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Antiproliferative effect of an analog of curcumin bis-1,7-(2-hydroxyphenyl)-hepta-1,6-diene-3,5-dione in human breast cancer cells

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Abstract. – BACKGROUND AND OBJECTIVES: Curcumin exhibits growth-suppressive activity against a variety of cancer cells, but low bioavailability prevents its use in chemotherapeutic applications. One strategy for circumventing this problem has been the creation of synthetic analogs. In this study we synthesized an analog of curcumin bis-1,7-2(hydroxyphenil)-hepta-1,6diene-3,5diore (BDMC-A) and investigated its anti-breast cancer property.

MATERIALS AND METHODS: We compared the impact of bis-1,7-2(hydroxyphenil)-hepta-1,6diene-3,5diore (BDMC-A) with that of curcumin in human breast cancer cell line MCF-7. MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] cell viability assay was used to examine the cell viability/proliferation. LDH assay and cell counts were performed to assess the cytotoxicity and anti-proliferative effects of the compound respectively. Flow cytometry followed by Western blot were performed to investigate the cell cycle distribution.

RESULTS: BDMC-A has an inhibitory effect on MCF-7 cells comparably equivalent to that of curcumin as determined by MTT assay. Cytotoxicity of the cells by both curcumin and BDMC-A were confirmed by LDH release assay and cell count assay. Flow cytometric studies showed accumulation of cells in the G2/M phase which confirms the cell cycle arrest. This was further confirmed by immunoblotting of the protein Cyclin D1, whose expression were found to be decreased in both curcumin and BDMC-A treatment.

CONCLUSIONS: The results indicate that the curcumin analog exhibit potent inhibitory activity which is comparable to that of curcumin in human breast cancer cells. Since the solubility of BDMC-A was higher in aqueous medium, it is expected to be more bioavailable, and hence more active in vivo. Further evaluation might reveal its role on various molecular targets.

Key Words:

Curcumin, MCF-7, BDMC-A, Breast cancer, MTT.

Introduction

Cancer is currently the second leading cause of death in the world. Combating cancer is of paramount importance today. Multidisciplinary scientific investigations are making the best efforts to combat this disease, but the perfect cure is yet to be brought into the world of medicine. Many anticancer therapies currently in use are inadequate not only in terms of their therapeutic efficacy but also because they have undesirable side effects. However, certain dietary constituents known as phytochemicals have been shown to exhibit growth-suppressive activity and chemopreventive properties against various types of cancers without the adverse side effects normally associated with current chemotherapies[1].

Curcumin, commonly called diferuloyl methane, is a hydrophobic polyphenol derived from rhizome (turmeric) of the herb *Curcuma longa*[2]. It is one of the plant derived promising chemopreventive agents studied for its anticarcinogenic and antioxidant properties. The mechanism of action of curcumin has been extensively studied at the molecular level. Several studies over the past decades have confirmed that curcumin targets multiple biochemical pathways[3].

Curcumin interferes with the transcriptional activation induced by transcription factors[4]. Curcumin has been shown to arrest the cell cycle at G_0-G_1 or G_2-M through up-regulation of the cyclin-dependent kinase inhibitors p21 and p27 and down-regulation of Cdc2 and cyclin B1[5].

In spite of its characteristics for chemoprevention and safety, curcumin's therapeutic use has been hindered by its low bioavailability[6]. One of the ways for the increased bioavailability of curcumin for its greater absorption into the blood stream is through the preparation of curcumin analogs[3]. Isomerization in the curcumin structure has found to enhance its antioxidant activity[7].

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Recently, a series of analogs of curcumin were synthesized and evaluated for their use in cancer chemotherapy[8].

We aimed at testing the anticancer efficacy of an analog of curcumin i.e. BDMC-A (bis-1,7-(2-hydroxyphenyl)-hepta-1,6-diene-3,5-dione). It can be more effective than curcumin as it possess hydroxyl group in the ortho position. The introduction of hydroxyl group in ortho position is known to increase the therapeutic activity. Several investigators have shown that ortho substitution with electron donor increases the stability of aryloxyl radical and thus antioxidant activity[9]. Hence, in the present study, we investigated the anticarcinogenic property of BDMC-A, on a breast cancer cell line (MCF-7) and compared its efficacy with that of curcumin.

Materials and Methods

Chemicals

Curcumin (1,7-bis-(4-hydroxyl-3-methoxyphenyl)-1,6-hepatadiene-3,5-dione; diferuloylmethane), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], and propidium iodide (PI), were purchased from Sigma-Aldrich, Bangalore, India. Monoclonal antibodies against cyclin D1 (SC-246) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Dulbecco's Modified Eagle Medium (DMEM), antibiotics and fetal bovine serum (FBS) were obtained from Sigma-Aldrich (Bangalore, India).

Synthesis of Curcumin Analog

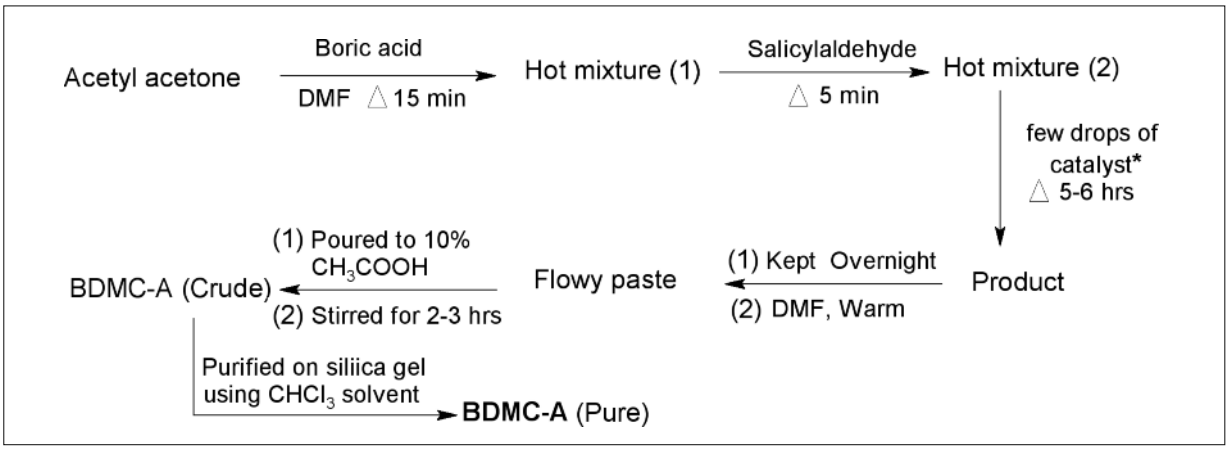
Curcumin analog BDMC-A (bis-1,7-(2-hydroxyphenyl)-hepta-1,6-diene-3,5-dione) was synthesized as per the method described by Dinesh Babu and Rajasekaran[10].

Cell Lines and Culture Conditions

The MCF-7 cell line was obtained from NCCS, Pune, India. The cancer cells were maintained in 1 × Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in CO_2 incubator in an atmosphere of humidified 5% CO_2 and 95% air. The cells were maintained by routine sub culturing in tissue culture flasks. The culture medium was changed every 48 h and the cells were split when they reached confluence. A range of concentrations of BDMC-A and curcumin were used for the determination of IC_{50}.

MTT Assay

The MTT colorimetric assay was performed as described by Mossman[11]. This test is based on the selective ability of living cells to reduce the yellow soluble salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide), to a purple- blue insoluble formazan precipitate. Experiments were performed in six replicates in 96-well flat-bottomed culture plates (Falcon BD Biosciences, San Jose, CA, USA). MTT was dissolved in phosphate buffered saline (PBS) at 5 mg/ml. After 24h of incubation of MCF-7 cells with different concentrations of curcumin and BDMC-A, 20 l of a 5 mg/ml MTT solution was added and the plate was incubated for 4 h, which were dissolved in 100 l of dimethylsulfloxide (DMSO) and plates were incubated at 37°C for 4 h. The amount of colored formazan metabolite formed was determined by absorbance at 570 nm



*Catalyst: 2:1 mixture of glacial acetic acid and diethanol amine. BDMC-A was synthesised and the yield was 78% and found to be 98% pure (HPLC, NMR: impurities were less than 2%).

in a VersaMax ELISA Microplate Reader, Molecular Devices Inc., Sunnyvale, CA, USA.

Estimation of Lactate Dehydrogenase

Lactate dehydrogenase activity was determined by the colorimetric method as described by King[12]. After incubation of curcumin and BDMC-A with cells for 24 and 48h, the medium (without cells) was removed without disturbing cells for LDH assay. Briefly 1 ml of buffered substrate and 0.1 ml of conditioned media were taken and 0.2 ml of water was added and maintained at 37°C. 0.2 ml of NAD^+ solution was added and incubated at 37°C for 15 min. 1 ml of DNPH reagent was added and incubated for further 15 min. Finally, 10 ml of sodium hydroxide (0.4 N) was added and the absorbance was read at 440 nm. The result was expressed as percentage leakage, which is proportional to the number of dead cells.

Cell Count Assay for Antiproliferative Effect

The cell suspension was mixed with trypan blue thoroughly and allowed to stand for 2 minutes. A cover-slip was placed over the two chambers of the haemocytometer. Using a Pasteur pipette. 10 l of the cell suspension was transferred to one of the haemocytometer chambers. The solution was passed under the cover slip by capillary action and made to fill the chamber. The haemocytometer was placed on the stage of an inverted microscope (Olympus America Inc., Center Valley, PA, USA) at 10X magnification and focused. Both viable (opaque) and non-viable (blue-stained cells) cells were counted separately.

Cell Cycle Analysis

To determine the effect of curcumin and BDMC-A on the cell cycle, MCF-7 cells (2×10^6 cells) were treated with curcumin and BDMC-A indifferent doses for 24h and fixed with 70% ethanol. After overnight incubation at −20°C, cells were washed with PBS prior to staining with propidium iodide (10 mg/ml PI; 0.5% Tween-20; 0.1% RNase in 0.01 M phosphate buffered saline pH 7.2). The cells were analyzed using BD FACS Canto™ (BD, Singapore) and data were analyzed using the BD FACSDiva software (BD Biosciences, San Jose, CA, USA).

Western Blot Analysis for Cyclin D1 expression

Western blot analysis was carried out using crude lysates of human breast cells, MCF-7 treated with selected concentration of curcumin and BDMC-A. Cells were homogenized and lysed in RIPA buffer (150 mMNaCl, 50 MTris, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4). Protein concentration was determined using Bradford reagent (Sigma-Aldrich Co, Saint Louis, MO, USA) and lysates were resolved on 12% sodium dodecyl sulphate (SDS)-polyacrylamide gels (PAGE). The proteins were then electro-transferred onto polyvinylidenedifluoride (PVDF) membrane. After blocking with 5% non-fat milk in Tris-buffered saline (TBS, 0.1M, pH 7.4), blots were incubated with primary antibody (mouse monoclonal Cyclin D1 antibody) at 4°C overnight. Protein abundance of b-actin served as a control for protein loading, and was determined with mouse monoclonal b-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were incubated with secondary antibody, HRP-conjugated goat anti-IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted at an appropriate dilution in 1% BSA, for 2h at room temperature. After each step blots were washed thrice with Tween (0.2%)-Tris-buffer saline (TTBS). Protein bands were detected by enhanced chemiluminescence method (ECL, Pierce, Rockford, IL, USA). Chemi Doc Imaging System (Bio-Rad, Hercules, CA, USA) was used to detect signal.

Statistical Analysis

All the data were analyzed using the SPSS 7.5-Windows Students version software (SPSS Inc., Chicago, IL, USA). For all the measurements, one-way ANOVA followed by Tukey's test was used to assess the statistical significance between groups. A statistically significant difference was considered at the level of $p \leq 0.05$.

Results

Cell Viability by MTT Assay

Human breast cancer cells were grown in the presence of varying concentrations of curcumin (10-50 M) and BDMC-A(15-75 M) for 24 h and 48h and cell viability was measured by MTT assay (Figures 1A, B). The inhibitory concentration at 50% (IC_{50}) on MCF-7 was 30 M for BDMC-A and 30 M for curcumin. For further experiments, a sub-optimum cytostatic dose of 30 M for BDMC-A and 30 M for curcumin was used.

In vitro Cytotoxicity in MCF-7 by LDH Assay

MCF-7 cells were cultured for 24 and 48h with different concentrations of curcumin (10 µM-50 µM) and BDMC-A (15 µM-75 µM). There was an increase in LDH leakage when compared to control and the cytotoxicity by BDMC-A (at the concentration of 30 µM) was comparable to that of curcumin (Figures 2A, B).

Cell Count Assay

The results showed that the cell viability declines as the concentration is increased. The cell viability declined to about 50% at a concentration of 30 µM in both curcumin and BDMC-A treated cells. The effect of BDMC-A was equivalent to that of curcumin (Figures 3A, B).

Cell Cycle Distribution Through Flow Cytometry

The cell cycle analysis was performed by a flow cytometry technique. MCF-7 cells were treated with 15 µM of both curcumin and BDMC-A and the cell cycle distribution was examined after 12h of drug treatment. Cell cycle analysis revealed a progressive accumulation of cells in the G2/M phase of the cell cycle (Figure 4). Flow cytometric analysis of the curcumin treated cells showed a decrease in the percentage of cells in the G1 phase from 67 to 58% and an increase in the percentage of cells from 12 to 18% in the S phase. Whereas in BDMC-A treated cells, it showed a decrease in the percentage of cells in the G1 phase from 67 to 62% and an

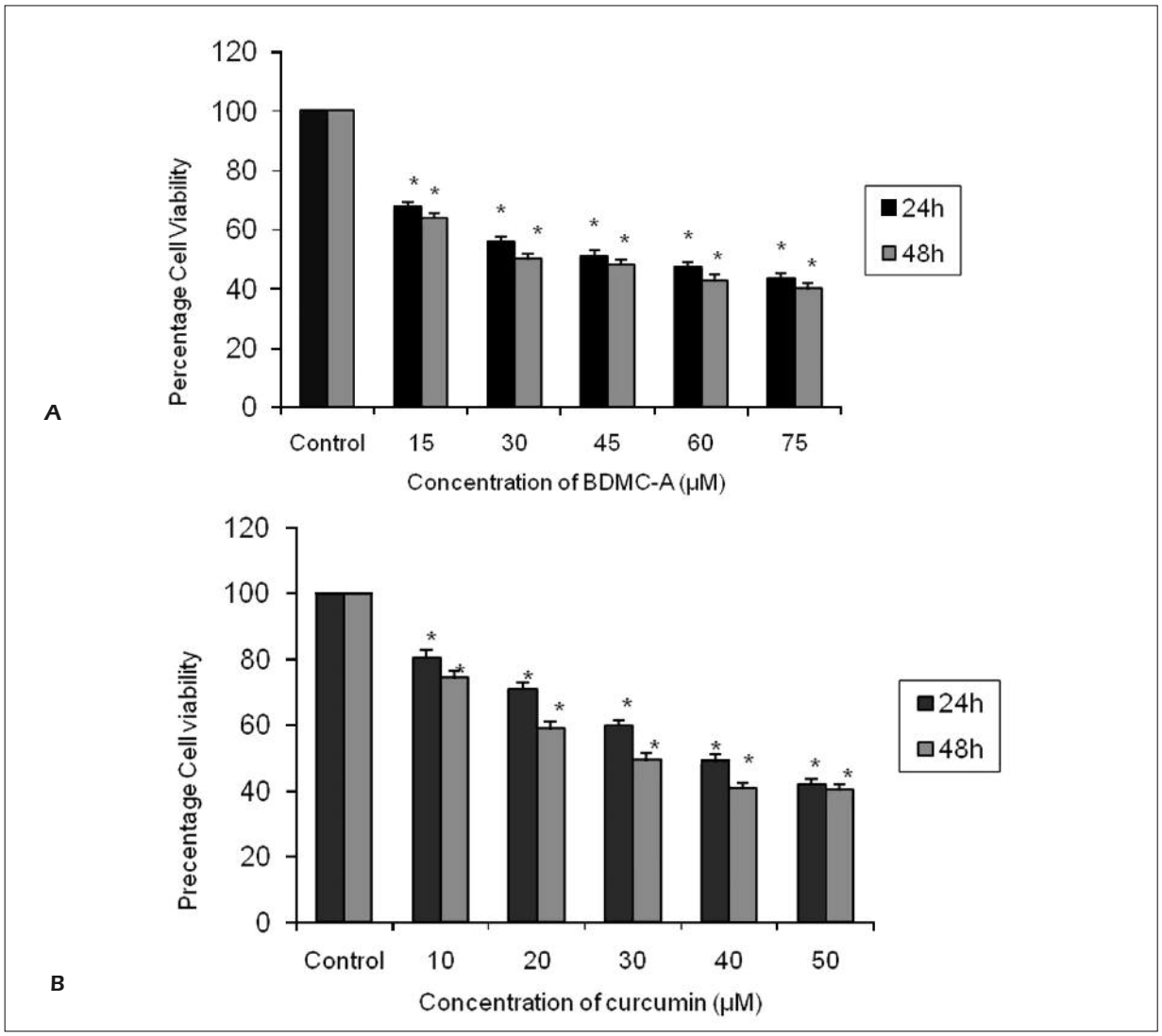


Figure 1. ***A,*** Growth inhibition of MCF-7 cells in vitro by BDMC-A. ***B,*** Growth inhibition of MCF-7 cells *in vitro* by curcumin.

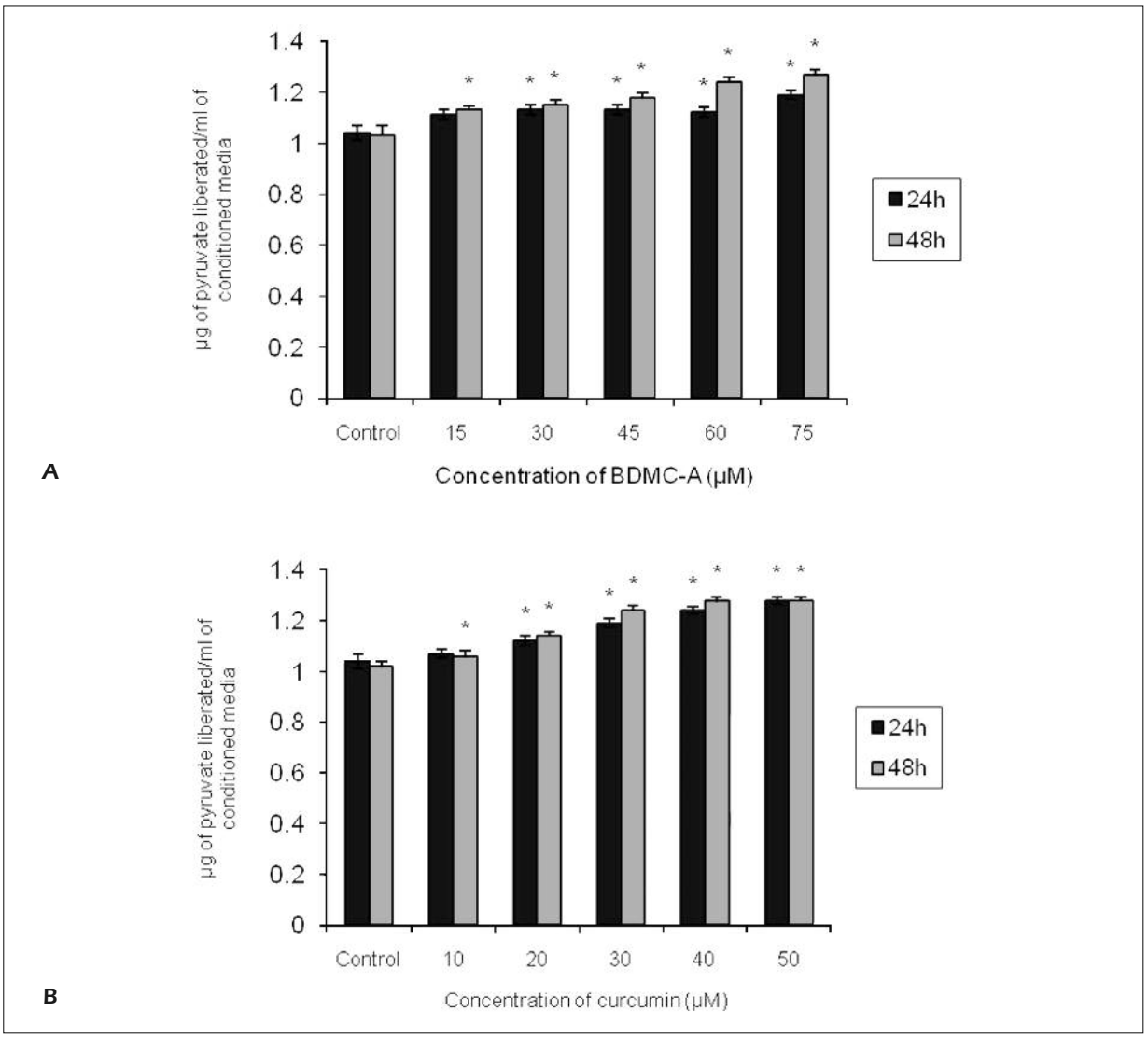


Figure 2. ***A,*** Effects of BDMC-A on MCF-7 cells on LDH release. ***B,*** Effects of curcumin on MCF-7 cells on LDH release.

increase in the percentage of cells in the S phase, from 12 to 17%, within 24h of curcumin treatment.

Western Blot of Cyclin D1

The results showed that there was a decrease in the expression of cyclin D1 in both curcumin and BDMC-A treated cells compared to that of control (Figure 5A). The densitometric readings showed that there was a significant decrease in the expression of cyclin D1 in both curcumin and BDMC-A treated cells but significant variation was not observed between curcumin and BDMC-A treated cells (Figure 5B).

Discussion

Bioactive compounds, known as phytochemicals, have been found to exhibit growth-suppressive activity as well as chemopreventive properties against various types of cancer[13]. Curcumin is one of the most widely characterized phytochemicals. Many studies have demonstrated inhibitory effects of curcumin on tumorigenesis and tumor growth *in vitro* and *in vivo*. It is less toxic and has an ideal potential to down-regulate the critical genes activated in cancer. Curcumin has been shown to have chemopreventive effects on breast cancer[14]. In the present study the anti-

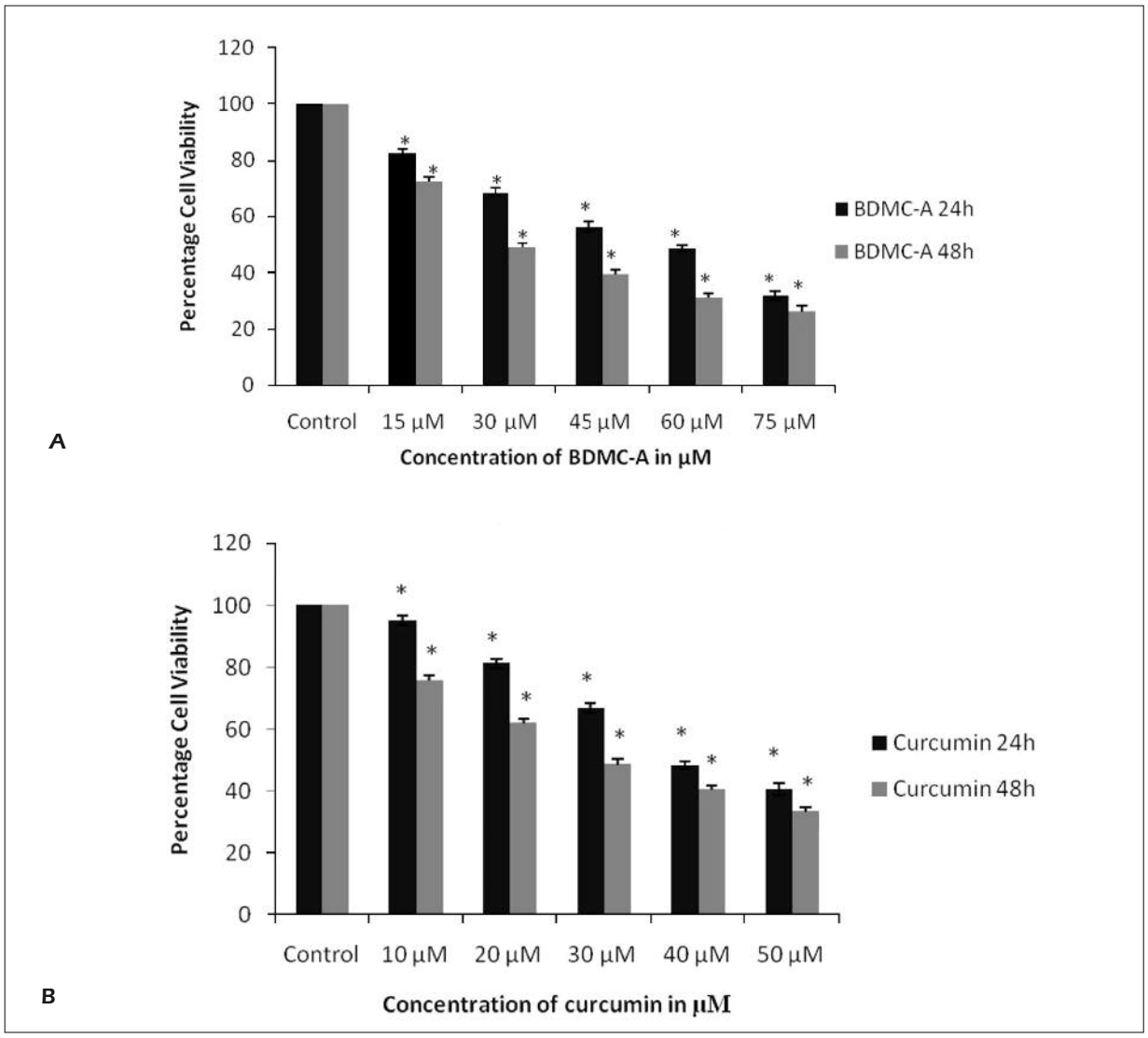


Figure 3. ***A,*** Effect of BDMC-A on MCF-7 cell viability. ***B,*** Effect of curcumin on MCF-7 cell viability.

carcinogenic effect of BDMC-A, an analog of curcumin, has been evaluated on MCF-7, a model for human breast cancer cells.

The MTT assay which measures the mitochondrial activity is an indicative of cell viability. Anti-proliferative study of curcumin and its analog BDMC-A by MTT assay revealed that BDMC-A inhibits cell proliferation at an equal concentration (IC_{50} at 30 µM) to that of curcumin (IC_{50} at 30 µM). The effect of curcumin on cell proliferation is consistent with previous reports demonstrating that curcumin affects cell proliferation in breast cancer cell lines[15,16]. LDH release assay, cell count assay and flow cytometry analysis confirmed the death of MCF-7 cells which were treated with curcumin and BDMC-A. The flow cytometric studies confirmed an accumulation of cells in the G2/M phase. Both Curcumin (15 µM) and BDMC-A (15 µM) at 12 h arrested the cells in G2/M phase. The cell cycle arrest at G2/M phase by curcumin has already been reported which correlates with our present findings[17]. This arises by a mechanism involving aberrant mitotic spindle assembly and function[18].

Cyclin D1 is a protein which is involved in the cell cycle regulation. It is required to mediate the G_1 to S transition, in turn leading to DNA synthesis and cell cycle progression[19]. The *cyclin D1* gene has been shown to play a critical role in breast carcinogenesis. It is possible that the antiproliferative effects of curcumin are due to inhibition of cyclin

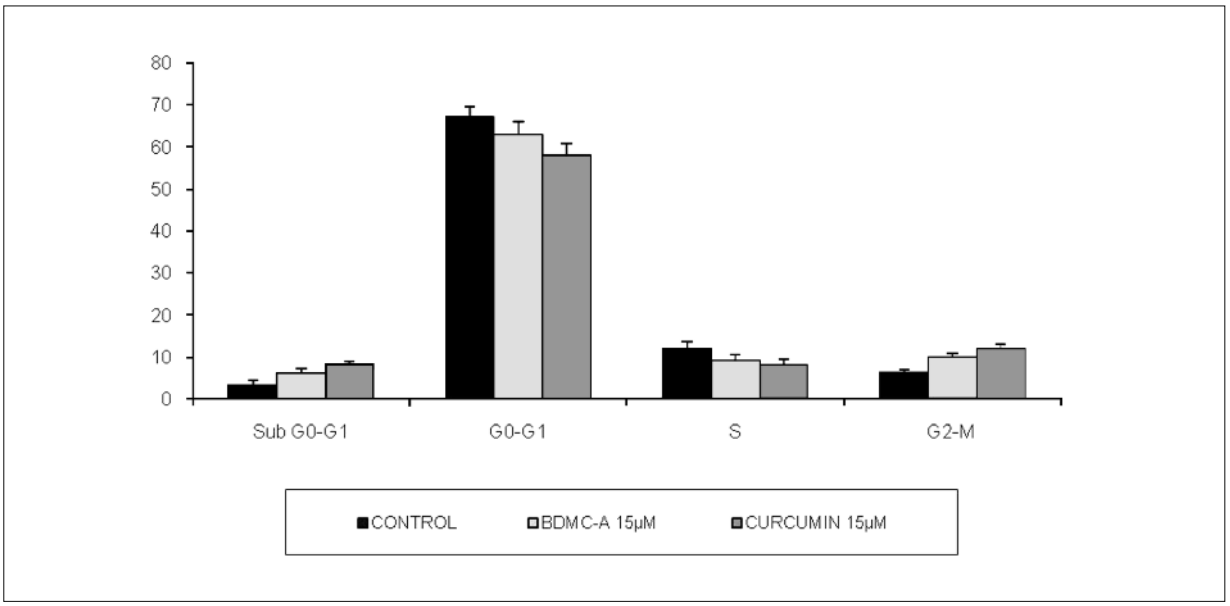


Figure 4. Cell cycle distribution through flow cytometry measured with propidium iodide assay.

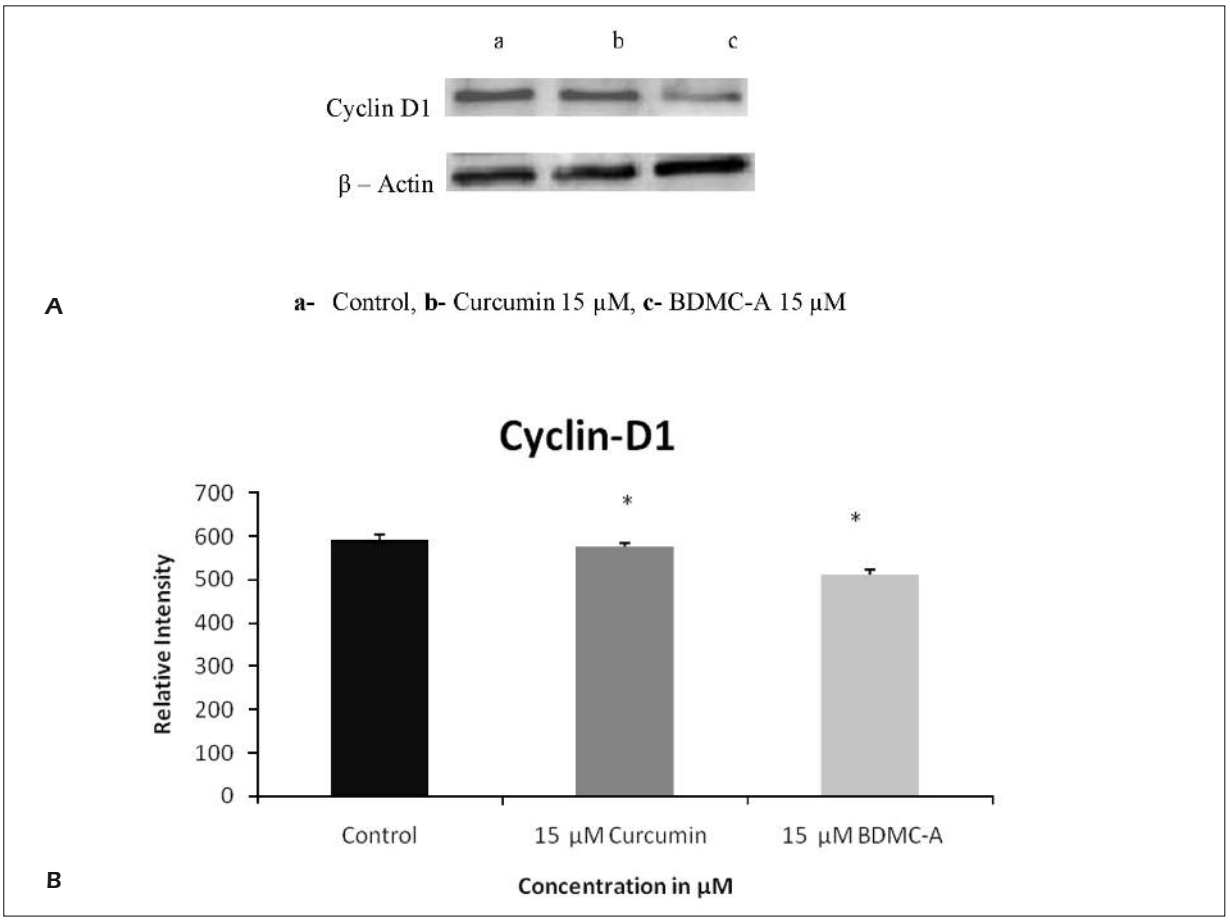


Figure 5. ***A,*** Effect of curcumin and BDMC-A on the expression of cyclin D1 protein in MCF-7. ***B,*** Intensity based quantification of western blot bands were analysed using Bio-Rad ECL Imager using Quantity One software.

D1 expression[20]. In the present study there was a significant decrease in the cyclin D1 protein expression in BDMC-A treatment and it was comparable to that of the decrease in curcumin.

The effective anti-proliferatory effect of BDMC-A may be due to the presence of the ortho hydroxyl group. It is reported that the existence of ortho hydroxyl group in the benzene ring of BDMC-A is critical for the prevention of TPA-induced skin cancer in mice[9]. In one of the reports, BDMC-A has been shown to possess anticarcinogenic property against a model for colon cancer[21].

The inhibition of cell proliferation and induction of cytotoxicity in breast cancer cells by BDMC-A in our study provide evidence for its potential role in breast cancer treatment. Moreover, the solubility of BDMC-A was higher in aqueous medium compared to curcumin. Hence it is expected to be more bioavailable and more active than curcumin. Further evaluation might reveal its role on various molecular targets.

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