



# Bacoside A - A Potent Phytomedicine Attenuates Epithelial - Mesenchymal Transition in HCT 116 Colon Cancer Cells

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## ABSTRACT

**Objective:** Epithelial mesenchymal transition (EMT) is the one of the significant events in the cancer metastasis. The active compounds Bacoside A from *Bacopa monnieri* good in antioxidant, anti-inflammatory, neuroprotective and anticancer properties. The present study focuses to check the efficiency of Bacoside A on EMT in HCT 116 colon cancer cell lines and to explore its potential cancer therapeutics.

**Material and Methods:** An *in vitro* study was designed with HCT 116 colon cancer cell lines. The cytotoxic effect of Bacoside A was carried out with the different concentrations ranges from 0-50 µg/mL. The free radical scavenging activity, the membrane stability assay, lipid peroxidation assay, protein denaturation assay and metal chelating activity were assessed. Cell migration and colony forming capacity was assessed with different concentrations of Bacoside A. Cell apoptosis was checked with Acridine Orange - Propidium Iodide staining. The gene expression was performed with epithelial markers and mesenchymal markers E-Cadherin, Snail and Vimentin.

**Results:** The results of the present work states that the Bacoside A potentially inhibit the cancer cell proliferation and the IC<sub>50</sub> value was found be 32 µg/mL. Further investigation on membrane stability the lipid peroxidation and protein denaturation, concentration dependent inhibition was found upon Bacoside A treatment. Migration assay results conclude that the higher concentration inhibits cell growth and the lower concentration slows down the cell migration. The results of colony forming units by HCT 116 colon cancer cells were effectively inhibited by Bacoside A treatment. The EMT induction studies indicates the EMT was attenuated on Bacoside A treatment. The results of apoptosis study clearly indicate the Bacoside A treatment induced cancer cell death. The gene expression analysis further confirms that the epithelial protein markers E-Cadherin was upregulated and intermediary protein -snail and mesenchymal marker - vimentin were down regulated.

**Conclusion:** The results of present work clearly states that phyto compound Bacoside A had a potent anticancer and anti-metastatic activity for HCT 116 colon cancer cell lines tested *in vitro*. Bacoside A effectively inhibits cell viability, colony formation, cell migration and induced apoptotic cell death in colon cancer cell lines.

**Keywords:** *Bacopa monnieri*; Bacoside A; cancer diagnosis and treatments; E-cadherin; EMT; M-cadherin

## INTRODUCTION

Colorectal cancer (CRC) ranks as the second leading cause of cancer death overall in both males and females, and it is the most commonly observed cancer in individuals younger than 50 years. The risk factors and causes of CRC include lifestyle factors such as an unhealthy diet, high alcohol intake, smoking, reduced physical activity, and excess body weight.<sup>1</sup> The CRC metastasis includes various mechanisms in which different intrinsic, factors, such as heterogeneity of tumour cells,

genetic abnormalities, and epithelial mesenchymal transition (EMT), are involved. All initiate the metastatic process and lead to cancer invasion and spread.<sup>2</sup> In EMT, cells lose their epithelial characteristics, such as cellular integrity and cell-cell attachment; gain mesenchymal properties, such as increased cell motility; and become associated with an invasive or metastatic phenotype.<sup>3</sup> EMT is a key event in understanding cancer progression, development, and pathogenesis. More attention has been focused on EMT as a target in cancer therapy. During EMT, epithelial cells are transformed into

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mesenchymal cells, changing from stationary, polarized cells to motile, spindle-shaped cells. This transition is believed to be a significant mechanism underlying cancer metastasis. Once these cells have disseminated to different sites, cancer cells tend to regain epithelial properties through the reversal of EMT, termed mesenchymal-epithelial transition (MET).<sup>4</sup> The process EMT expresses the intermediate filaments, changes the intracellular junctional composition, and alters cell morphology. Other important markers of EMT include membrane-bound E-cadherins, transcription factors, and epithelial-specific markers. The important mesenchymal markers include fibronectin, N-cadherin, and vimentin. Upregulation of epithelial markers and downregulation of mesenchymal markers are considered significant controlling events in cancer metastasis.<sup>5</sup> The transcription factors Snail 1, Snail 2, Twist, and ZEB1 are involved in the epigenetic expression of epithelial markers, transcription activation of matrix metalloproteins and cytoskeleton remodelling. EMT confers metastatic potential to cancer cells. EMT as a target in cancer therapeutics is considered a meaningful approach to preventing cancer progression.<sup>6</sup> Researchers are focusing on discovering and developing medications to combat cancer. The limitations of anticancer drugs include non-specificity, wide biological distribution, short half-life, and toxicity.<sup>7</sup> Several anticancer drugs have been identified from natural compounds of plant, animal, and microbial origin. There has been increasing interest in identifying medications derived from natural resources to treat CRC by acting on various specific targets. The benefits of medicinal herbs for CRC prevention include induction of apoptosis, cell-cycle arrest at various stages, and alteration of multiple cell-signalling pathways.<sup>8</sup> *Bacopa monnieri* (*B. monnieri*) is a medicinal herb found in India, commonly used in the Ayurvedic system of medicine for various treatments because of its pharmacological activities, such as neuroprotective, antioxidant, anti-ulcer, antimicrobial, analgesic, and anticancer properties.<sup>9</sup> Bacoside A is an amphiphilic mixture found in *B. monnieri*, consisting of both sterol and sugar moieties and including different saponin glycosides, namely bacopaside II, bacopaside X, Bacoside A3, and bacopasaponin C. Bacoside A possesses anti-cancer properties and effectively inhibits the growth of breast, colon, and liver cancers, as well as glioblastoma, as demonstrated by various studies.<sup>10</sup> Bacoside A can effectively interact with various signaling pathways, such as epidermal growth factor receptor/Ras/Raf/mitogen-activated protein kinase, Notch signaling, and Wnt/ $\beta$ -catenin signaling, as demonstrated by gene expression analysis and molecular docking studies in neuroblastoma cells.<sup>11</sup>

Bacoside I and II, reported to have tumour-suppressive effects specifically block transmembrane proteins, alter tumour cell

migration, and affect metastasis in colon and breast cancer cells. Drugs targeting transmembrane alter cell migration, invasion, and growth of cancer cells.<sup>12</sup> Bacoside A significantly arrests the cell cycle and induces apoptosis in glioblastoma cells, which are highly metastatic and malignant, and for which temozolomide is insufficient to improve patient outcomes and survival.<sup>13</sup> Transmembrane proteins play a crucial role in cancer metastasis by influencing cell-cell adhesion, migration, and invasion. They act as signaling receptors, adhesion molecules, and transporters. Different transcriptomic and proteomic studies have attempted to elucidate their role in cancer metastasis.<sup>14</sup> Certain proteins, such as E-cadherin, M-cadherin, vimentin, Snail1, Snail2, and fibronectin are considered good indicators because they are directly involved in cancer metastasis through EMT.<sup>15</sup> Several external and internal factors can activate these proteins, stimulate the process of EMT, and lead to cancer metastasis. Recently, membrane proteins have increasingly been targeted by phytochemicals for cancer therapy to overcome chemoresistance. Most membrane-bound proteins possess receptors with subunits that receive signals; these proteins regulate the basal surface of cancer cells and lead to changes in cytoskeletal organization.<sup>16</sup>

Based on the above findings, the present study aimed to assess the potential of the phytochemical Bacoside A to modulate genes regulating EMT through *in vitro* gene expression analysis in HCT 116 colon cancer cell line. This preliminary study was aimed to identify the anti-inflammatory, apoptosis and EMT expression analysis and could be opening the way to recognize the therapeutic activity of phyto compound Bacoside A to treat against cancer spreading, invasion and the associated problems during the course of disease.

## MATERIAL AND METHODS

**Cell line maintenance:** The human colon cancer cell line HCT 116 was propagated in Dulbecco's Modified Eagle Medium (DMEM) (Himedia) with glucose, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin; cultures were maintained in a 5% CO<sub>2</sub> incubator at 37°C. Cell counts were performed using a hemocytometer, and a density of 5.0×10<sup>3</sup> cells/well was used for the cytotoxicity assay. Sri Ramachandra Institute of Higher Education and Research Ethics Committee approval was obtained for conducting the cell line studies (approval number: IEC-NI/22/JUL/83/85, date: 14.10.2022).

**Cytotoxic Assay:** Cytotoxicity was evaluated using the MTT assay in the HCT 116 colon cancer cell line. MTT enters mitochondria of viable cells and is reduced to insoluble purple formazan crystals. The cells were solubilized with dimethyl sulfoxide, and the released formazan reagent was measured

spectrophotometrically at 570 nm. Different concentrations (5, 10, 20, 40, 80, 160, 320  $\mu\text{g}/\text{mL}$ ) of Bacoside A (Natural remedies, Bangalore, India) were dissolved in methanol and used to treat HCT 116 cells for a cytotoxicity assay. The optical density was measured at 570 nm on a microplate reader. The fifty percent inhibitory concentration ( $\text{IC}_{50}$ ) of the drug was calculated from the dose-response curves.

### Free Radical Scavenging Assay

**DPPH Assay:** Different concentrations of Bacoside A were mixed thoroughly with 1 mL of methanol and 0.1 mM DPPH, allowed to stand for 30 min in the dark, and the optical density was measured at 523 nm using a ultraviolet (UV)/visible (VIS) spectrophotometer. The standard and the blank were processed simultaneously.<sup>17</sup> The scavenging activity was calculated using the following formula:

$$\% \text{ Scavenging Activity} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$$

where  $A_{\text{sample}}$  is the absorbance of the test sample

$A_{\text{control}}$  is the absorbance of the control.

**Hydroxyl Radical Scavenging Activity:** Assessment of hydroxyl radical scavenging activity was carried out with the reaction mixture contains, 1 mL of ferric chloride, 1 mL of hydrogen peroxide and 1 mL of ethylene diamine tetra acetic acid and 1 mL of stock solution of salicylic acid. Bacoside A at different concentrations (25-200  $\mu\text{g}/\text{mL}$ ) were added along with the reaction mixture allowed for the incubation at 37 °C for 1 hour. 1 mL of 2.8% sodium hydroxide was added to arrest the reaction and the optical density were measured at 510 nm using a UV-VIS spectrophotometer.<sup>18</sup> The percentage of scavenging activity of the sample was calculated by following formula:

$$\% \text{ Scavenging activity} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

**FRAP Assay:** A working solution of 2,4,6-Tripyridyl-s-triazine (TPTZ) was prepared in acetate buffer. TPTZ solution and ferric chloride stock solution were mixed in a 1:1 ratio to prepare the Ferric reducing antioxidant power (FRAP) reagent. The test solution, Bacoside A, at concentrations of 25-200  $\mu\text{g}/\text{mL}$ , was mixed thoroughly with FRAP reagent and incubated at room temperature for 30 minutes. The absorbance was measured at 593 nm using a spectrophotometer.<sup>19</sup> The antioxidant capacity of Bacoside A was calculated using the following formula:

$$\text{Antioxidant capacity} = (A_{\text{sample}} - A_{\text{control}}) / \text{Slope}$$

### In vitro membrane stability Assay

**Lipid Peroxidation Assay:** The lipid peroxidation assay, the cells were treated with different concentration (25-200  $\mu\text{g}/\text{mL}$ ) of Bacoside A, 20  $\mu\text{L}$  of 2 mM ascorbic acid and 4 mM  $\text{Fe}_2\text{SO}_4$  were added to the mixed solution and incubated for 60 min at 37 °C. Subsequently, 200  $\mu\text{L}$  of TBARS reagent (40%

trichloroacetic acid, 1.4% thiobarbituric acid, and 8% HCl) was added to the mixture, which was then incubated at 90 °C for 60 min. At the end of incubation, the mixture was allowed to stand at room temperature and was centrifuged at 10,000 rpm for 5 min at 4 °C. The supernatant was then collected and measured at 530 nm using a microplate reader. The lipid peroxidation status was calculated relative to the control.<sup>20</sup> The peroxidation status was assessed using the following formula:

$$\text{Concentration of MDA (nmol/mL)} = (S_A / S_V) \times \text{DF}$$

$S_A$  = Amount of MDA in sample (nmol)

$S_V$  = Sample volume (mL)

DF = Sample dilution factor

**Protein Denaturation Assay:** Protein denaturation assay was performed using Bacoside A at concentrations of 10-50  $\mu\text{g}/\text{mL}$  with 0.5 mL of 1.5 mg/mL bovine serum albumin, followed by incubation at 37 °C for 20 min. This reaction mixture was further heated for 3 min at 57 °C. Added 250  $\mu\text{L}$  of 0.5 M phosphate buffer (pH=6.3). 100  $\mu\text{L}$  of each mixture was transferred into separate test tubes; alkaline copper reagent and 1% Folin-Ciocalteu reagent were added in the same proportion. After 10 min of incubation at 55 °C, absorbance was measured at 650 nm using a spectrophotometer.<sup>21</sup>

**Metal Chelating Assay:** Metal chelating assay measures the ability of test samples to chelate free ferrous ions in solution thereby inhibiting Fe(II) binding to ferrozine which generates a highly colored complex. EDTA acts as a positive control, capable of chelating ferrous ions in a dose-dependent manner.<sup>22</sup>

$$\text{Metal chelation (\%)} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$$

**Cell Migration Assay:** Cells grown in DMEM medium with 10% FBS were seeded in a 6-well tissue culture plate. After 24 hours, the cells reached 80% confluence, forming a monolayer. The monolayer was scratched across the center of the well using a 10  $\mu\text{L}$  tip, with the axis of the tip perpendicular to the bottom of the well. The cells were viewed under a phase-contrast microscope. The culture dish was placed in an incubator, and an image was captured every 12 hrs. 100,000 cells were incubated for 12 hours in a 5%  $\text{CO}_2$  incubator at 37 °C. After 24 hours, cells were treated with the drug at the respective concentrations. A scratch was made in all wells using a sterile 10  $\mu\text{L}$  pipette tip. The images were observed under a fluorescence microscope, and photographs of each well were captured and labeled at 0, 12, 24, and 36 hours, respectively.<sup>23</sup> The percentage of wound closure area was calculated using the following formula:

$$\% \text{ Wound Closure Area} = \frac{A(0) - A(t)}{A(0)} \times 100$$

Where A(t) - Wound area at time t, A(0) - Initial wound area

**Clonogenic Assay:** Mono layered cultured cells in six-well plates in a range of  $5 \times 10^6$  cells/well and allowed to adhere. The cell culture medium was refreshed (2 mL/well), and cells were treated with Bacoside A. Depending on the proliferation rate, cells were then incubated at 37 °C for 36 hours; cell growth in all six-well plates was stopped by fixation and staining with 80% ethanol containing 8% methylene blue. Colonies of  $\geq 50$  cells were counted under the microscope. Based on the colony size and cell morphology, the magnification was adjusted.<sup>24</sup>

% Plating Efficiency (PE) % = No. of colonies formed/No. of colonies seeded X100

% Survival Fraction (SF) = No. of colonies formed after treatment/No. of colonies seeded X PE X100

**EMT induction Analysis :** The induction assay was performed with the warm culture media at 37 °C. All other parameters will be the same as in the standard culture protocols. Cells ( $1 \times 10^6$  per well) in a 6-well plate and were then treated with the  $IC_{50}$  concentration of Bacoside A. The treated plates were incubated at 37 °C in a carbon dioxide incubator, and the cell morphology was monitored at 0, 24, 36, and 48 h by inverted light microscopy.<sup>25</sup>

**Cell apoptosis Assay:** Cell apoptosis analysis was carried out using a combination of the fluorescent dyes acridine orange (AO) and propidium iodide (PI). When using the AO-PI mixture, it will stain the cells: live cells emit green fluorescence, and dead cells emit reddish-orange when exposed to an appropriate light source. HCT 116 cells were seeded at a density of  $1 \times 10^6$  cells per well in a 35-mm dish and incubated for 24 hours. The cells were treated with 10  $\mu$ g/mL, 20  $\mu$ g/mL, and 30  $\mu$ g/mL of Bacoside A. After incubation, the medium was removed and 100  $\mu$ L of AO/PI stain was added to the plate. The viability of the cells were determined based on the emission of the fluorescent colour using an inverted fluorescent microscope.<sup>26</sup>

**RNA Isolation, cDNA Synthesis and Quantitative Polymerase Chain Reaction (qPCR) Analysis:** HCT 116 colon cancer cell lines ( $3 \times 10^5$  cells) were treated with control and Bacoside A inhibitory concentration ( $IC_{50}$ ) for 48 hours. Every 12 hrs during the treatment, RNA was extracted using the Trizol method. The quantity and purity of RNA were assessed using a NanoDrop spectrophotometer. Reverse transcription reactions for the production of cDNA were carried out using the RevertAid First Strand cDNA Synthesis Kit. Genes specific for epithelial characteristics (E-cadherin), mesenchymal characteristics (vimentin), and the transcription factor (Snail - 1) were selected for expression analysis. Quantitative RT-PCR reactions were performed with High ROX Amplicon

SYBR Green Master Mix and specific primers for target genes. Gene-specific forward and reverse primers are designed for the gene amplification reaction. Expression values for qPCR products were processed using the housekeeping gene  $\beta$ -actin as a reference, and ratios relative to untreated samples were calculated. The relative expression fold change was calculated by the  $2^{-\Delta\Delta Ct}$  method. The primer sequences used are as follows.

Primers	Forward Primer (5'-3')	Reverse Primer (5'-3')
E-Cadherin	CGACAAAGGACAGCCTATT	AGTTGGAAATGTGAGCAAT
Snail	ATACCACAACCAGAGATCCTCA	GACTCACTCGCCCAAAGATG
Vimentin	GCTCGTCGTCGACAACGGCT	CATTTCACGCATCTGGCGTTC
$\beta$ -actin	GCTCGTCGTCGACAACGGCT	CAAACATGATCTGGGTCTCTCTC

By the comparative Ct method, the qPCR data were analysed, and the expression of target genes was normalized to that of  $\beta$ -actin. A 1.2% agarose gel was used to visualize the PCR products.

**Western Blot Analysis:** Proteins were extracted from the control and Bacoside A-treated groups after 48 hours. The PBS-washed cells were scraped off with a cell scalpel in 1 mL of cold PBS and centrifuged for 5 min at 4 °C and 5,000 g. Total protein was extracted from the supernatant using RIPA buffer. The protease and phosphatase inhibitors were added, and the proteins were kept on ice for 15 min. Then the lysates were centrifuged at  $12,000 \times g$  for 10 min. The supernatants were collected and used for total protein determination. The extracted proteins were separated by 10% gel electrophoresis and subsequently transferred to PVDF membranes (EMD Millipore). The membranes were blocked in 5% skim milk for 1 h at room temperature. Antibodies against  $\beta$ -actin, E-cadherin, vimentin, and Snail1 were incubated overnight at 4 °C. The secondary antibodies were added at a dilution of 1:1,000 and incubated for 2 h at 25 °C. The enhanced chemiluminescent substrate reagent (ECL) was applied to the film, and the film was analyzed on a Quant LAS 4010 imaging system (Ultra-Violet Products Ltd.).

### Statistical Analysis

All the experiments were done in triplicates. Statistical analysis were performed using SPSS 16.0. Data were expressed as mean  $\pm$  standard error of the mean. Chi-square test was used to assess associations between EMT-related gene expression and other parameters. An independent t-test was used to analyze differences between two unrelated groups. Statistical significance was accepted if  $p < 0.05$ .

## RESULTS

**Cytotoxicity Assay:** Cytotoxicity studies were performed using the MTT assay, which measures cell viability. The viable

cells capable of reducing MTT to purple formazan crystals were quantified. This is the most representative and sensitive method for assessing cell proliferation and growth. Percent growth inhibition at different concentrations of Bacoside A was observed in HCT 116 colon cancer cell lines. At concentrations up to 20  $\mu\text{g}/\text{mL}$ , there was little growth inhibition, and above that concentration there was a marked decline in the growth of cancer cells. The cytotoxicity results showed that cell death was concentration-dependent. From the cytotoxicity linear curve, the fifty-percent  $\text{IC}_{50}$  was determined to be 32  $\mu\text{g}/\text{mL}$  (Table 1, Figure 1).

**In vitro free-radical scavenging activity:** A dose-dependent scavenging response of Bacoside A against free-radical generation was assessed. Bacoside A showed the strongest percentage inhibition and radical scavenging activity compared with the reference standard, gallic acid. In the DPPH assay, scavenging activity exceeded 60% at 40  $\mu\text{g}/\text{mL}$  of Bacoside A. Results of the hydroxyl radical scavenging assay

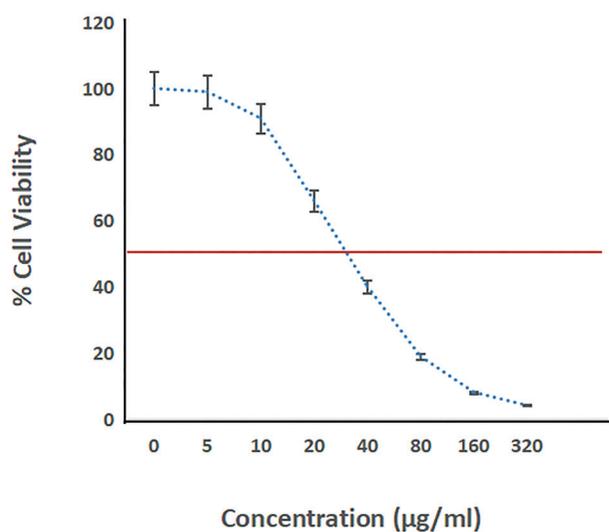


FIGURE 1: Cytotoxicity assay.

Values are mean  $\pm$  standard error of the mean of triplicates (n=3).

No.	Concentration ( $\mu\text{g}/\text{mL}$ )	% of viable cells
1	Control	100 $\pm$ 0.01
2	5	99.01 $\pm$ 0.02
3	10	91.92 $\pm$ 0.04
4	20	66.11 $\pm$ 0.003
5	40	40.11 $\pm$ 0.002
6	80	18.89 $\pm$ 0.003
7	160	8.04 $\pm$ 0.002
8	320	4.04 $\pm$ 0.003

Values are mean  $\pm$  standard error of the mean of triplicates (n=3).

showed that Bacoside A exhibited 98% scavenging activity at a concentration of 50  $\mu\text{g}/\text{mL}$ , which is almost equal to that of the potential standard free radical scavenger, gallic acid. Similarly, the ability to reduce ferric ions was determined by the FRAP assay, which indicates concentration-dependent linearity in the percentage reduction of ferric ions. At a concentration of 50  $\mu\text{g}/\text{mL}$ , Bacoside A reduced ferric ions by 90% (Figure 2).

The HCT 116 cancer cells were further tested for the radical generation, and the scavenging role of Bacoside A at different concentrations were also tested using the above-mentioned assay. The results confirm that the phytomedicine Bacoside A is a potential radical scavenger that shows 15-35% radical-scavenging activity across all tested methods. This decrease may be due to continued rapid proliferation of cancer cells, which was also significantly inhibited ( $p < 0.05$ ) by Bacoside A at concentrations above 50  $\mu\text{g}/\text{mL}$  (Figure 3).

**In vitro Membrane Stability Assay:** Oxidation of macromolecules is a common process in cancer cells due the increase in the cellular deterioration by various mechanism. Thereby it is valid to analyse the molecular stability in the presence of Bacoside A, the results clearly proves that the Bacoside A effectively inhibit lipid peroxidation, significant ( $p < 0.05$ ) suppression of malondialdehyde levels were identified in dose dependent manner. Figure 4 shows that concentrations of Bacoside A higher than 50  $\mu\text{g}/\text{mL}$  have a greater inhibitory effect on lipid peroxidation in colon cancer cell lines. Inhibition of protein denaturation also gradually increases with increasing concentrations of Bacoside A, reaching 80% inhibition at 50  $\mu\text{g}/\text{mL}$  in bovine serum albumin. The generation of free ions has been the main cause of peroxidation in biological systems, and the metal-chelating activity was also tested on HCT 116 colon cancer cell lines, both with and without Bacoside A. A linear graph was observed, indicating that Bacoside A exhibited strong chelating activity with values above 85% and 50% in the absence and presence of HCT 116 colon cancer cell lines, at concentrations of 50  $\mu\text{g}/\text{mL}$  and 200  $\mu\text{g}/\text{mL}$ , respectively (Figure 4).

**In vitro cell migration activity:** Cell migration was assessed using a scratch-wound assay and observed under an inverted phase-contrast microscope. Compared with the control, cell migration was observed in the treated group, but faster and maximal migration was observed in the control group at 24 hrs and 36 hrs. Wound closure in the test group was calculated to assess cancer cell migration at Bacoside A concentrations of 10  $\mu\text{g}/\text{mL}$  and 32  $\mu\text{g}/\text{mL}$ . The images clearly showed the rapid migration of the cancer cells in the Bacoside A-treated groups at the lower concentration. Almost 80% of the scratched area was covered by cells within 12 hrs, and complete closure was observed within 24 hrs. Cells treated

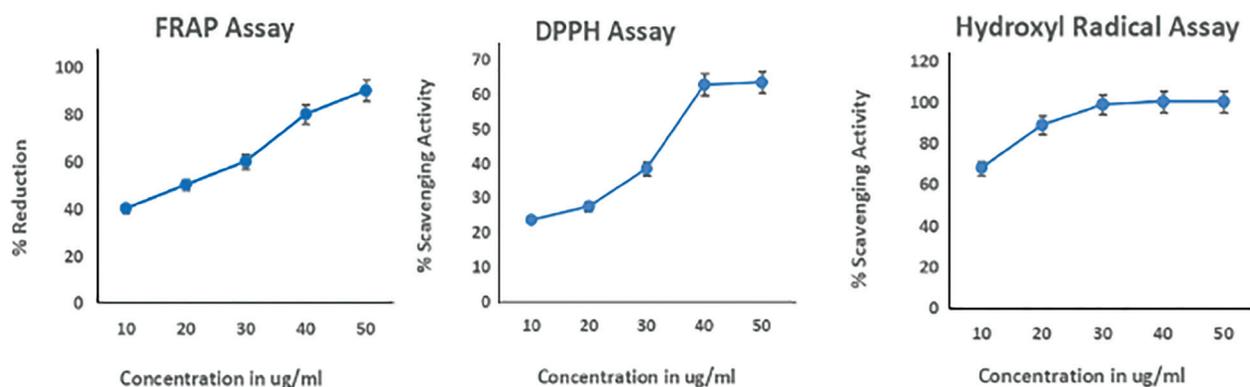


FIGURE 2: *In vitro* free radical scavenging assay.

Values are mean  $\pm$  standard error of the mean of triplicates (n=3).

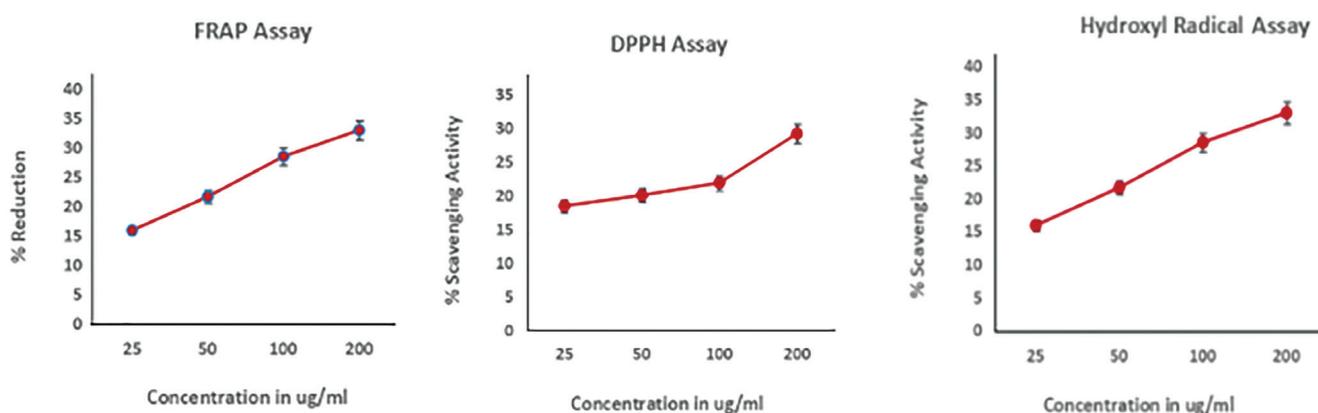


FIGURE 3: Free radical scavenging assay by Bacoside A on HCT 116 cell lines.

Values are mean  $\pm$  standard error of the mean of triplicates (n=3).

with the lower concentration (10  $\mu\text{g}/\text{mL}$ ) covered 60% of the scratched area within 12 hours ( $p < 0.01$ ), indicating that the concentration used was insufficient to stop migration completely. In contrast to this result, the  $\text{IC}_{50}$  value of Bacoside A demonstrates a significant inhibitory effect ( $p < 0.001$ ) on cancer cell migration; only about 20% of cells were observed in the scratched area within 12 hrs of treatment (Table 2, Figure 5).

***In vitro* Clonogenic Assay:** The effect of Bacoside A on colony forming capacity of HCT 116 was carried out, the cancer cells usually grows in colonies by communicating with the neighbouring cells. Multiple proteins are involved in colony formation with adjacent cells. The results of the clonogenic assay show that treatment with Bacoside A at concentrations of 10-50  $\mu\text{g}/\text{mL}$  significantly ( $p < 0.001$ ) reduces the colony-forming potential of cancer cells compared with the untreated control group. Calculation of colony-forming units (50 cells) after Bacoside A treatment showed that colony

formation decreased gradually between 10 and 30  $\mu\text{g}/\text{mL}$ , whereas concentrations of 40 and 50  $\mu\text{g}/\text{mL}$  caused a rapid reduction to approximately one-fifth of the control (Figure 6). The surviving fraction calculation also correlates with this result and shows a significant decline as the concentration of Bacoside A increases above the  $\text{IC}_{50}$  values (Figure 7).

#### ***In vitro* Epithelial - Mesenchymal transition induction Assay:**

The EMT inducing cell culture conditions were applied to check the status of the epithelial to mesenchymal transition on Bacoside A treatment. Observations under phase-contrast microscopy demonstrated that cells lost their classical epithelial morphology and acquired a mesenchymal, spindle-shaped morphology over time. At 0 hr, both the induced and uninduced groups exhibited densely packed, spindle-shaped cells. EMT-induced cells treated with Bacoside A appeared less densely packed and exhibited a variant, spindle-shaped morphology and the number of cells also declined at 24 and 36 hours of observation. The results clearly indicate that the

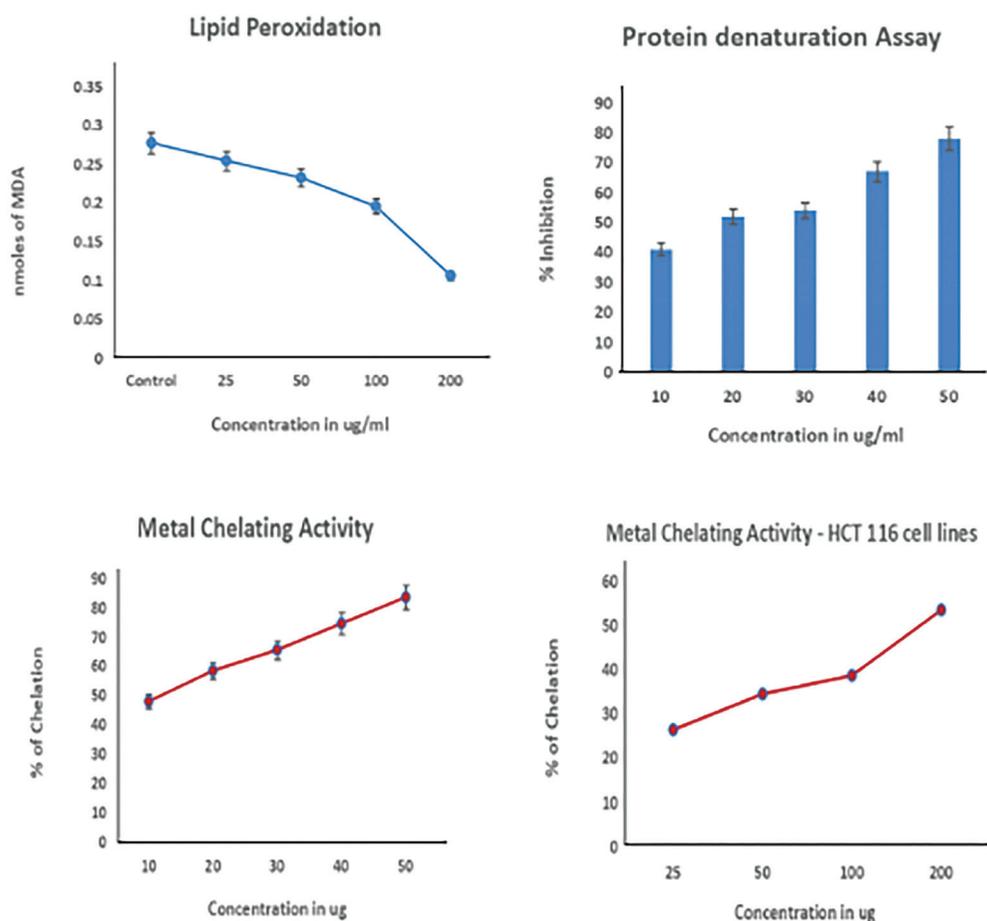


FIGURE 4: *In vitro* membrane stability assay.

Values are mean  $\pm$  standard error of the mean of triplicates (n=3).

TABLE 2: *In vitro* cell migration activity.

Time (hrs)	Concentration					
	Control		10 ( $\mu\text{g/mL}$ )		32 ( $\mu\text{g/mL}$ )	
	Wound distance (mm)	Wound closure (%)	Wound distance (mm)	Wound closure (%)	Wound distance (mm)	Wound closure (%)
0	30.37 $\pm$ 0.002	-	28.04 $\pm$ 0.001	-	30.75 $\pm$ 0.030	-
12	5.94 $\pm$ 0.002	80.44	10.85 $\pm$ 0.004	61.30**	23.95 $\pm$ 0.002	22.11***
24	-	100	7.84 $\pm$ 0.004	72.03**	20.74 $\pm$ 0.003	32.55***
36	-	100	3.84 $\pm$ 0.003	86.30*	18.73 $\pm$ 0.001	39.08***

The values are mean  $\pm$  standard error of the mean (n=3); \*\*\*p<0.001, \*\*p<0.01, \*p<0.05 statistically Significant compared with control.

cancer cell may have transitioned from the epithelial to the mesenchymal state by losing its intercellular integrity and converting into a loosely distributed form, suggesting that Bacoside A can effectively act on cancer cells and induce cell death (Figure 8).

**Apoptosis Assay:** The apoptotic assay was done with double staining of AO/PI in which the live and dead cells were identified based on the emission of green and reddish orange

fluorescence. As shown in Figure 9, the untreated HCT 116 cancer cells appeared healthy and green, with intact nuclei. Treatment with Bacoside A at different concentrations (10-30  $\mu\text{g/mL}$ ), followed by incubation for 24 hours, resulted in an increased number of dead cells observed under a fluorescent microscope. Apoptotic results show that Bacoside A-treated cells at concentrations of 30  $\mu\text{g/mL}$  and above exhibited increased late apoptotic features, with a reddish-orange

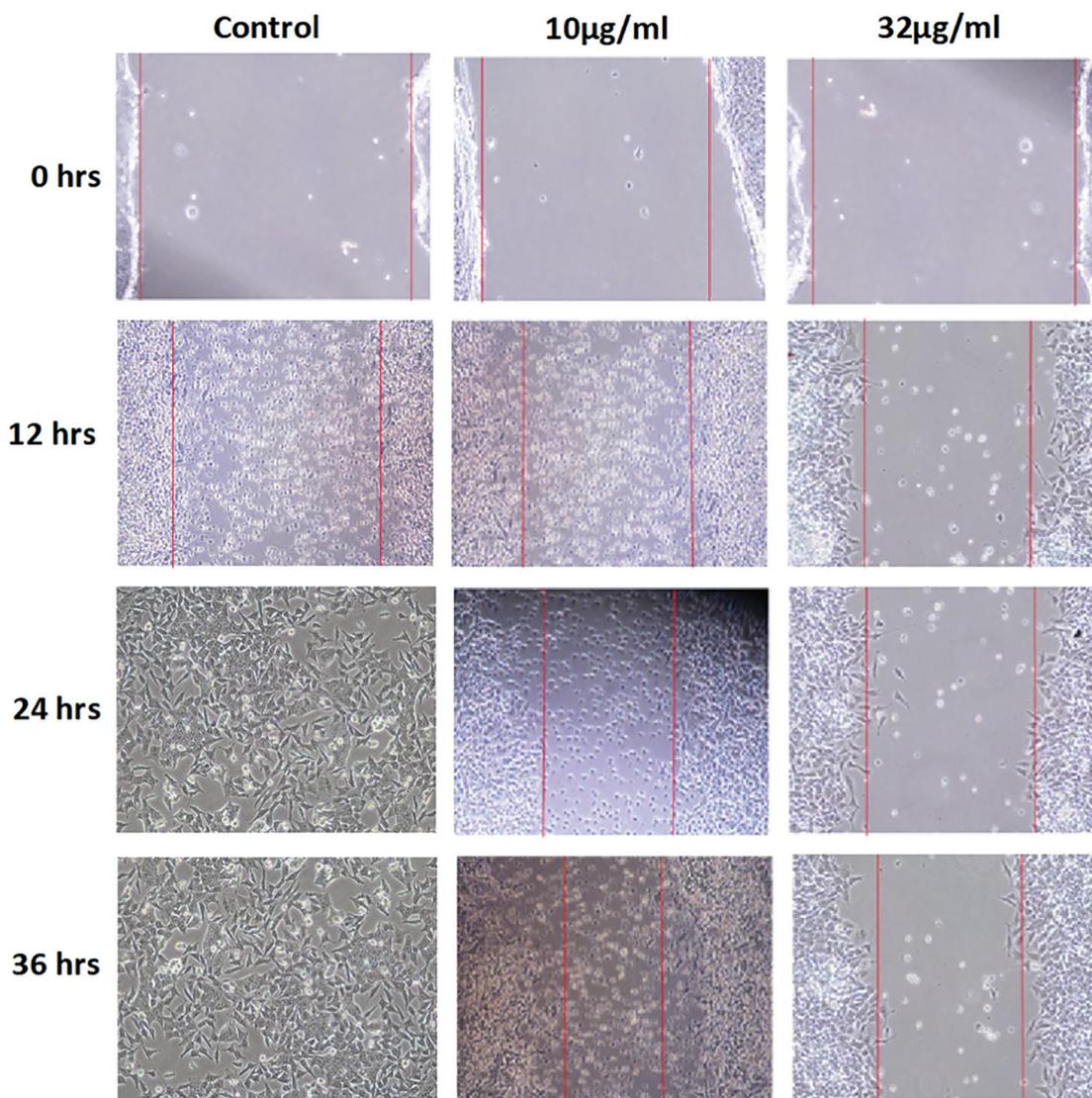


FIGURE 5: *In vitro* cell migration activity.

appearance attributable to PI-positive staining of denatured DNA within 12-24 hours. Lower concentrations (10 µg/mL and 20 µg/mL) can also induce apoptosis in cancer cells, as indicated by the relatively early apoptotic appearance of yellowish-orange fluorescence. Prolonged incubation induces the cellular necrosis observed in HCT 116 cells double-stained with AO/PI.

**RNA Isolation, cDNA Synthesis and qPCR Analysis:** The real time PCR analysis was carried out to analyse the expression pattern responsible genes involved in EMT mechanism and the changes observed under the treatment of IC<sub>50</sub> dose of Bacoside A on HCT 116 cancer cells. The gene expression profile was evaluated using the epithelial marker E-cadherin, the EMT transcription factor Snail1, and the mesenchymal marker vimentin. The qPCR analysis of mRNA expression showed that the mRNA level of the epithelial marker

E-cadherin was high and was up-regulated by Bacoside A during 48 hrs of treatment. The results further confirm that the mRNA expression levels of the mesenchymal marker vimentin and the transcription factor Snail1 were decreased and their gene expression was downregulated (Table 3, Figures 10, 11).

## DISCUSSION

Cancer development from primary cancer cells involves several molecular mechanisms in our system. Metastasis is the ability of cancer cells to move away from the site of origin. Metastasis of cancer cells is the major cause for failure of therapy and disease management. The molecular mechanism of metastasis being poorly understood. EMT is one of the significant events that lead to invasion, stress resistance, and dissemination. Various *in vitro* and *in vivo* studies have demonstrated that EMT is associated with cancer metastasis.<sup>27</sup>

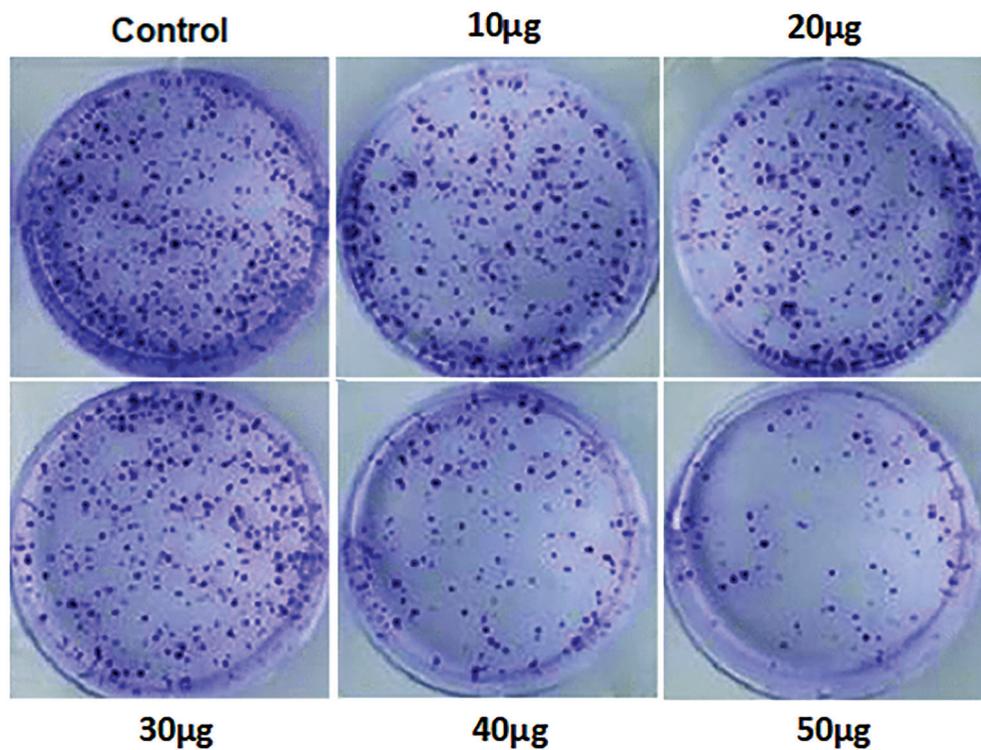


FIGURE 6: *In vitro* clonogenic assay.

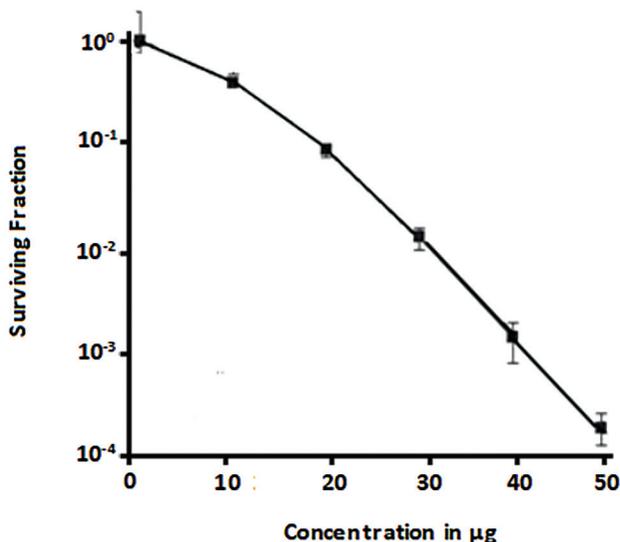


FIGURE 7: Percent survival fractions of colonies.

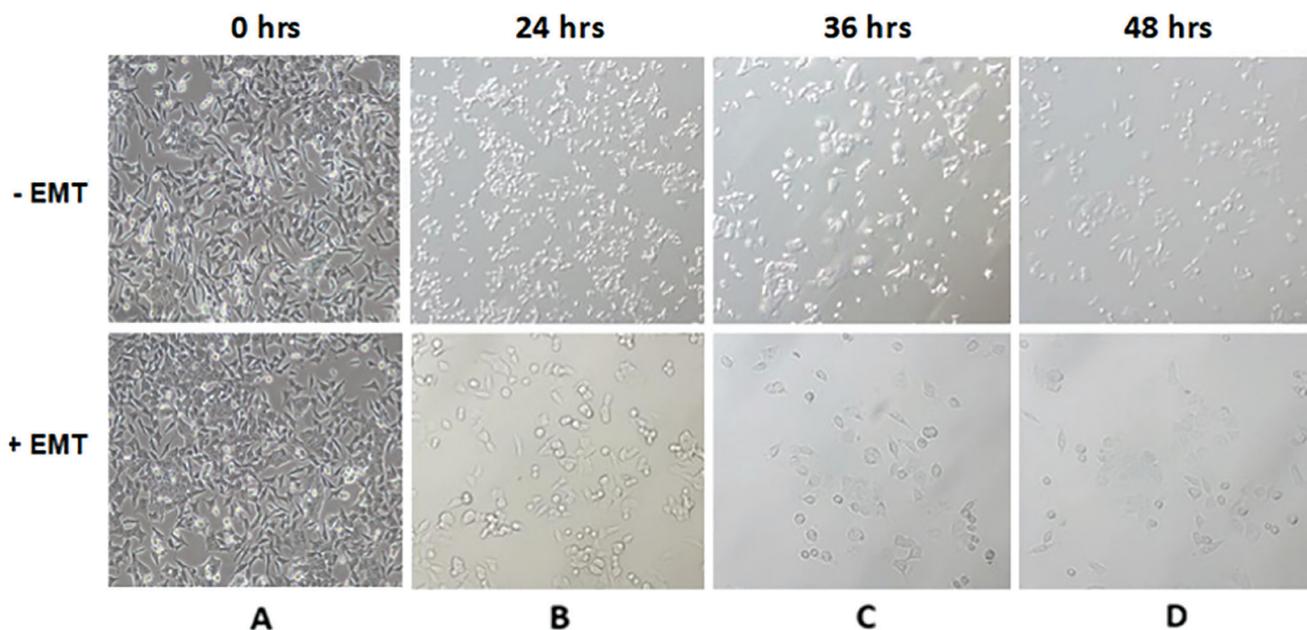
Values are mean  $\pm$  standard error of the mean of triplicates (n=3).

In the present *in vitro* study, the effect of Bacoside A on EMT and the prevention of cancer metastasis was evaluated. Bacoside A demonstrated cytotoxicity against HCT 116 colon cancer cells, indicating that it is a promising anticancer agent. Similar studies on Bacoside A have demonstrated its cytotoxic effect via induction of apoptosis against various cancers, such

as oral squamous cell carcinoma,<sup>28</sup> and its ability to inhibit cellular proliferation in C6 glioma cells.<sup>29</sup>

The study also finds the beneficiary effect of Bacoside A on free radical generation during cancer progression. The results of the present investigation clearly indicate that Bacoside A possesses substantial potential to inhibit the production of various types of free radicals. The hydroxyl radical produced in the body is highly reactive and is one of the most potent oxidizing agents; it reacts with almost all biomolecules in living cells at a high rate. It is involved in many pathological processes. A similar identification was also made for free-radical scavenging and antioxidant roles of bacosides against liver and nerve cells.<sup>30</sup> Another finding indicates that Bacoside A acts as an effective antioxidant, has a therapeutic role and is a potential anticancer, and analgesic agent.<sup>31</sup>

Cell membrane integrity maintains cellular stability. The results support that chronic inflammation accompanied by protein denaturation, is mainly caused by the loss of bonding interactions, especially hydrogen and disulphide bonds. When assessing membrane stability, the results of a lipid peroxidation and protein degradation study support that Bacoside A suppresses lipid peroxidation, effectively inhibits protein denaturation, and exerts a membrane-strengthening effect under inflammatory and disease conditions. It is



**FIGURE 8:** *In vitro* epithelial - mesenchymal transition induction assay.

Epithelial mesenchymal transition (EMT) induction on treatment of Bacoside A in HCT 116 colon cancer cells (A) Untreated cells showed viable cells with epithelial morphology (B) Lost on epithelial morphology and dead cells were observed in with less density (C, D) Structurally disrupted and dead cells were identified on EMT induction within 24 hours of bacoside A treatment.

**TABLE 3:** Fold changes in the gene expression analysis.

Genes	Time (hrs)	Average CT	$\Delta$ CT	$\Delta\Delta$ CT	$2^{-\Delta\Delta$ CT}
E-Cadherin	0	35.85	17.18	-0.0183333	1.012788761
	12	34.74	16.21	-0.9883333	1.98389174
	24	33.73	15.065	-2.1333333	4.38729982
	36	32.64	14.09	-3.1083333	8.62385727
	48	32.23	13.85	-3.3483333	10.1847121
Snail1	0	32.615	13.945	-3.1117	8.644005559
	12	31.925	13.395	-2.5617	5.904029787
	24	30.92	12.255	-1.4217	2.679010062
	36	29.6	11.05	-0.2167	1.162072436
	48	28.605	10.225	0.6083	0.655969208
Vimentin	0	24.78	6.11	-1.1633333	2.239743156
	12	25.385	6.855	-0.4183333	1.133638278
	24	26.355	7.69	0.4166667	0.749153521
	36	26.385	7.835	0.5616667	0.0677519
	48	26.655	8.275	1.0016667	0.449422699

CT: Computed tomography.

therefore expected that the free radicals are scavenged by Bacoside A and that their attack on the macromolecules such as lipids, proteins, and nucleic acids is also mimicked. Research findings also support the present work, in an *in vivo* study in which dichlorvos induced oxidative stress and membrane instability, these effects were reversed by Bacoside A. research

findings with the combinations of bacosides treatment proved they have the ability of membrane stabilization and give significant protection of cell membranes against injurious substances and thereby exhibit anti-inflammatory activity. These findings correlated with compounds inferred to stabilize lysosomal membranes, one of the mechanisms by

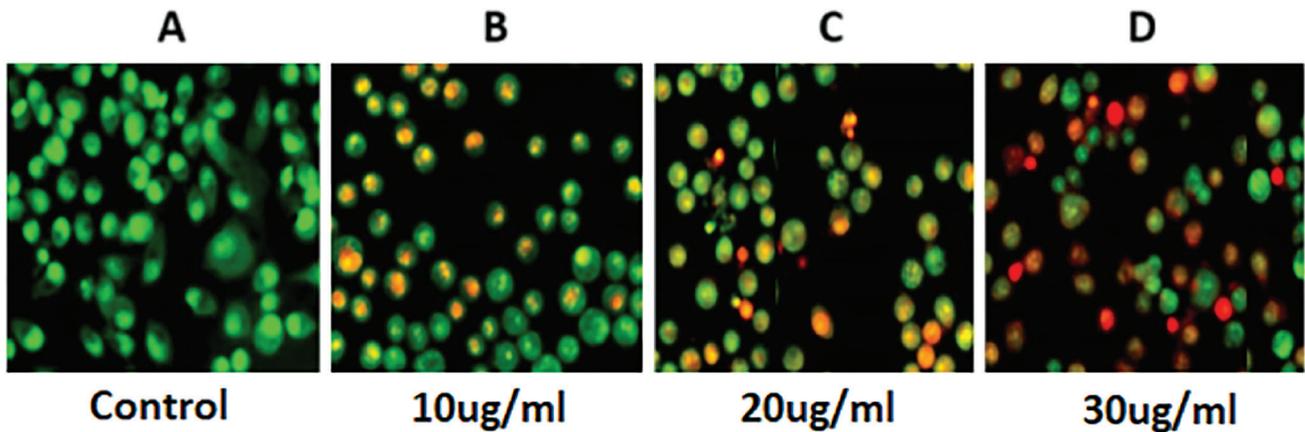


FIGURE 9: Apoptosis assay.

Apoptotic characteristics on treatment of Bacoside A in HCT 116 colon cancer were observed on 24 hours of treatment (A) Viable cells indicated by green fluorescence, (B, C) Early apoptotic features, namely, blebbing and chromatin condensation as well as late apoptotic cells were detected after 24 hours of treatment with bacoside A indicated by orange fluorescence, (D) Late apoptosis and cell necrosis were observed after 24 hours of Bacoside A shown by red fluorescence.

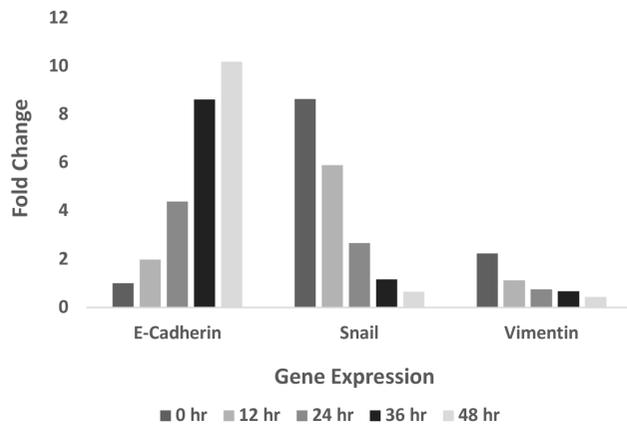


FIGURE 10: mRNA expression analysis.

which the test herb mediates its anti-inflammatory action.<sup>32</sup> Another study supports the present investigation states that the Bacoside A is a high potential compound that maintains the membrane potential and strengthen the molecular stability in colon cancer, mammary cancer, pancreatic cancer and in other illness such as Alzheimer's disease and Parkinson's disease.<sup>10</sup>

The membrane-bound proteins play an important role in maintaining membrane stability and integrity in cancer cells. In colon cancer, the ECM of the cell increases stiffness and gains the ability to hold the cells together and to communicate biochemical signals and information for its action. Alterations in this mechanism lead to variations in structure and function. Such proteins—MMPs, cadherins, integrins, fibronectin, and laminin—may be affected by oncogenic activation of canonical signalling pathways primarily involved in

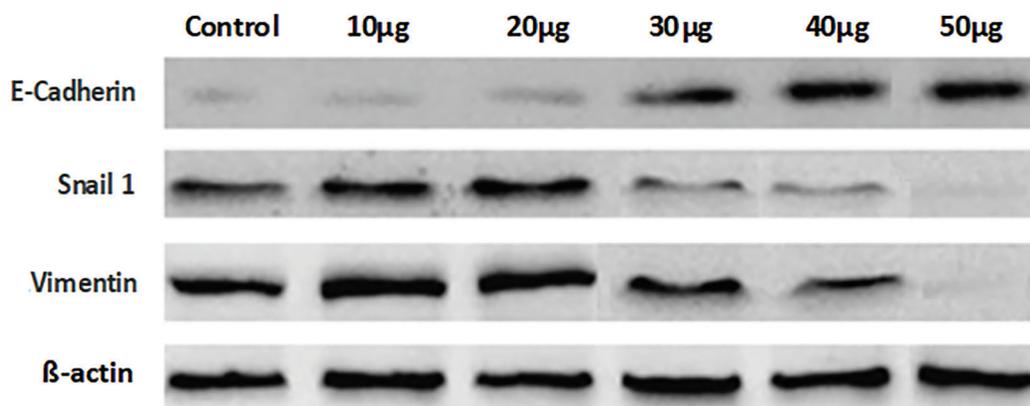


FIGURE 11: mRNA expression analysis.

cytoskeletal modification during adhesion and migration. The results of the present study's cell migration analysis indicate that rapid migrations observed in cancer cells may be due to disturbances in cell adhesion proteins, which effectively promote cancer cell mobility and lead to metastasis. Bacoside A effectively reverses this process by up to 75% at concentrations above 32  $\mu\text{g/mL}$ . Studies conducted by Hai-Yun Liu (2024) reported that Bacoside A inhibits migration-associated matrix metalloproteinases MMP2 and MMP9.<sup>12</sup> Another study supports that combined doses of bacopside I and II possess a synergistic action in inhibiting endothelial migration and inducing apoptosis in CRC cells.<sup>33</sup>

The present study clearly demonstrates the inhibitory effect of Bacoside A on the colony-forming capacity of cancer cells. Colony formation is the capacity of individual cancer cells to form large colonies in which they exist as undifferentiated cancer stem cells. During treatment with Bacoside A at  $\text{IC}_{50}$  and above, the number of colony-forming units of cancer cells is effectively reduced. This may be due to increased cell death induced by Bacoside A treatment, which reduces cell integrity and disrupts the coordinating actions of related proteins required for colony formation. Studies using 3D clonogenic assays can facilitate understanding of the mechanisms underlying CSC stemness, notably drug resistance, pro- and anti-apoptotic mechanisms, and pathways involved in self-renewal. Cancer cells protease activity improve its invading capacity to other tissues by degrading the basement membrane and the surrounding ECM.<sup>34</sup> The studies conducted by Ishikawa et al.<sup>35</sup> state that clonogenic assays evaluate the ability of single cells to proliferate and form colonies.

The EMT induction assay was performed to assess the ability of the cancer cells: How they can separate from each other, adapt, and migrate to other areas. Signals from the tumor microenvironment stimulate cancer cells to undergo EMT and adopt an invasive phenotype. In the present study, lower-concentration Bacoside A-treated groups showed comparatively higher cell counts, whereas higher-concentration groups had significantly fewer cells, although individual cells were still observed. This indicates that Bacoside A can effectively reduce the number of cancer cells, and its effects on cell migration and invasion may be due to altered signaling pathways. A Literature review also supports that the study conducted in lung cancer provides promising evidence that certain drugs can induce changes in epithelial characteristics and mesenchymal conversion, which are involved in a wide variety of processes such as invasion, metastasis, and drug resistance in cancer cells.<sup>36</sup> Another noteworthy finding from the analysis of the EMT transition in breast cancer cells reveals that the combinatorial involvement of collagen and pro-inflammatory cytokines plays an important role in the phenotypic changes.<sup>37</sup>

The apoptotic studies in the present investigations clearly show that Bacoside A is an effective agent that destroys cancer cells and induces apoptosis. At lower concentrations, cell death is minimal, whereas at the  $\text{IC}_{50}$  a greater number of dead cells was observed. Several reports have suggested that the anticancer potential of plant-based chemotherapeutic agents triggers apoptosis by inducing functional changes in mitochondrial energy transfer in cancer cells through alterations in various signalling and cell-cycle arrest pathways. Further supporting evidence indicates that the aqueous fraction of the ethanolic extract of *B. monneri* inhibits cell viability, colony formation, cell migration, and induces apoptotic cell death in Cal33 and FaDu cells.<sup>28</sup>

EMT is induced during cancer progression and contributes to the formation of metastatic colonies. It is the process by which epithelial cells lose their differentiated characteristics and acquire mesenchymal traits. In this process, cancer cells acquire metastatic properties by increasing their mobility, invasiveness, and resistance to apoptosis. Furthermore, EMT-derived tumor cells acquire stem cell characteristics and become resistant to therapy. Protein expression levels related to migration, apoptosis, and autophagy were assessed by Western blotting. Findings revealed that, in the untreated group, the epithelial marker E-cadherin was downregulated, while the transcription factor Snail1 and the mesenchymal marker vimentin were upregulated; however, the expression of these proteins was modified during Bacoside A treatment. Here, the reciprocal interference between an altering tumor microenvironment and the EMT phenotype was investigated *in vitro*, suggesting that Bacoside A can promote the expression of epithelial markers such as E-cadherin, which is often down-regulated in EMT, thereby helping to maintain cell-cell adhesion and prevent cell migration. The anti-EMT effects of Bacoside A are thought to involve modulation of signaling pathways such as the Wnt/ $\beta$ -catenin pathway, which plays a crucial role in regulating EMT. Studies support that the EMT program is activated by autocrine and paracrine signals from the tumor microenvironment, which include a variety of cytokines, interleukins, and growth factors that stimulate signaling pathways in tumor cells and converge on the activation of a set of transcription factors. In addition to EMT-TF regulation of genes associated with epithelial and mesenchymal states, other regulatory mechanisms contribute to the control of these cell states.<sup>38</sup>

The overall finding of the present study clearly demonstrates the efficacy of Bacoside A against cancer cell migration. In addition, suppression of other transcriptional markers that support mesenchymal expression, such as Snail, Slug, and ZEB1, should be assessed. The extension studies related to the significant pathways that regulate the process of cancer metastasis in other cancers, such as liver and pancreatic cancers, in combination with the evaluation of the efficacy of Bacoside A administration

in the cancer-induced liver models, need to be analyzed. Further analysis of hypoxia-mediated arrest of cell migration can also be performed to strengthen the present findings.

## CONCLUSION

EMT is recognized as playing a key role in cancer development, metastasis, and chemotherapy resistance, and its crucial roles throughout cancer progression have recently been investigated. Although there is still debate about whether EMT causes cancer metastasis, its importance in cancer chemoresistance is becoming more widely recognized, with many EMT-related signaling pathways implicated in cancer chemoresistance. Targeted cancer treatment has been an emerging field over the past decade. Several monoclonal antibody therapies and small-molecule compounds, particularly kinase inhibitors, have been discovered or synthesized and are undergoing clinical trials, demonstrating improved anticancer efficacy. While many targeted therapies demonstrated encouraging preliminary clinical outcomes, such as improved overall survival, a significant proportion of patients who received targeted therapies developed drug resistance after long-term treatment.

## Ethics

**Ethics Committee Approval:** Sri Ramachandra Institute of Higher Education and Research Ethics Committee approval was obtained for conducting the cell line studies (approval number: IEC-NI/22/JUL/83/85, date: 14.10.2022).

**Informed Consent:** Retrospective study.

## Footnotes

**Conflict of Interest:** No conflict of interest was declared by the author.

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