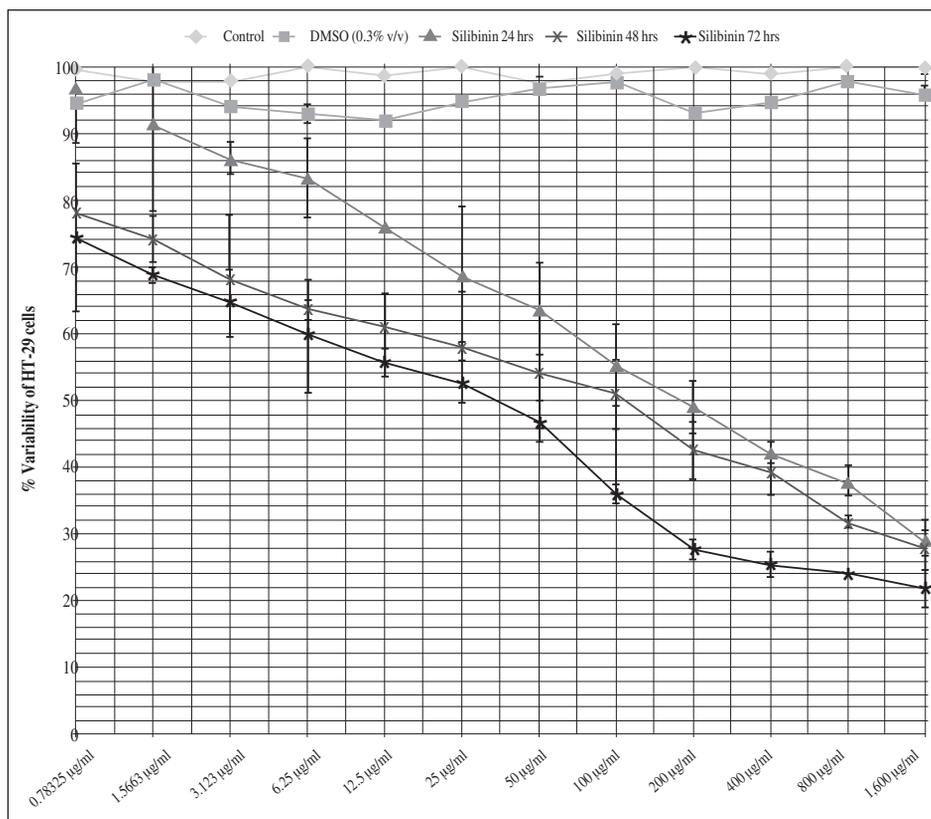


Fig. 3.—MTT assay of HT-29 cells in presence of different concentrations of silibinin. Cells were incubated with silibinin for 24, 48 or 72 h. After washing the cells with PBS, the cell viability was assessed by MTT assay, as described under Materials and Methods. All assays were carried out in triplicate. Vertical bars indicate the SD of the mean at various points. Similar experimental conditions were employed as for results given in figure 2.

**Table I**  
*IC<sub>50</sub> and % cell viability values for silibinin at different time in HT-29 cell line*

Incubation time (hrs)	No. of cells/well	IC <sub>50</sub> (µg/ml)	% cell viability
24	1 x 10 <sup>6</sup>	180	29
48	1 x 10 <sup>6</sup>	110	27
72	1 x 10 <sup>6</sup>	40	22

earlier studies of Agarwal et al.<sup>6</sup>, who reported cell-cycle arrest and apoptosis of the colon cancer cells. Similar to earlier reports, the potency of silibinin to inhibit cell growth was augmented with increase in time the cells were allowed to interact with the drug. Although Silibinin inhibits the growth of a number of cancer cell lines *in vitro*, but the degree of inhibition varies with severity of the carcinogenicity. Present data show, IC<sub>50</sub> values for HT-29 cells was 40 µg/ml at 72 h, whereas other investigators reported IC<sub>50</sub> values of 75 µg/ml for HT-29 cells using FACS analysis (4), 150 µM for bladder papilloma RT4 cells (5), and 5 µg/ml for FET cells and GEO cells and 38 µg/ml for HT116 cells<sup>8</sup>. Noh et al.<sup>15</sup> have reported IC<sub>50</sub> value for silibinin in MCF-7 human breast cancer cells. Thus, the efficacy of silibinin in inhibiting the growth of different cancer lines is quite different. Such differences in the potency of the drug in arresting cell growth may be due to differences in the experimental conditions used, the cell type and potential carcinogenicity of the cell lines.

Although the underlying mechanism by which silibinin inhibits the cell growth of cancer lines is essentially unknown. But it was apparent, that the dead cells produced an aggregated massive cell mass. Agarwal et al.<sup>6</sup> have reported the up regulation of certain cell cycle bio-markers, such as Kopl/p27 and Cipl/p21 proteins, as well as the mRNA levels encoding these proteins. However, a decrease in the expression of CDK2 and CDK4, cyclin E and Cyclin D1 proteins was also observed. It was reported that 15% of the cells showed apoptotic cell death after 48 h of silibinin treatment of the cells. Present data showed 78% of the cells died after 72 h, when incubated with 1,600 µg/ml of silibinin. The apparent discrepancy in the two studies may be attributed to different concentration of the drug used in the two studies (100 versus 1,600 µg/ml).

Hogan et al.<sup>8</sup> have described that silibinin markedly inhibited the cell proliferation by causing cell cycle arrest, which involved a decrease in CDK expression, which is a fundamental cell cycle regulatory protein.

The effect of silibinin was more potent in HCT116 cells, which exhibit more malignancy compared to FET and GEO cancer cell lines. Inhibition of human prostate cancer PC-3 tumour xenograft in athymic nude mice by silibinin treatment has also been reported by Singh et al.<sup>18</sup> The observed decrease in tumour growth was associated with reduced Vascular Endothelial Growth Factor (VEGF) expression. An increase in apoptosis of human bladder papilloma RT-4 cancer cells by silibinin has been reported by Tyagi et al.<sup>17</sup> both *in vivo* and *in vitro* conditions. Silibinin exposure elevated the expression of P53 levels in RT-4 cells and increased phosphorylation of ser-15 activated caspase cascade and caused bid cleavage for apoptosis. Enhanced apoptosis of MCF-7 human breast cancer cells in presence of silibinin has been described by Noh et al.<sup>11</sup>. A 60% decrease in cell viability was observed by these investigators in presence of 200 µM silibinin. Silibinin has been reported to exhibit strong anti-oxidant activity in intact animal systems<sup>2</sup>. This property of the compound could be the underlying basis of its anti-tumour activity in a variety of cancer cell lines, including HT-29 cells, as shown in the present study. Hadi et al.<sup>19</sup> have proposed that anti-cancer property of plant polyphenols involves the arrest of cell-cycle by inducing cyclin A and E and inactivation of cell cycle regulator cdc2. It was shown that polyphenols with anti-cancer and pro-apoptotic properties are able to sequester the endogenous copper ions in the nucleus, which lead to an inter-nucleosome DNA breakage, since such regions of DNA are more labile to cleavage by reactive oxygen species (ROS).

In conclusion, the present findings support the earlier anticancer properties of silibinin that has great potential in inhibiting the growth of HT-29 cancer cells *in vitro*. The drug could be useful as a potential chemo preventive and therapeutic agent in the treatment of colon cancer.

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