



Effect of natural compounds curcumin and isothiocyanate on breast cancer cell lines MCF-7 and MDA –MB 231

Reddy sirigiripeta 1, Sudhakar chekuri 1, Raju sandupatla 2, Rojarani anupalli 1

Department of Genetics, Osmania University, Hyderabad-1

Department of chemistry, Osmania University, Hyderabad-2

1. Abstract:

Breast cancer is a malignant, uncontrolled growth of epithelial cells lining the ducts or lobules of breast tissue arising through a series of molecular mutations at the cellular level. It is the most commonly occurring cancer and the leading cause of cancer death in females worldwide. In 2008 alone, 1.38 million new cases of breast cancer were diagnosed and 458,400 women died of breast cancer. The causes of most breast cancer cases remain to be unknown. However, recent years have shown numerous advances in the identification of various risk factors including gender, environment, hormones, genetic factors, and lifestyle. The incidence of breast cancer among women may increase with advancing age, late age at first birth (≥ 30), the use of postmenopausal hormone replacement therapy, prior family history, higher dietary fat intake, obesity, and alcohol intake (Michaud et al., 2008). *Curcumin* is the principal active component of Indian curry spice turmeric and has been found to exert preventive and therapeutic effects in various cancers. Apart from this cruciferous vegetable compound isothiocyanates shown therapeutic effects. This is, in part, due to its ability to influence a diverse range of molecular targets and signaling pathways the effect of the dimethoxycurcumin and benzyl isothiocyanates will be tested on MCF-7 and MDA MB231 cell lines.

Keywords: benzyl isothiocyanate, dimethoxy curcumin , MDA-MB-231

2. Introduction:

Cancer is a class of diseases or disorders characterized by uncontrolled cell growth and/or defects in critical control mechanisms (like growth arrest or ability to undergo apoptosis). Furthermore, cancer cells are characterized by their ability to spread, either by direct growth into adjacent tissue through invasion, or by implantation into distant sites by metastasis. The unregulated growth that characterizes cancer is caused by mutations in genes that encode proteins controlling cell homeostasis. In recent years, cancers reported in females have become more common in developing countries. Annually, 10.9 million people suffer from breast cancer worldwide that result in about 6.7 million deaths from the disease.

Breast cancer is a malignant, uncontrolled growth of epithelial cells lining the ducts or lobules of breast tissue arising through a series of molecular mutations at the cellular level (Lippmann, 2005). It is the most commonly occurring cancer and the leading cause of cancer death in females worldwide. In 2008 alone, 1.38 million new cases of breast cancer were diagnosed and 458,400 women died of breast cancer (Jemal et al., 2011). The causes of most breast cancer cases remain to be unknown. However, recent years have shown

numerous advances in the identification of various risk factors including gender, environment, hormones, genetic factors, and lifestyle (Michaud et al., 2008). The incidence of breast cancer among women may increase with advancing age, late age at first birth (≥ 30), the use of postmenopausal hormone replacement therapy, prior family history, higher dietary fat intake, obesity, and alcohol intake (Michaud et al., 2008). **Curcumin** is the principal active component of Indian curry spice turmeric and has been found to exert preventive and therapeutic effects in various cancers. This is, in part, due to its ability to influence a diverse range of molecular targets and signalling pathways. **Isothiocyanates** are the horizon of next generation targeted therapy with curcumin in breast cancer. Isothiocyanates (ITC), originating from the enzymatic degradation of glucosinolates (GSL), which are secondary metabolites of Brassicaceae plants, exhibit anticarcinogenic properties due to their ability to induce cytoprotective genes. Effect of the curcumin and isothiocyanates will be tested on MCF-7 and MDA-MB-231 cell lines. Morphological Assessment and cytotoxic nature of these compounds and cell viability was done using cell viability assays like MTT assay.

3. METHODOLOGY:

a) Materials:

The MCF-7 and MDA MB 231 cell lines were purchased from NCCS (PUNE) whereas the reagents for cell line growth namely phosphate-buffered saline (PBS), Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin mix (10000 units/mL penicillin and 10000 $\mu\text{g/mL}$ streptomycin), Fungizone Amphotericin (250 $\mu\text{g/mL}$), Trypsin in ethylenediaminetetraacetic acid (EDTA) solution (0.05%) were purchased from Global services (Hydrabad, India). The cell grade plastic wear and chemical reagents were purchased from Fisher Scientific (Loughborough, UK) or Sigma-Aldrich (Dorset, UK). Cell culture procedures were carried out in a micro-airflow biosafety cabinet level II. The cells were maintained using carbon dioxide (CO_2) incubator maintained at 37°C , 5% CO_2 under humidified conditions and cells were centrifuged using REMI (at 1500rpm) centrifuge. Dimethyl sulfoxide (DMSO) $>99.9\%$ purity (HPLC grade) used for stock preparation/dilution. The plant compounds dimethoxy curcumin and benzyl isothiocyanates were purchased from HiMedia.

The other reagents like DAPI (4',6-Diamidino-2-phenylindole) for morphological assessment of cells was also purchased from Himedia. Major equipments like inverted digital microscope (Life Technologies EVOS FL) and Microplate reader iMark (Biorad) were used.

b) Preparation of media and reagents:

Preparation of media:

Media was prepared by adding 50mL FBS, 5mL penicillin/streptomycin mix and 5mL Fungizone Amphotericin into each of the 500mL phenol red free and phenol red DMEM containing 4.5g/L D-glucose, L-glutamine and pyruvate.

Preparation of drug stocks:

Stock of 10mM was prepared in 1mL DMSO which was then serially diluted to give stocks of 0.1mM 1 μM , 3 μM , 5 μM , 10 μM and 15 μM are prepared. Like this different concentrations for both dimethoxy curcumin and benzyl isothiocyanates were prepared. and stored at -20°C .

Preparation of reagents for cell staining:

Paraformaldehyde, 4% w/v, was prepared by dissolving 4g of paraformaldehyde in 100mL of PBS at 60°C . Sodium hydroxide solution of 1M concentration was then prepared by dissolving 0.4g in 10mL which was then added dropwise into the paraformaldehyde solution until the pH was 7.4.

Triton X-100, 0.2% v/v, was prepared by adding 100 μL of Triton X-100 into 50mL double distilled water. DAPI stock was prepared by dissolving 1mg of DAPI in 1mL DMSO. It was then further diluted by 100 fold in glycerol.

c) Cell Culture:

ER positive cell line, MCF-7, and MDA MB-231 were maintained in T-75 flasks with DMEM containing 10% heat inactivated FBS, 1% Fungizone and 1% penicillin/streptomycin at 37°C in a humidified 5% CO_2 atmosphere. For subculturing, on reaching a confluence of $\approx 80\%$, the media was removed from the flask and 2 ml PBS was added. After discarding the PBS, 2mL trypsin was added and the flask was kept in the incubator for 2min at 37°C in a humidified 5% CO_2 atmosphere after which 3mL DMEM was added and mixed thoroughly. The cell suspension was then placed in a fresh tube and centrifuged at 1200rpm for 5min. The pellet was then re-suspended in 1mL of phenol red free DMEM and seeded into two new T-75 flask

containing 15mL DMEM, each with splitting ratio of 1:2 by adding 500 μ L of the cell suspension. The flasks were then incubated at 37°C in a humidified 5% CO₂ atmosphere.

Cell harvesting and counting:

On reaching a confluence of \approx 80%, cells from the flask were placed in a fresh tube and 1mL of cell suspension made following the procedure described above. The cell suspension of 10 μ L was added to 20 μ L trypan blue solution in a separate tube and mixed thoroughly, placed under the glass slide on the haemocytometer and cells counted under light microscope to determine the cell concentration.

Optimisation of cells and drug concentrations:

96 well plates were set up with MCF-7 and MDA MB 231 cell lines concentrations of 5X10⁴ cells/mL/well and 1X10⁵ cells/mL/well in 4mL DMEM with E2 (DMSO concentration was less than 1% in the media). The plates were then incubated at 37°C in a humidified 5% CO₂ atmosphere and checked for confluence under light microscope.

To evaluate the optimum drug concentration to be used, 96 well plate was set up at cell concentration of 1X10⁵ cells/mL/well with different drug concentrations of 1 μ M, 2 μ M, 3 μ M, 5 μ M, 10 μ M, 15 μ M and control without any drug.

Optimisation of incubation time:

To optimise the incubation time, 96 well plates with MCF-7 cells and MDA MB231 cells were incubated at 37°C in a humidified 5% CO₂ atmosphere for 24 hrs and 48 hrs with treated and untreated control in duplicate. The cells were replenished with media after every 48 hours.

Cell proliferation study:

96 plates were set up with MCF-7 and MDA MB231 cells with concentrations of 1X10⁵ cells/mL/well in 4mL phenol red free DMEM with both benzyl isothiocyanate and dimethoxy curcumin with different concentrations and control in duplicate. Cells were allowed to adhere for 24h before being exposed to both drugs in different plates. After 2 days of incubation at 37°C in a humidified 5% CO₂ atmosphere, media from the wells was removed and 600 μ L trypsin added for 2min followed by 1mL phenol red free DMEM and mixed thoroughly. The cell suspension was placed in a new tube and centrifuged at 1200rpm for 5min. The pellet was then re-suspended in 1mL media from which 10 μ L of the cell suspension was added to 20 μ L trypan blue solution in a separate tube and cell count determined following the procedure described above.

d) Morphological assessment of cells

Morphological assessment was done by using DAPI stain (Cell permeable fluorescent minor groove-binding probe for DNA. Binds to the minor groove of double-stranded DNA (preferentially to AT rich DNA)

A six well plate was set up with each well containing a cover slip and final drug concentrations of 1 μ M, 2 μ M, 3 μ M, 5 μ M, 10 μ M, 15 μ M as described above. The plate was then incubated for 96h after which media was aspirated and cells rinsed three times with 1mL PBS. After incubating in 1mL 4% paraformaldehyde for 10min cells were then rinsed again in 1mL PBS for three times each lasting 5min. Following this, cells were permeabilised by immersing in 1mL 0.2% Triton X-100 for 5min and rinsed twice with 1mL PBS. Three glass slides were prepared with a drop of DAPI solution for each sample and the cover slip was then placed over the drop upside down using forceps. The slides were kept in the dark overnight and viewed under the inverted fluorescence microscope at a magnification of X20.

e) Cell viability assay by using MTT:

Plated and cultured MCF-7 and MDA MB231 cells (100 μ L per well) in a clear bottom 96-well tissue culture plates. (The number of cells were 10⁵ cells per well). Added test samples in different concentrations ranging from 5 to 50 μ M in triplicate after 24 hr of seeding and incubated the cells for 24 hr's, of time. A volume of 20 μ L culture medium was used for the all test samples. Removed the medium and washed cells with PBS twice. Added 15 μ L MTT reagent per well which was made up in PBS medium to a final concentration of 0.5 mg/mL. The volume of the reagent should be adjusted depending on the volume of cell culture. Cells were incubated for 3 hours at 37°C until intracellular purple formazan crystals are visible under microscope. Removed MTT reagent and added 100 μ L of the DMSO to each well and mixed gently on an orbital shaker

for one hour at room temperature. The volume of DMSO should be adjusted depending on the volume of cell culture. Measured the absorbance at OD570nm for each well on an absorbance plate reader.

4. Results:

To determine the growth inhibitory effects of curcumin and benzyl isothiocyanate on MCF-7 and MDA MB 231 cells and to evaluate the IC₅₀ value, MTT assay was performed and different concentrations of dimethoxy curcumin and benzyl isothiocyanate (0, 10, 20, 30, 40 and 50 (In micro molar concentration)) were used.

4. A) Morphological assessment of cells by using DAPI

Figure 1

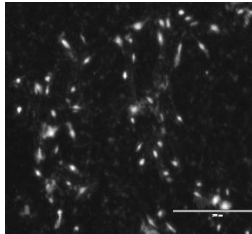


Figure 2

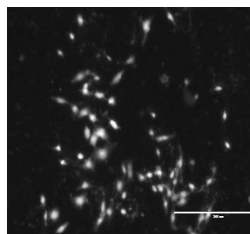


Figure 3

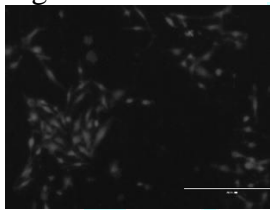


Figure 4

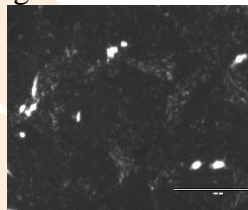


Figure 1 – Showing apoptosis when MCF-7 cells treated with dimethoxy curcumin (20 μM)

Figure 2- MCF-7 cells treated with benzyl isothiocyanate (10 μM)

Figure 3 – MDA MB 231 cells treated with dimethoxy curcumin 20 μM

Figure 4 - MDA MB 231 cells treated with benzyl isothiocyanate (10 μM)

Figure 5

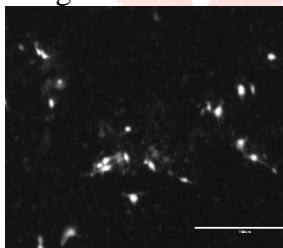


Figure 6

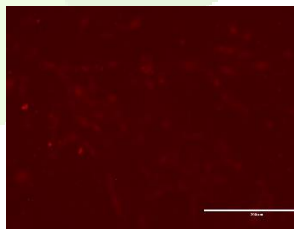


Figure 7

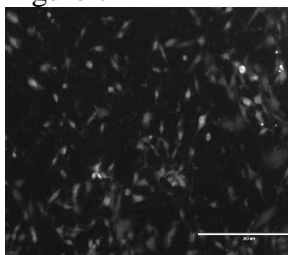


Figure 8

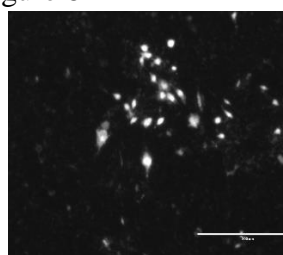


Figure 5- showing apoptosis when MCF 7 Cells treated with Dimethoxy curcumin (15 μM)

Figure 6- MDA MB 231 with benzyl isothiocyanate

Figure 7- MCF 7 cells without drug (control)

Figure-8- MDA MB 231 with benzyl isothiocyanate

Morphology of MCF-7 cells

Figure 9

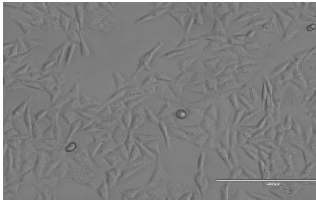


Figure 10

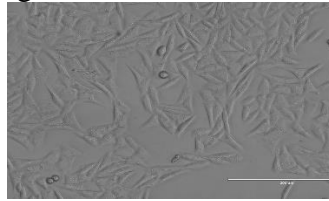


Figure 11

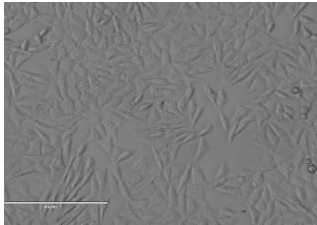


Figure 12

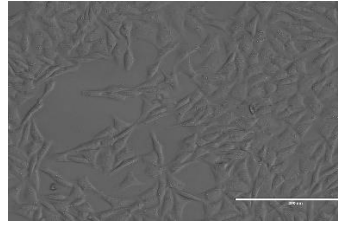


Figure 9- MCF-7 Cells after 24 hrs incubation

Figure 10- MCF-7 Cells after 24 hrs incubation

Figure 11- MCF 7 Cells after 48 hrs incubation

Figure-12- MCF-7 Cells after 48 hrs incubation

Morphology of MDA MB -231 Cells

Figure 13

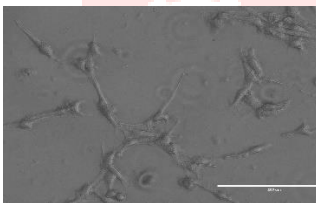


Figure 14

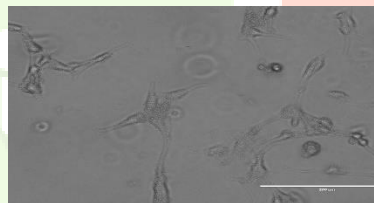


Figure 13- MDA MB 231 Cells after 24 hrs incubation

Figure 14- MDA MB 231 Cells after 24 hrs incubation

After addition of compound

Figure 15

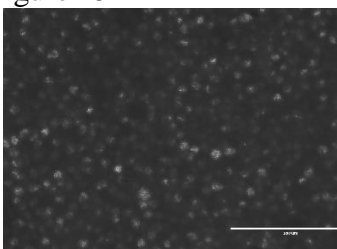


Figure 16



Figure 17

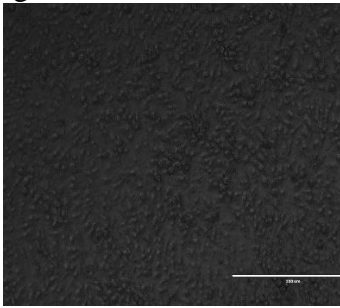


Figure 18

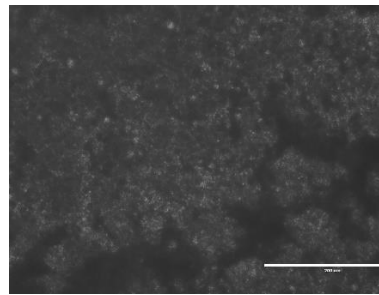


Figure 15- MCF-7 cells treated with dimethoxy curcumin (30µM)
 Figure 16- MDA MB 231 Cells treated with dimethoxy curcumin (20µM)
 Figure 17- MCF-7 cells treated with benzylisothiocyanate(15µM)
 Figure-18- MDA MB 231 Cells treated with benzylisothiocyanate(30µM)
Formation of formasan crystals after addition of MTT dye in the wells

Figure 19

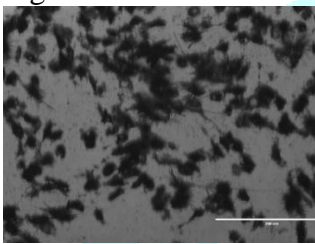
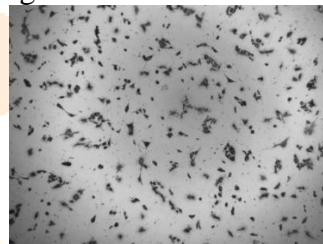


Figure 20



4. B) cell viability determination:

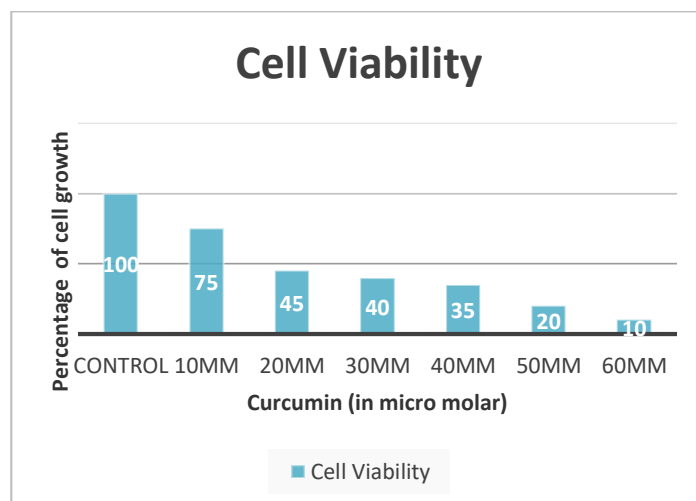
MCF-7 Cells treated with dimethoxy curcumin in different concentrations

(After 24 hours)

Table 1

DimethoxyCurcumin	Cell Viability (%)
Control	100
10µM	75
20µM	45
30µM	40
40µM	35
50µM	20
60µM	10

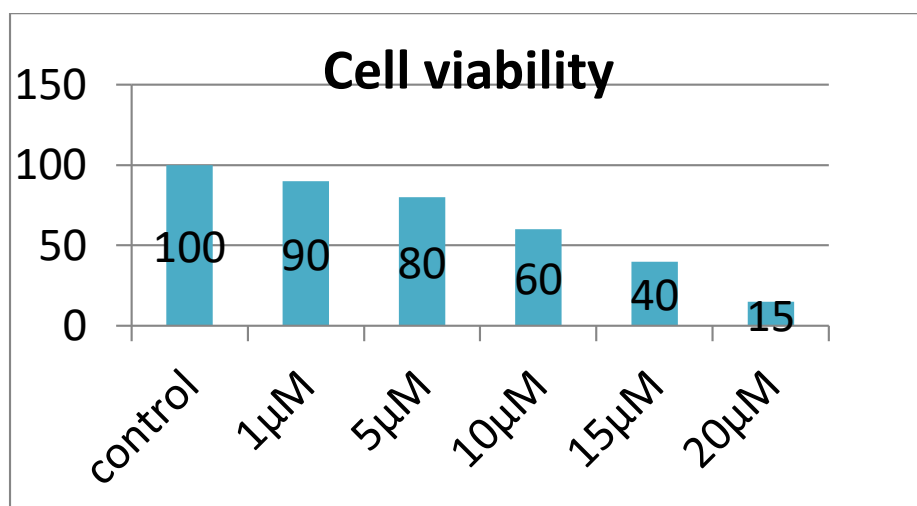
Figure 21: describes the cytotoxic effect of dimethoxy curcumin on MCF- Cells (24h)



MCF-7 Cell lines treated with benzyl isothiocyanates**Table2**

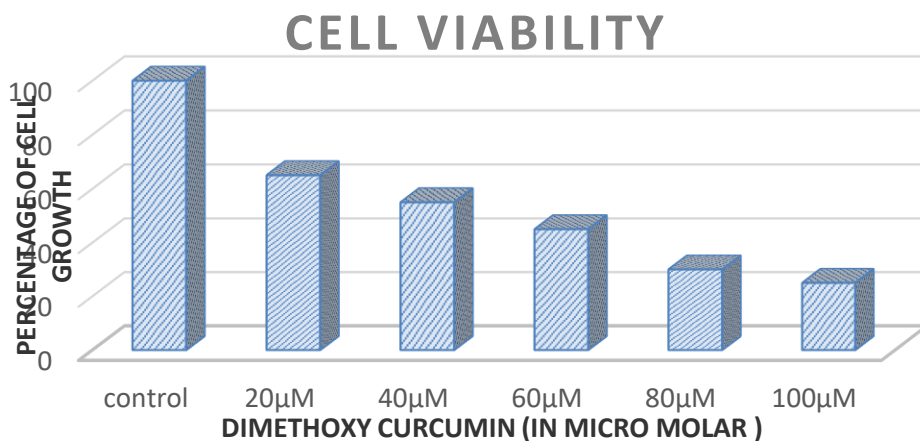
Isothiocyana te in micro molar	Cell viability
control	100
1 μ M	90
5 μ M	80
10 μ M	60
15 μ M	40
20 μ M	15

Figure 22: Describes the cytotoxic effect of isothiocyanate compounds on MCF-7Cell proliferation (24h)

Benzyl isothiocyanate in combinations (in μ M concentration)**MDA MB-231 cells treated with dimethoxy curcumin in different concentrations (After 24 hrs)Table 3**

Drug concentration (in micro molar)	Cell viability (in %)
control	100
20 μ M	65
40 μ M	55
60 μ M	45
80 μ M	30
100 μ M	25

Figure 23: cytotoxic effect of dimethoxy curcumin on MDA MB 231 Cells (24h)

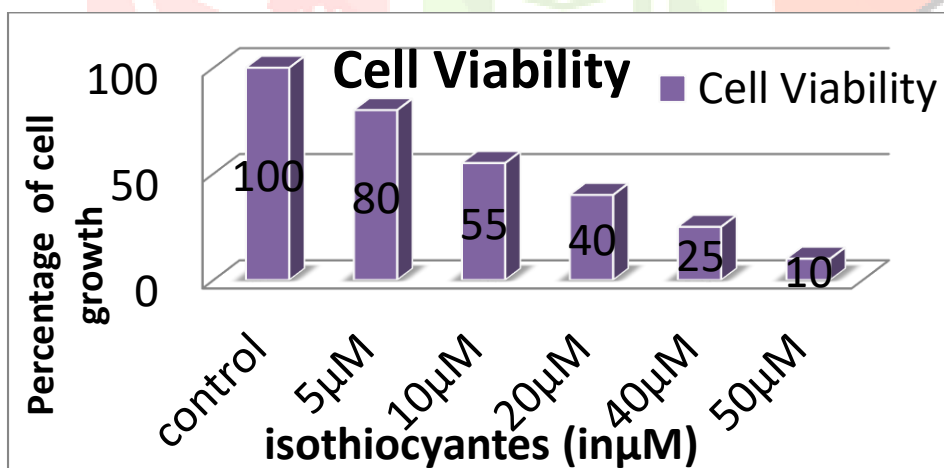


MDAMB-231 Cells treated with benzyl isothiocyanates for 24 h

Table 4

Curcumin	Cell Viability (%)
control	100
5µM	80
10µM	55
20µM	40
40µM	25
50µM	10

Figure 24: Describes the cytotoxic effect of isothiocyanate compounds on MDAMB-231 Cell proliferation(24h)



Discussion:

From the first objective we now know the importance of cruciferous vegetable compound benzyl isothiocyanate and dimethoxy curcumin in its crucial role in breast cancer treatment. this study has shown that moderate concentration of dimethoxy curcumin and low concentration of both benzyl isothiocyanate can induce apoptosis in the breast cancer cell line MCF-7 and MDA MB231, but the combinations of both the compounds benzyl isothiocyanate and dimethoxy curcumin at a low concentrations was found to stop the proliferation and triggers more apoptosis in breast cancer cell lines, which is a new property of these compounds to be uncovered showing its potential application as therapeutics in the treatment of breast carcinoma. This should be an important issue for further research evaluating the clinical benefit. MCF-7 and

MDA MB231 cells at passage 3 with continuous drug exposure for 24 hrs had significantly down regulated its proliferation when compared to both control and positive control (doxorubicin).

In the second objective morphological assessment was carried out by using DAPI (4',6-diamidino-2-phenylindole) which binds to minor groove (AT rich regions) of DNA. By using DAPI Stain the apoptosis was clearly observed in breast cancer cell lines treated with different concentrations. Untreated cells were found to possess normal morphology with intact nuclei whereas the cells at low concentrations (10 μ M and 15 μ M) exhibited the characteristics of apoptosis with condensed and fragmented nuclei.

Conclusion:

From the above graphs it is concluded that the IC 50 (concentration of a particular drug at which 50 % of desired target is inhibited) values of dimethoxy curcumin and benzyl isothiocyanates are as follows

IC 50 values of dimethoxy curcumin are 15 μ M and 20 μ M

IC 50 value of benzyl isothiocyanate is 10 μ M

In summary, curcumin and benzyl isothiocyanate induce cytotoxic potential in both MCF-7 and MDA MB-231 cell lines and observed morphological changes. From the above work it is concluded that the selected drugs showing anti-cancer activity on breast cancer cell lines MCF-7 and MDA-MB 231 and the selected drugs can be preferred for clinical trials of breast cancer.

Acknowledgments

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Ethical issues

No ethical issues to be declared.

Competing interests

The authors declare no competing interests.

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