THERAPEUTIC OPTIONS FOR THE TREATMENT OF BREAST CANCER: USING CYTOREG AND GENISTEIN ISOFLAVONE

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by

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This thesis was prepared under the directions of the candidate's thesis advisor, Dr. James Kumi-Diaka, Department of Biological Sciences, and has been approved by the members of her supervisory committee. It was submitted to the faculty of The Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

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Date

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ABSTRACT

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Title:	Therapeutic Options for the Treatment of Breast Cancer:
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In spite the heavy investments in therapeutic research breast cancer still impacts the lives of women globally. The projected incidence of new cases in USA for 2008 is 67,770, with estimated 40,480 deaths. In this study, we investigated the therapeutic efficacy of Cytoreg®-genistein combination treatment on MCF-7 human breast cancer cells. MCF-7 cells were treated with genistein and Cytoreg® single and combination treatments for 24-48hr; and the chemosensitivity assessed using bioassays: Trypan Blue and MTT for cell viability; Ethidium bromide/Rhodamine123 to assess apoptosis induction; FAM Poly-Caspase binding assay for mechanism of action. The overall data indicated dose- and time-dependent cell death in the MCF-cells and that apoptosis was the major means of treatment-induced growth inhibition. There was evidence of Cytoreg®-induced autophagy in the cells. The overall findings indicated that genistein-Cytoreg® combination was more efficacious than either genistein or Cytoreg® alone. Cytoreg® enhanced the phytosensitivity of MCF-7 cells to genistein isoflavone.

To Thomas, Vinnie, Bromley (grandpa), and Mavis you will remain in my heart always,

I am grateful to have had you all in my life.

Mummy I owe all that I am to you

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INTRODUCTION

Breast cancer is the most frequently diagnosed cancer in women and is three times more common than all gynecologic malignancies combined. The incidence of breast cancer has been increasing steadily from 1:20 in 1960 to1:7 women in 2006 and presently projected to increase to 1:8 women in 2008 (Parkin and Fernandez, 2006; Basler, 2008). While the risk of breast cancer increases with age, all women are at risk of getting breast cancer. Of women who live to age 85, one woman in eight will develop breast cancer during her lifetime (Parkin and Fernandez, 2006).

According to the American Cancer Society and SEER (Surveillance Epidemiology and End Results), the overall incidence of breast cancer in men was 1.2 per 100,000, compared to 128 per 100,000 in women (Ries et al., 2006; Smith et al., 2006). The differences in mortality were equally wide: 0.3 per 100,000 in men; 26 per 100,000 in women (Ries et al., 2006; Smith et al., 2006). An estimated 182,460 women will be diagnosed with breast cancer in 2008 (Ries et al., 2006; Smith et al., 2006; Jemal, 2008). Of this number, approximately 67,770 new cases of in situ breast cancer are expected to occur and an estimated 40,480 women will die of the disease in 2008 (Smith et al., 2006; Jemal 2008). Ductal carcinoma in situ (DCIS) is the most frequently expressed form of breast cancer, constituting approximately 85% of the new cases diagnosed today.

The United States age-adjusted cancer mortality rate was 25.8 per 100,000 women per year (Parkin and Fernandez, 2006; Smith et al., 2006; Hwang et al., 2007; Jemal et al., 2008). This rate is based on mortality data from 2000 through to 2003. Death rates by race were: 25.3 per 100,000 Caucasian women, 34.3 per 100,000 Black women, 12.6 per 100,000 women of Asian/Pacific heritage, 13.4 per 100,000 American Indian/Alaska Native women and Hispanic ~ 16.2 per 100,000 women of Hispanic descent (Parkin and Fernandez, 2006; Ries et al., 2006; Smith et al., 2006; Hwang et al., 2007; Jemal et al., 2008).

An estimated 19,010 new cases of breast cancer are expected to occur among African American women in 2008 (Jemal et al., 2008). The incidence of breast cancer in African American women is approximately 12% lower than in Caucasian women yet, African American women have a 36% higher mortality rate than Caucasian women (Jemal et al., 2008). Conversely, the incidence of breast cancer among African American women under the age of 40 is higher than among Caucasian women of the same age (Jemal et al., 2008). Approximately 5,830 deaths are projected to occur from breast cancer among African American women in 2008 (Jemal et al., 2008). Contributing factors offered to explain the higher mortality among African American women compared to Caucasian women include: dissimilarities in access to and utilization of early detection and treatment technology, race associated biological differences, and risk factors which are distributed differentially by race or socioeconomic status (Parkin and Fernandez, 2006; Ries et al, 2006; Smith et al., 2006; Hwang et al., 2007; Jemal et al., 2008).

The breast is a glandular organ which consists of connective tissue, fat, and breast tissue that contains mammary glands necessary for milk production. The main functional purpose of the breast in any mammal is milk production for the offspring. Each breast has a nipple which is surrounded by an areola. The areola's color is determined by the individual's skin-tone and consequently, it may vary from pink to brown. Mammary glands are comprised of lobules. Each breast has approximately 10-20 lactiferous ducts that drain milk from the lobules to the nipple, where each duct has its own opening (McKinley and O'Loughlin, 2006).

The most common type of breast cancer originates either in the mammary ducts (ductal carcinoma) or in the lobules (lobular carcinoma). Breast cancer and cancer in general are actually a complex family of diseases. The clinical definition of cancer is a large group of diseases which vary with age of onset, growth rate, status of cellular differentiation, diagnostic detectability, invasiveness, metastatic potential, treatment response, and prognosis (Ruddon, 1995; Conklin et al., 2007). Cancer arises from a series of mutations of cellular DNA, leading to unregulated growth and proliferation of cells.

The point of origin of a tumor is determined by the microscopic appearance of the cancer cells from a biopsy. Obtaining cancer cells from a biopsy is essential in determining the type of cancer and consequently, selecting the appropriate treatment (Ruddon, 1995). If cancer is present and is localized with its origination, this is referred to as "in situ" breast cancer. Two forms of "in situ" carcinomas exist: Ductal Carcinoma in Situ (DCIS) which is considered to be a precancerous condition and Lobular Carcinoma in Situ (LCIS) which, if present, increases the risk of developing breast cancer (Ruddon, 1995).

Another form of breast cancer is invasive breast carcinoma. Here, the cancer cells metastasize from their point of origin and invade the surrounding tissues that normally lend support to the ducts and lobules of the breast. This form of breast cancer is capable of spreading to other areas of the body, mainly the lymph nodes. Two types of invasive carcinoma exist: (i) invasive ductal carcinoma (IDC) which can spread through the blood stream or lymphatic system and (ii) invasive lobular carcinoma (ILC) which is similar to IDC with respect to method of infiltration (Arpino et al., 2004; Anderson et al., 2004). However, ILC may not be easily detectable and consequently is more difficult to diagnose by conventional methods such as mammography. Other forms of breast cancer include: inflammatory breast cancer, medullary carcinoma, mucinous (colloid) carcinoma, Paget's disease of the breast, and tubular carcinoma (Ruddon, 1995; Anderson et al., 2004).

The factors predicting the development of breast cancer are poorly understood and it is difficult to determine why one individual is more susceptible to developing breast cancer than another (Ruddon, 1995; Ramanakumar, 2007). However, potential risk factors have been identified which may contribute to the increased likelihood of a woman developing breast cancer. Possible risk factors include: environmental factors, lifestyle, race/ethnicity, age, genetic predisposition (only 5-10% of breast cancer cases are genetic) and medical (Ruddon, 1995). Epidemiologists have also demonstrated an apparent relationship between cancers and exposures to extreme weather conditions, proximity to chemical and fertilizer poisons and potential mechanical hazards (Ramanakumar, 2007).

Environmental factors are many and may include high doses of radiation to the chest before age 35, exposure to chemical/fertilizer poisons or contact with carcinogenic

materials introduced either through accidental exposure, treatment or occupational exposure ((Ruddon, 1995). Lifestyle factors include smoking, excessive alcohol intake, poor nutrition (eating foods high in saturated fats) and inactivity (limited or no exercise). Women of higher socioeconomic status are more likely to develop breast cancer. This is attributed to several lifestyle factors including: nulliparity, having children after age 30, or not breast feeding. Additional factors that increase the likelihood of developing cancer are early menarche (before age 12 years) or late menopause (age 55 and older) (McKinley and O'Loughlin, 2006; Goss and Strasser-Weippl, 2004).

Only 5-10% of the breast cancers that develop are a result of genetic predisposition (Bouker and Hilakivi-Clarke, 2000; Rowell et al., 2005; Anderson et al., 2006). Current research is directed towards studying genes that are associated with aggressive forms of breast cancer. The presence of these genes is thought to contribute to an increased risk for breast cancer. Mutations in the BRCA1 and BRCA2 genes are the most common and specific genetic factors in breast cancer (Ruddon, 1995). Although women with BRCA mutations have an increased risk of developing breast cancer it is important to note that some women with the BRCA gene mutation never develop breast cancer. This indicates additional unknown factors exist which contribute to the development of cancer in those individuals with the BRCA mutations (Ruddon, 1995).

Another gene of potential causative importance is the HER2 gene. HER2/*neu* (c-erbB-2) is a protooncogene which is amplified and/or over-expressed in approximately 30% of human breast cancer (Mukai et al., 2002; Kumi-Diaka et al., 2006). Studies have shown that like the BRCA1 and BRCA2 genes, the HER2/*neu* gene is associated with biologically aggressive cells (Mukai et al., 2002; Kumi-Diaka et al., 2006).

Breast cancer is considered a heterogeneous disease meaning the cancer varies between women of different age groups and cell populations within the tumor itself. The incidence of breast cancer increases with age; plateaus at the age of forty-five, then increases dramatically after age fifty (Ozanne et al., 2006). Generally, older women have a less aggressive form of the disease than younger women. Fifty percent of breast cancer is diagnosed in women over sixty-five; indicating the ongoing necessity of yearly screening throughout a woman's life. Autopsy studies show 2% of the population has undiagnosed breast cancer at the time of death (Parkin and Fernandez, 2006; Ozanne et al., 2006; Hwang et al., 2007).

Chemotherapy, Radiation therapy and Surgery are conventional forms of treatment today. However, these treatments come with mild, severe and or fatal side effects due to cytotoxicity. Currently attention is given to phytochemicals as phytotherapeutics or adjuvants to chemotherapy and radiation therapy (Hamelers et al., 2003). Two therapeutic agents currently under study are genestein isoflavone and the chemical Cytoreg®. A brief discussion of genistein is presented to demonstrate its anti-tumor activities.

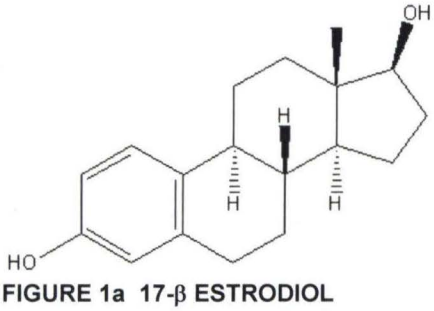

FIGURE 1a 17-β ESTRODIOL

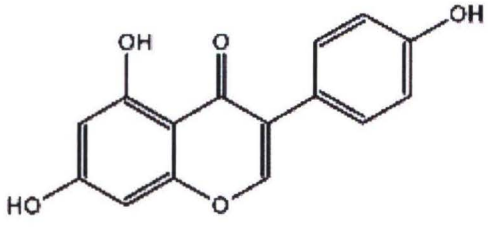

FIGURE 1b GENISTEIN

Genistein is an isoflavone found most commonly in soy products and holds great promise as a natural cancer preventative agent. Isoflavones are multipurpose biochemicals that have several functions in the soy plant. They contribute color to the soybean, protect the plant against bacterial and fungal infections, and serve a hormone-like role (as a phytoestrogen) in plant cell regulation. Isoflavones benefit humans in four ways: as estrogens and antiestrogens, as cancer-enzyme inhibitors, as antioxidants, and as immune system enhancers or stimulants (Chen et al., 2003; Conklin et al., 2006). Isoflavones also possess non-hormonal properties that are associated with the inhibition of cancer cell growth (Messina et al., 2006). Of the numerous isoflavones found in soy products, genistein is the most potent inhibitor of cancer cell growth and proliferation, as evidenced by clinical and epidemiological studies (Bouker and Hilakivi-Clarke, 2000). The mechanism by which genistein induces cell proliferation is still unclear. Studies involving genistein have shown that phytochemicals can act in conjunction with other agents to inhibit the growth of cancer cells (Sakamoto, 2000; Khoshyomn et al., 2002). The focus of the present study is on the phytochemical genistein isoflavone.

Genistein is a solid substance that is insoluble in water. The molecular formula for genistein is $C_{15}H_{10}O_5$, and its molecular weight is 270.24 daltons. Genistein is also known as 5, 7-dihydroxy-3- (4-hydroxyphenyl)-4*H*-1-benzopyran-4-one, and 4', 5, 7-trihydroxy- isoflavone. Genistin is the 7-beta glucoside of genistein, and has greater water solubility than genistein (Fukutake et al., 1996; Dixon and Daneel, 2002; Steer et al., 2003). Genistein displays structural similarities to estrogen as seen in the molecular structures in the figures above. As such, genistein can bind to estrogen receptors, regulate gene expression and display both estrogen agonist and antagonist properties (Chen et al.,

2003; Maggiolini et. al., 2002; Yang et al., 2007). This ability may allow genistein to control cell growth, thus preventing growth and proliferation of hormone dependent tumors (Kumi-Diaka et al., 2006).

Genistein displays several mechanisms of action which may contribute to its anticancer properties, namely: apoptosis upregulation, angiogenesis inhibition, DNA topoisomerase II inhibition and protein tyrosine kinase inhibition (Chen et al., 2003). Existing data shows that genistein also exhibits a concentration dependent bi-phasic effect of ER-α positive/estrogen-dependent cells such as MCF-7 (Yang et al., 2007).

The MCF-7 cell line is a HER2/*neu* positive human breast cancer cell. Genistein has been shown to cause dose- and time- dependent increases in BRCA1 and BRCA2 protein levels in MCF-7 (the estrogen-dependent breast cancer cell line) (Fan, 2006). The implication of this is that caspase-3-deficiency in MCF-7 cells could partly account for MCF-7 resistance to genistein (Yang et al., 2007). A significant association between genistein-induced apoptosis and caspase protease expression in cancer cells has also been reported (Li et al., 1999; Nakagawa et al., 2000; Linford and Dorsa, 2002). The caspases are a group of cysteine proteases that regulate apoptotic execution (Porter and Janicke, 1999). Eleven caspases have been recognized in humans, three of which are effector caspases that result in programmed cell death. They include CASP3, CASP6 and CASP7. CASP-3 is an essential member of the caspase family. Functional caspase-3 is vital to genistein-induced apoptosis. An important note is CASP-3 can be activated by apoptotic signals from both death receptor and intracellular pathways (Riedl and Shi, 2004). Cytochrome c release appears associated with apoptosis initiated caspase-3 activation (Pei, 2003). The knowledge of caspases and their role in genistein-induced apoptosis is

essential in explaining the apoptotic pathway elicited by MCF-7 breast cancer cells when challenged with genistein.

Another potential anti-tumor agent of interest is a synthetic chemical, Cytoreg®. Cytoreg® is a novel, synthetic, anti-tumor, pro-apoptotic and therapeutic agent with great potential as an anticancer drug. Cytoreg® is a pharmacologically active, low pH acid mixture (pH < 1.0) of which hydrofluoric (HF), hydrochloric and sulfuric acids (H_2SO_4) are active principles (Hassanhi and Izbicka, 2005; Kumi-Diaka et al., 2006).

In addition to being an apoptotic agent; Cytoreg® also acts as a cellular regulator and antioxidant agent (Wilga et al., 2000; Kumi-Diaka et al., 2006; Villalobos, 2005). The ions contributed by Cytoreg® are not crystals and as such are independent of their size with respect to intra and extra cellular transportation. Greater penetration and increased regulation of cellular activity is achieved because Cytoreg® has a low molecular weight and high oxidative activity with consequent, greater efficacy (Villalobos, 2005; Kumi-Diaka et al., 2006).

Cytoreg® functions to stimulate the immune system by the presence of the fluoride ion (Lockshin and Zakeri, 2004; Kumi-Diaka et al., 2006). The drug induces the production of IL-1, IL-6, TNF-α as well as stimulating factors of macrophages and granulocytes (Kumi-Diaka et al., 2006). Induction of IL1 activates T cells and other cytokines. IL-1 functions as a chemo-protective and radio-protective agent, consequently, as a protectant it prevents myelosuppression in animals (Kumi-Diaka et al., 2006). The capacity for destruction of tumor cells is increased with the activation of macrophages, making Cytoreg® an active agent which appears to have immunotherapeutic significance (Lockshin and Zakeri, 2004; Kumi-Diaka et al., 2006).

CASP-3 protease expression induced by Cytoreg® is consistent with apoptosis induction in tumor cells, thus implicating utilization of CASP-3 protease the apoptosis-induction death machinery (Kumi-Diaka et al., 2000).

A characteristic that makes Cytoreg® a desirable therapeutic agent is its ability to cross the cell membrane of both quiescent and proliferating cells. Presently, most therapeutic regimens are able to treat proliferating cells only (Kumi-Diaka et al., 2006). Existing data shows that Cytoreg® exerts a dose- and time-dependent growth inhibition in MCF-7 breast cancer cells with significant differences ($P < 0.05$) in chemosensitivity between the different breast cancer cell lines.

Consistent with the results of an earlier study, Cytoreg® significantly inhibits cell growth and proliferation, through apoptosis induction as the key death pathway in series of human adenocarcinoma cells (Hassanhi IV and Izbicka, 2005; Kumi-Diaka et al., 2006). Furthermore, both hormone sensitive and hormone independent tumors display susceptibility to Cytoreg®-induced growth inhibition, implying that the action of Cytoreg® does not appear to be directly related to hormone regulation activities (Kumi-Diaka et al., 2006).

Previous studies involving genistein and Cytoreg® combination have shown that the presence of Cytoreg® enhanced the therapeutic efficacy of genistein in an additive manner. Genistein-arrested cancer cell growth occurs at the G2/M phase of the cell cycle (Sakamoto, 2000). Cytoreg® enters both proliferating and non-proliferating cells, disrupting the mitochondrial transmembrane, leading to the release of cytochrome c and the initiation of apoptosis. The modus operandi of both treatments differ, and this difference allows them to achieve a higher therapeutic efficacy when used in combination

(Sakamoto, 2000; Khoshyomn et al., 2002). The present study is aimed at elucidating potential anti-tumor properties of Cytoreg® and genistein when used in combination.

HYPOTHESIS/OBJECTIVES

The combination of genistein with Cytoreg® will significantly potentiate the anticancer activity of genistein in MCF-7 breast cancer cells through apoptosis induction.

SPECIFIC AIMS

In Support of this Hypothesis Three Specific Aims were Proposed:

1) To determine the therapeutic efficacy of genistein and Cytoreg® in single treatment MCF-7 human breast cancer cells.
2) To determine the therapeutic efficacy of Cytoreg®-genistein combination compared to single treatments in MCF-7 human breast cancer cells.
3) To determine the mode of cell death and mechanism of action of growth inhibition in MCF-7 human breast cancer cells.

EXPERIMENTAL DESIGN

The cells were grown to 70-80% confluence, harvested by scraping and seeded in a 48 well plate. They were then incubated for 48hr at 5% CO_2, 37°C, and 89% humidity to allow growth and adherence. After incubation the supernatant was discarded and treatments of genistein and Cytoreg® were administered. Six (6) cytotoxic dose ranges were utilized for single and combination treatments. The plates were incubated for 24hr – 48 hr. The plates were prepared for the following bioassay analyses: Trypan Blue exclusion and MTT for viability determination; Ethidium Bromide/Acridine Orange for apoptosis detection; Ethidium Bromide/Rhodamine123 and FAM Polycaspase to determine mechanism of action.

MATERIALS AND METHODS

Cell lines: Normal Human Breast Epithelial Cells (NHBC), and MCF-7 human, breast, adenocarcinoma cell line (Complements of Dr. Kumi-Diaka).

Culture media: RPMI 1640, supplemented with 10% fetal bovine serum, 100 IU/ml of penicillin and 100µg/ml of streptomycin (Sigma Aldrich).

Bioassay materials: MTT, Ethidium Bromide (EtBr), Acridine Orange (AcrO), Rhodamine (Rh123) and FAM Polycaspase (Invitrogen).

Test reagents: Genistein isoflavone (Indofine); Cytoreg® (complement of Cytorex Biosciences Inc.).

Genistein isoflavone (purchased from Indofine Chemical Co, Summerville, NJ, USA) was constituted in DMSO (dimethylsulfoxide) solvent to make a stock solution of 10,000µg/ml, from which aliquots of working concentrations of 10, 20, 30, 40, 50 and 60µg/ml ($G_{10} - G_{60}$) were prepared and stored at –20˚C until used. Cytoreg® was supplied by Cytorex Biosciences Inc. as a stock solution. A series of dilutions were prepared at six cytotoxic dose ranges of 0.0005, 0.001, 0.002, 0.005, 0.01, and 0.02µg/ml ($Cyt_{0.0005} - Cyt_{0.02}$) and stored at 5˚C in the refrigerator, until needed. Combination treatments consisted of varying concentrations of genistein and Cytoreg® as shown below:

Table 1 Chart of combination treatment ($G_{10}/Cyt_{0.0005}$–$G_{60}/Cyt_{0.02}$)

Combinations	1	2	3	4	5	6
Genistein μg/ml	10	20	30	40	50	60
Cytoreg® μg/ml	0.0005	0.001	0.002	0.005	0.01	0.02

MCF-7 cells and NHBC cells were cultured at 37°C, 5% CO_2, and 89% humidity. The cell cultures were allowed to grow to 70-80% confluence for the experiments. MCF-7 cells are well-characterized estrogen receptor positive (ER+) cells, and therefore are useful in vitro model of hormone dependent breast cancer studies. MCF-7 cells are also HER2/*neu* positive, positive for cytokeratin and negative for desmin, endothelin, GFAP (Glial Fibrillary Acidic Protein), neurofilament and vimentin.

CELL CULTURE AND TREATMENT

The cells were allowed to grow to 70-80% confluence, harvested by scraping and seeded in a 48 well plate for 48hr. After incubation the supernatant was discarded and treatments of genistein and Cytoreg® were administered. Six cytotoxic dose ranges were used for single and combination treatments.

Briefly, 1.5 x 10^4 cells (MCF-7 or HBC) in 100μl of media was dispensed into the wells of a 48 well plate in duplicates. The cells were incubated at 5% CO_2, 37°C, and 89% humidity for a total of 36hr to allow growth and adherence. The supernatant was then removed and the cells were treated with varying concentrations of genistein (G_{10} – G_{60}) and Cytoreg® range ($Cyt_{0.0005}$ – $Cyt_{0.02}$). The plates were incubated for 24hr – 48hr and then prepared for bioassays analysis as discussed below:

i) TRYPAN BLUE EXCLUSION ASSAY

The purpose of using this assay was to determine the percentage of viable to non-viable cells before and after treatment at 24hr and 48hr.

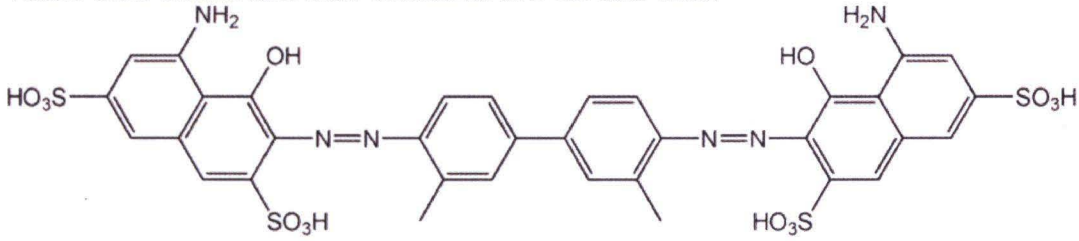

Figure 2 4-hydroxyphenylazobenzene (A diazo dye) (Trypan Blue Structure)

Trypan blue exclusion assay is a cell viability assay which works off the premise that viable cells with intact cell membranes are able to exclude the dye whereas dead or dying cells will absorb the dye because of compromised membrane integrity. Briefly, 20µl of cell suspension was pipetted into a 2ml vial to which 30µl of PBS and 50µl of trypan blue solution was added. The suspension was mixed and allowed to sit at room temperature (25°C) for 1 minute to allow dye uptake. Both chambers of a hemocytometer were loaded with the dye/cell suspension and counted in duplicates under a light microscope at 200x (Olympus BH2). Percentage of viable cells was calculated using the following formula:

$$\text{\% viable cells} = \frac{\text{Number of viable cells}}{\text{Total cell number (live and dead)}} \times 100$$

The percentage viable cells were graphed against the dosage of the treated agents and the LD50 was extrapolated from the graph.

ii) MTT ASSAY

This assay was used to analyze the metabolic state of MCF-7 breast cancer cells and normal human breast epithelial cells.

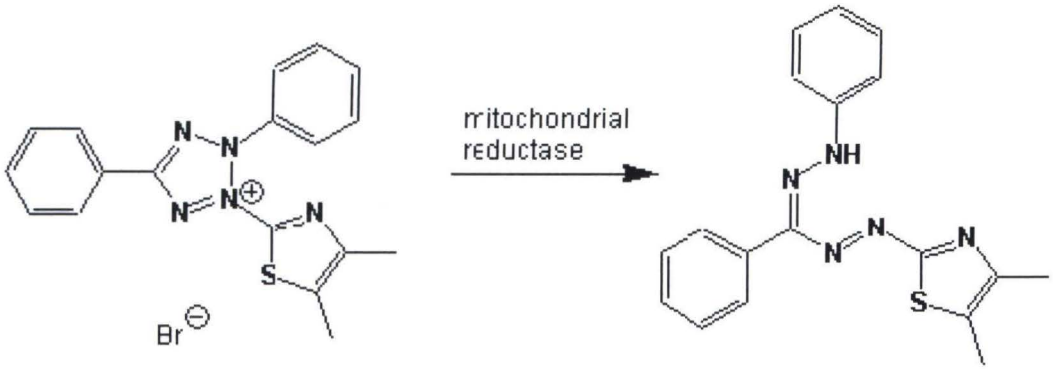

Figure 3 **MTT (soluble)** **FORMAZAN (insoluble)**

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay, assesses the metabolic status of cells. Normal cells contain mitochondrial dehydrogenase enzyme which when exposed to MTT, the enzyme cleaves the tetrazolium rings of the MTT dye and forms dark blue formazan crystals which are impermeable to cell membrane. Consequently, the formazan accumulates within the cells. A direct relationship exists between the number of live cells and the level of formazan production (intensity of the color) (Denizot and Lang, 1986). The intensity of the color can be quantified by using an ELISA plate reader.

Briefly, 50µl of MTT working solution was added to the cell suspension in each well. The cells were incubated for 4 hours at 37°C, 5% CO_2 and 89% humidity. After incubation the contents in each well were aspirated and the cell suspension transferred into microcentrifuge tubes. The cell suspensions were centrifuged for 10 minutes at <400x g. The unincorporated dye-supernatant was discarded and 100µl of MTT lysing solution was added to the pellets in each microcentrifuge tube to solubilize the pellets. A

100µl of the resulting solutions were transferred to a labelled 96-well microtiter plate. The absorbance of each sample was determined using Multiskan biochromatic automated microplate reader (Multiscan, DC) at 590nm. The percentage viable cells were calculated relative to the untreated control, using the formula:

$$\textbf{\% viable cells} = (A_T - A_B)/ (A_C - A_B) \times 100$$

Where A_C = Absorbance of the control (mean value)

A_T = Absorbance of the treated cells (mean value)

A_B = Absorbance of the blank (mean value)

A dose response graph was constructed and the IC_{50} (concentrations reducing the absorbance/OD by 50%) for each treatment was interpolated from the curve.

iii) ETHIDIUM BROMIDE/ACRIDINE ORANGE ASSAY

A fluorescent based assay used to definitively distinguish between viable, apoptotic and necrotic cells as it is based on loss of cell membrane integrity. Acridine orange is able to cross the plasma membrane of all cells as a result, when incorporated into the DNA it stains the nuclei of viable cells green. Conversely, ethidium bromide is excluded from viable and early apoptotic cells as their membranes are still intact. EtBr stains late apoptotic and necrotic cells orange as their plasma membranes are no longer intact. The acridine orange/ethidium bromide assay is more accurate at apoptosis detection than Trypan Blue exclusion as the combination of fluorescent dyes allows the comprehensive categorization of cells. Each component of the combination has a different emission spectrum.

Briefly, a mixture of ethidium bromide (10µl) and acridine orange (75µl) were combined then 1µl of this cocktail was transferred to 25µl of cell suspension. The mixture was incubated in the dark, at room temperature for 2 minutes. Briefly, 10µl of cell suspension was transferred onto a microscope slide and covered with a cover slip. Using a band-pass filter the cells were observed then analyzed under a fluorescent microscope. Apoptosis detection of the stained cells was determined using morphological and fluorescent characteristics. Quantitative analysis of apoptosis was determined by counting cells in several fields then recording the percentage of viable, apoptotic and necrotic cells.

iv) ETHIDIUM BROMIDE-RHODAMINE123 ASSAY

This combination assay was performed to determine treatment induced apoptosis and involvement of mitochondria. EtBr/Rh123 is frequently used as a nucleic acid stain. In this assay the EtBr/Rh123 combination were used as differential stains to distinguish between viable, apoptotic, and necrotic cells. In addition, the assay determines the transmembrane potential of active mitochondria. Rhodamine123 is a fluorescent dye which facilitates the study of cellular mitochondria in situ. It is used to analyze the morphology and function of the mitochondria in an intact viable cell. Rhodamine123 is a cationic fluorochrome that enters the cell through mitochondrial transmembrane potential. Therefore, viable cells containing active mitochondria will absorb the Rh123 dye and emit a green fluorescence, apoptotic cells displaying inactive mitochondria will be detected through definitive orange/brown staining patterns, whereas non-viable (dead)

cells will absorb the EtBr dye and will stain red, because this reagent only enters cells with ruptured membranes.

Briefly, 2µl of the Rh123 working solution was added to the cell suspension and incubated at 37°C, 5% CO_2 and 89% humidity for 5 minutes. After incubation 20µl of EtBr was added to the mixture and then incubated at room temperature for 5 minutes then 10µl of stained-cell mixture was pipetted onto a microscope slide, covered with a glass cover slip and studied under a fluorescent microscope with a band-pass filter. Apoptosis detection was determined by the morphology and fluorescent characteristics of stained cells. Viable cells fluoresce a vivid green, apoptotic cells are indicated by orange/brown and necrotic cells by red. Cell death was enumerated by counting an average of 50 cells distributed across five designated areas on each slide and then recorded as the percentage of viable, apoptotic and necrotic cells.

v) FAM POLY-CASPASE BINDING ASSAY

The present study employed FAM-VAD-FMK to measure caspase activation, in situ. The assay utilizes the fluorochrome labeled inhibitor of caspases (FLICA), which through the fluoromethylketone (FMK) binds to the active center of the activated enzymes. FLICA is a cell permeable, non-cytotoxic reagent which upon entering the cell, the labeled inhibitor binds covalently to active caspases (1, 3, 4, 5, 6, 7, 8 and 9) then achieves caspase inhibition by halting and thereby preventing further apoptotic activity. The remaining unbound FAM-VAD-FMK reagent disperses out of the cell and is washed away. FLICA bound cells showed morphological changes that were typical of apoptosis. This assay employs green fluorescent signaling to quantitatively measure the number of

active caspase enzymes present in each cell sample. The green fluorescent-labeled inhibitor, FAM-VAD-FMK, is the carboxyfluorescein (FAM) derivative of valylalanylaspartic acid (VAD) fluoromethylketone (FMK), an effective inhibitor of caspase activity. Cells which contain the bound probe produce a green fluorescence which can be analyzed by using fluorescent microscopy, fluorometry, or flow cytometry. Viable and necrotic cells remain unstained whereas apoptotic cells stain green. The number of activated caspases increases as apoptosis progresses consequently, cells in the earlier stages of apoptosis will stain a lighter green that those in the later stages of apoptosis.

Briefly, 300µl of cell suspension was aliquotted into sterile microcentrifuge tubes, to which 10µl of 30 X FAM reagent was added. The mixture was incubated at 37°C, 89% humidity, 5% CO_2 for 1hour; during which the cells were resuspended periodically and protected from light. After incubation, 2ml of 1X Wash buffer (1:10 ratio of de-ionized water) was added to each tube and mixed. The cells were then centrifuged at <400x g for 5 minutes at room temperature, and the supernatant was removed and discarded. The cell pellets were resuspended in 1ml of 1X wash buffer and vortexed to disrupt any cell to cell clumping. To assure removal of any unbound fluorescent dye, this process was repeated after which the cell pellets were resuspended in 300µl of 1X wash buffer and placed on ice. Quantitative analysis of apoptosis was determined by counting cells in several fields and recording the percentage of viable, apoptotic and necrotic cells. The cells were observed under fluorescent microscope using a band-pass filter (excitation 490nm, emission>520nm) to view green fluorescence signaling apoptotic cells.

STATISTICAL ANALYSIS

All experiments were performed in duplicates then repeated three times to ensure comparative results. Linear regression analysis was used to identify trends within treatments. Differences between treatments were assessed by ANOVA and significant differences were investigated with post-hoc analysis. Alpha was set at <0.05 for all analyses.

RESULTS

i) TRYPAN BLUE EXCLUSION ASSAY

Results showed a steady decline in the number of viable cells as the treatment concentration increased. Both treatment types (genistein and Cytoreg®) showed dose- and time- dependence (Graph not shown). The difference in the number of cells before and after treatment was significant ($P<0.05$).

ii) MTT ASSAY

Normal human breast epithelial cells showed no significant cytotoxic effects ($P>0.05$) after 48hr exposure to various concentrations of genistein. Similar results were observed for Cytoreg® (Figure 15). The percentage viable cells remained relatively constant between 10-50μg/ml concentration of genistein and 0.0005-0.001μg/ml concentration of Cytoreg® and only showed a slight decline at 60μg/ml concentration (Figure 15).

However, results for MCF-7 cells showed genistein induced growth inhibition as drug concentration increased. An initial increase in cell viability was observed between the 0-10μg/ml concentration, followed by a significant ($P<0.05$) decline in the percentage viable cells throughout all remaining cytotoxic dose ranges (Figure 4). A strong and direct correlation was observed between cell viability and treatment concentration ($r^2=0.981$). MTT showed a dose- and time- dependent relationship in cells treated with both genistein and Cytoreg® at 24hr and 48hr (Figure 4-14). This was consistent with the

data collected in the TB assay. Significant ($P<0.05$) differences were observed between the single and combination treatments at all dose levels (Figure 7 and 11). Furthermore, single treatment Cytoreg® showed a greater significant decrease in the total cell death of MCF-7 cells at a lower dose than the combination treatment. Results for the combination treatment at 48hr showed greater response at lower concentrations than those at corresponding concentrations of the single treatment (Figure 11).

iii) ETHIDIUM BROMIDE/ACRIDINE ORANGE ASSAY

Results indicated that apoptosis was the main form of cell death furthermore, the number of apoptotic cells increased as either genistein or Cytoreg® alone or a combination of both treatment concentrations increased. Additionally, the combination treatment revealed a direct correlation between increased drug concentration exposure and drug induced cell death ($r^2=0.992$) (Figure 16-18). The combination treatment showed greater effectiveness when compared to single treatments at 24hr ($P<0.05$).

iv) ETHIDIUM BROMIDE-RHODAMINE123

Results obtained showed apoptosis as the primary form of cell death (Figure 19-21). The data observed for genistein and Cytoreg®, for single and combination treatments indicated a concentration-dependent effect. The percentage apoptosis induced by combination treatment was significantly higher than either of the single treatments ($P<0.05$).

v) FAM POLY-CASPASE BINDING ASSAY

The data indicated a strong correlation between caspase activity and increased apoptotic induction ($r^2=0.981$) for genistein and Cytoreg®, for single and combination treatment. Increased caspase activity was associated with increased apoptosis induction

along the various cytotoxic dose ranges (Figure 22-23). Similar results were observed for Cytoreg® (Figure22). However, where genistein and Cytoreg® single treatments showed greatest percentage caspase activity at the higher therapeutic dosages the combination treatment showed greatest caspase activity at the lower cytotoxic dose ranges.

DISCUSSION

The purpose of this study was to investigate if the combination of genistein with Cytoreg® would significantly enhance the anti-tumor activity of genistein in MCF-7 breast cancer cells through apoptosis induction. The MTT assay showed a dose-and time-dependent effect of all three treatment regimen in MCF-7 cells. Single treatments of genistein and Cytoreg® at 24hr and 48hr showed an overall decrease in cell viability. Single treatments at 48hr indicated the number of viable cells was significantly ($p<0.05$) lower than 24hr treatments. However, at each treatment dosage level, Cytoreg®-induced growth inhibition was significantly higher than genistein-induced growth inhibition as indicated by the MTT assay. These results confirm findings in earlier studies which revealed single treatment groups achieved growth inhibition through apoptosis induction in MCF-7 breast cancer cells (Fan, 2006; Yang et al., 2007). Combination treatment trends were similar to the single treatments. However the inhibitory effect of genistein-Cytoreg® combination on MCF-7 cell growth response was significantly ($p<0.05$) greater than either genistein or Cytoreg® single treatments. It appears that the addition of Cytoreg® to genistein, induced an inhibitory effect that was additive in the MCF-7 cells. This was more evident at higher combination cytotoxic dose ranges. The ID50 obtained for combination treatments was lower than that from the single treatments. This implies greater therapeutic potential of the combination treatment over single treatments (Sakamoto, 2000; Khoshyomn et al., 2002). Furthermore, this study has provided

evidence of autophagic cell death. Autophagy (self-eating) is a form of cellular degradation that leads to lysis of cellular components which in turn may lead to karyorrhexis (Levine, 2007; Vicencio et al., 2008). Cytoreg®-treated and Cytoreg®-genistein combination-treated cells showed evidence of autophagy, which was not observed in cells treated with genistein alone; implying that one of the mechanisms by which Cytoreg®-induced cell death may be initiation of autophagy, leading to apoptosis and/or necrosis in the cells. To our knowledge, this is the first time treatment-induced (specifically Cytoreg®-induced) autophagic cell death is being reported. Here, we are hypothesizing that autophagy releases apoptotic inducing factors/proteins with subsequent induction of apoptosis; by-passing the caspase protease signaling pathway. The alternate possibility is that such released factors could cause caspase protease cascade to induce apoptosis. In previous studies in our laboratory, blocking of caspase did not totally abrogate Cytoreg®-induced apoptosis in the cells. Further research is on-going. Normal Breast Epithelial Cells showed no significant ($p>0.05$) cytotoxic effect over all dose ranges when compared to all other treatment types.

The AcrO/EtBr analysis revealed apoptosis was the main mode of cell death in MCF-7 cells induced by all treatment groups (single and combination). This observation was in conformity with previous studies (Li et al., 1999; Nakagawa et al., 2000; Linford and Dorsa, 2002). Genistein-induced apoptosis in MCF-7 cells was significantly higher than Cytoreg®-induced apoptosis; although total cell death induced by Cytoreg® was higher than total cell death induced by genistein. Combination induced the highest percentage of apoptosis in the cell. Dose- and time- dependent apoptosis induction by genistein and Cytoreg® combined has been reported in previous studies (Fan, 2006;

Kumi-Diaka, 2006) Genistein has the ability to mimic 17-β-estrodiol partly due to structural similarities; and can regulate gene expression by binding to estrogen receptors (Chen et al., 2003; Maggiolini et. al., 2002; Yang et al., 2007). This characteristic partially accounts for its ability to influence cell growth, thus inhibiting growth and proliferation of hormone dependent carcinomas (Kumi-Diaka et al., 2006). The MC-7 cell line is a HER2/*neu* positive, estrogen dependent human breast cancer cell line. Genistein demonstrates increased effectiveness against MCF-7 cells whereby when genistein induced apoptosis occurs this triggers a dose- and time- dependent increase in BRCA1 and BRCA2 protein levels in MCF-7 cells (Fan, 2006).

Caspase protease activation was confirmed with data obtained from the results. A strong, positive correlation ($r^2 = 0.981$) between caspase expression and apoptosis induction was identified. Of the three treatment groups, combination treatment showed the most significant apoptotic response with respect to caspase activity. The combination treatment group was more sensitive to caspase expression than either single treatment group. At lower caspase expression levels apoptosis induction was manifested. However, the overall results indicated caspase protease as the major pathway in apoptosis induction. These results were in agreement with findings in previous studies (Porter andanicke, 1999; Pei, 2003).

CONCLUSION

The present study indicated that Cytoreg® enhanced the sensitivity of MCF-7 carcinoma cells to genistein isoflavone thus making the combination treatment more effective than the single treatment. The overall data indicated that growth inhibition occurred through apoptotic induction in all treatment groups. The findings suggest that the combination of genistein with Cytoreg® could be a more effective therapeutic regimen than single treatments for hormone-dependent breast cancer.

Future studies are on going to explore Cytoreg®-genistein combination as potential therapeutic option for breast cancer.

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ILLUSTRATIONS

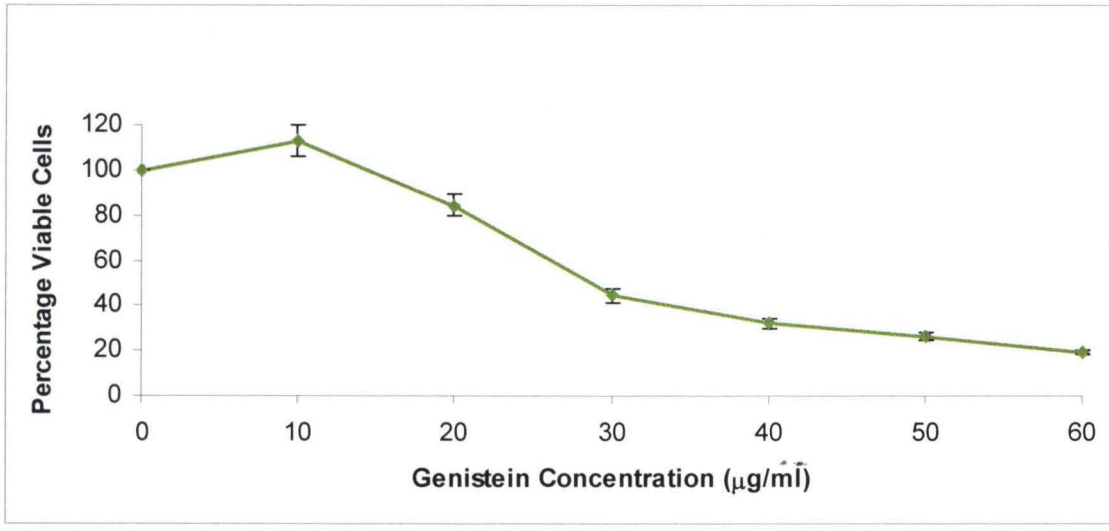

Figure 4 Growth and viability of MCF-7 cells was assessed using the MTT assay. The cells were treated with varying concentrations of (Gn_0 – Gn_{60}) for 24hr. Percentage viability at all dosage levels were significantly different($P<0.05$). Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

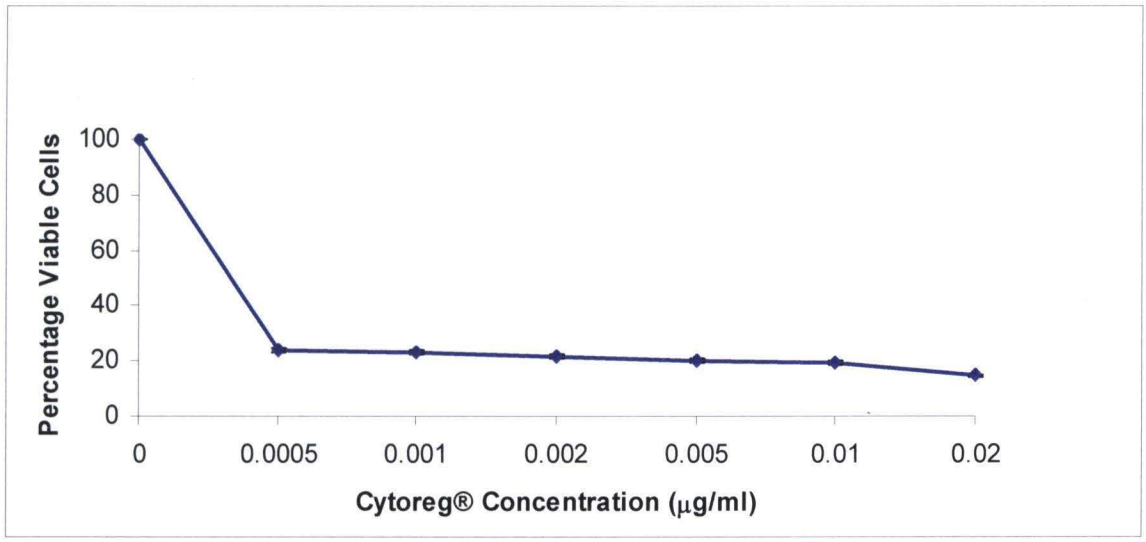

Figure 5 Growth and viability of MCF-7 cells was assessed using the MTT assay. The cells were treated with varying concentrations of Cytoreg ($Cyt_{0.0005}$ – $Cyt_{0.02}$) for 24hr. Percentage viability at all dosage levels were significantly different($P<0.05$).Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

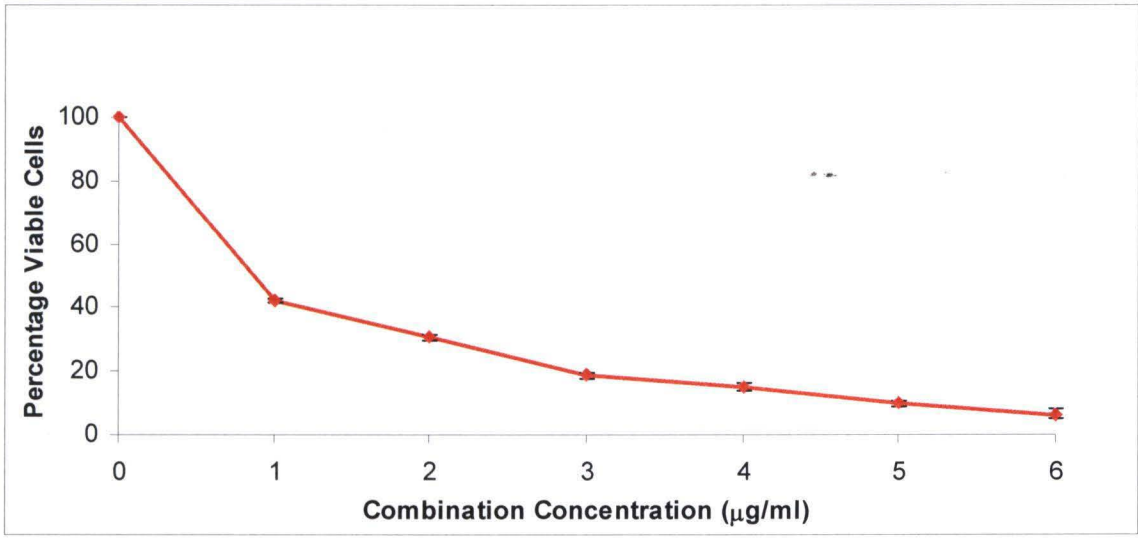

Figure 6 Growth and viability of MCF-7 cells was assessed using the MTT assay. The cells were treated with varying concentrations of genistein and Cytoreg® combined (Gn0/Cyt0.0005 – Gn60/Cyt0.02) respectively for 24hr. Percentage viability at all dosage levels were significantly different($P<0.05$). Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

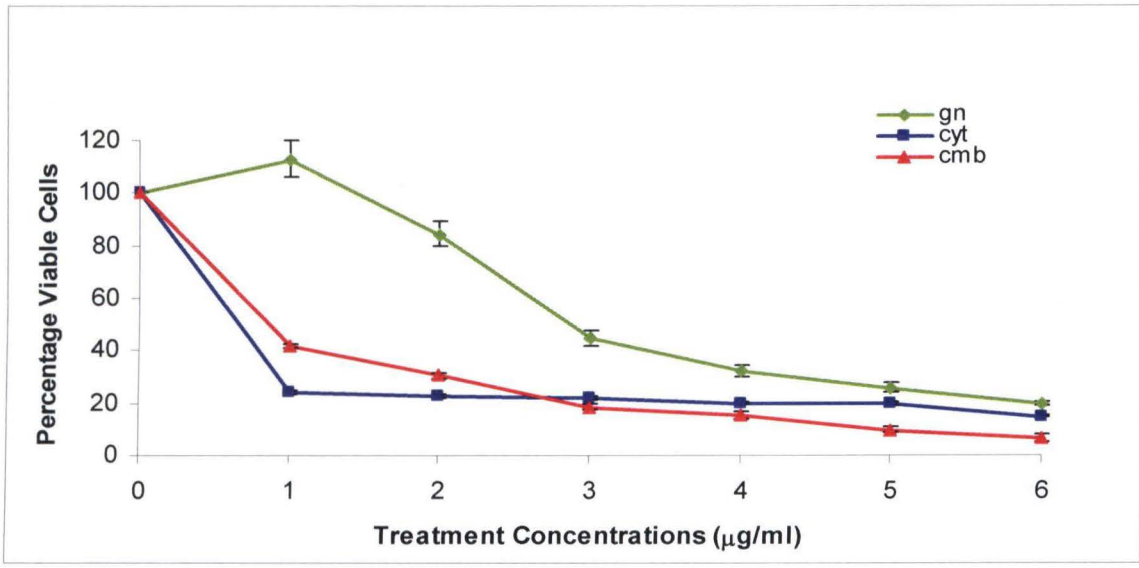

Figure 7 Growth and viability of MCF-7 cells was assessed using the MTT assay. The cells were treated with varying concentrations of genistein (Gn_0 – Gn_{60}), Cytoreg® ($Cyt_{0.0005}$ – $Cyt_{0.02}$) and genistein-Cytoreg® combination for 24hr as described in the experiment. Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

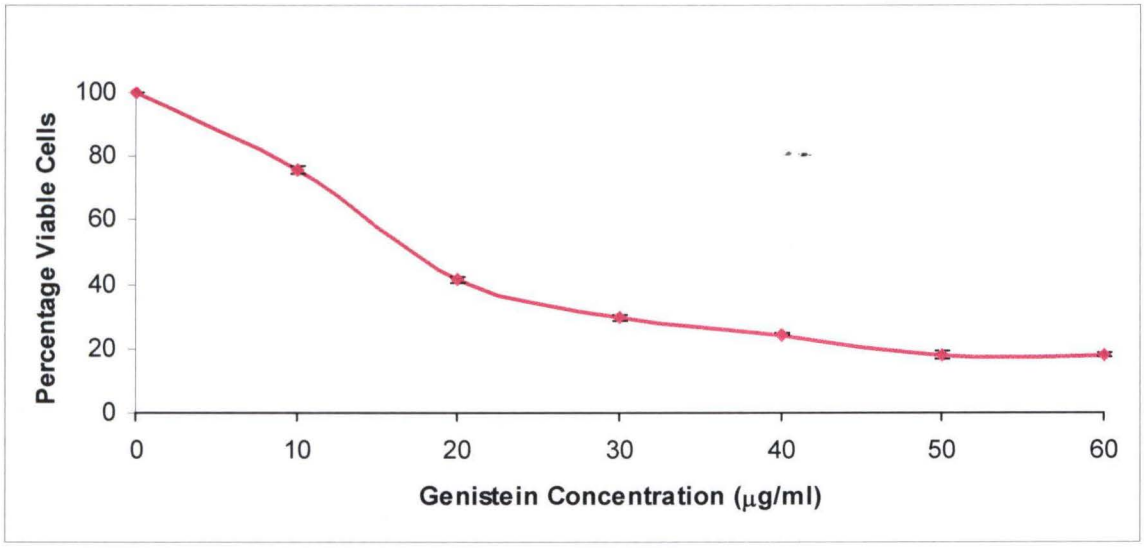

Figure 8 Growth and viability of MCF-7 cells was assessed using the MTT assay. The cells were treated with varying concentrations of genistein (Gn_0 – Gn_{60}) for 48hr. Percentage viability between the dosage levels were significantly different($P<0.05$). Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

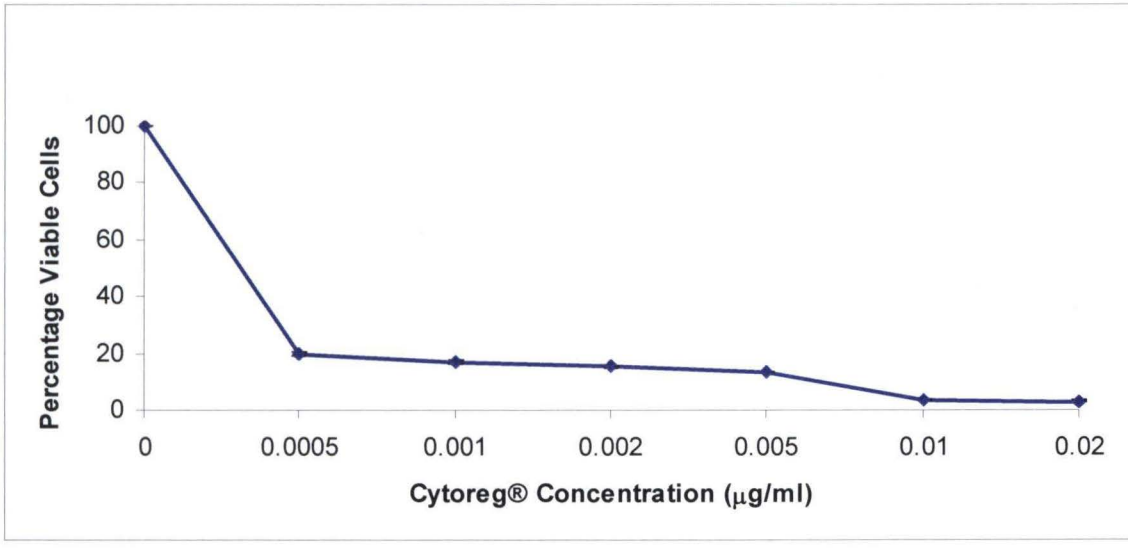

Figure 9 Growth and viability of MCF-7 cells was assessed using the MTT assay. The cells were treated with varying concentrations of Cytoreg® (Cyt0.0005 – Cyt0.02) for 48hr. Percentage viability between the dosage levels were significantly different($P<0.05$). Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

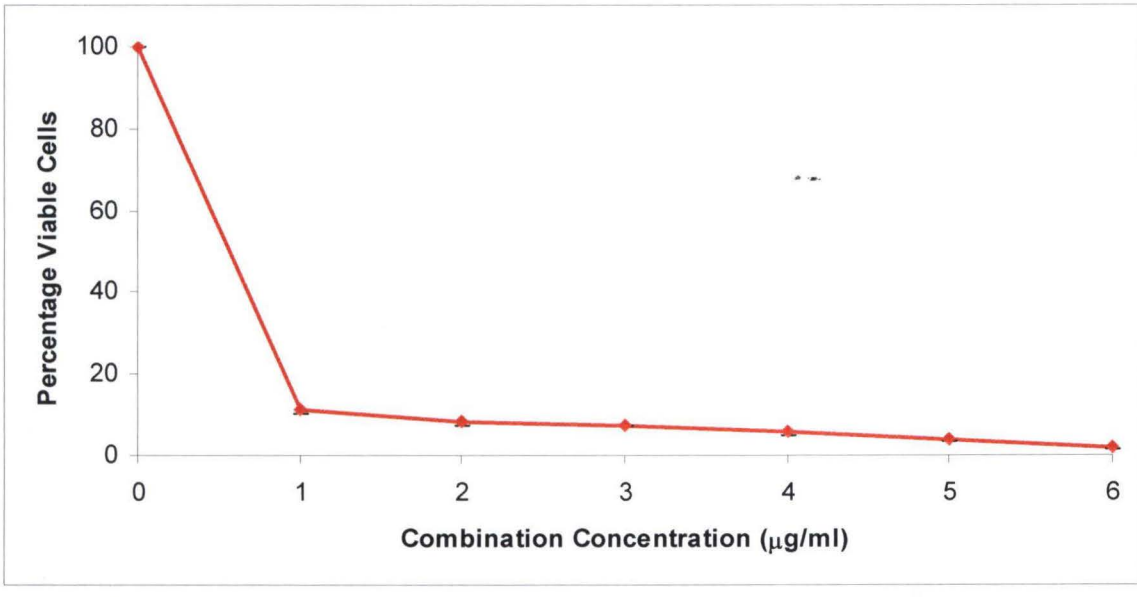

Figure 10 Growth and viability of MCF-7 cells was assessed using the MTT assay. The cells were treated with varying concentrations of genistein and Cytoreg® combined (Gn0/Cyt0.0005 – Gn60/Cyt0.02) respectively for 48hr. Percentage viability between the dosage levels were significantly different($P<0.05$), except at dosage levels 2 and 3. Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate.Bar = SEM.

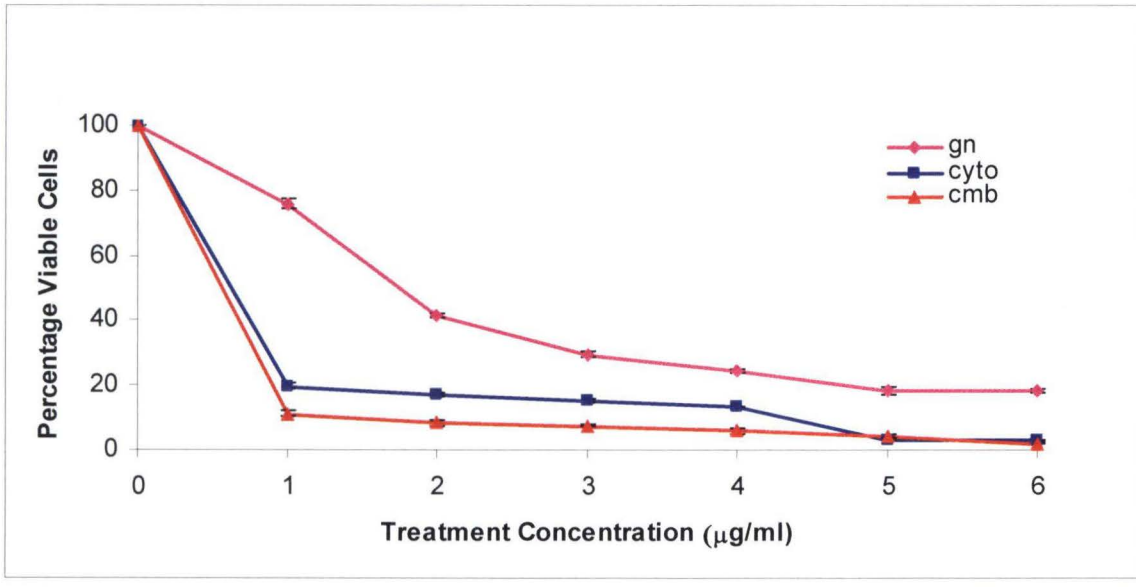

Figure 11 Growth and viability of MCF-7 cells was assessed using the MTT assay. The cells were treated with varying concentrations of genistein (Gn_0 – Gn_{60}), Cytoreg® ($Cyt_{0.0005}$ – $Cyt_{0.02}$) and genistein-Cytoreg® combination for 48hr as described in the experiment. Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

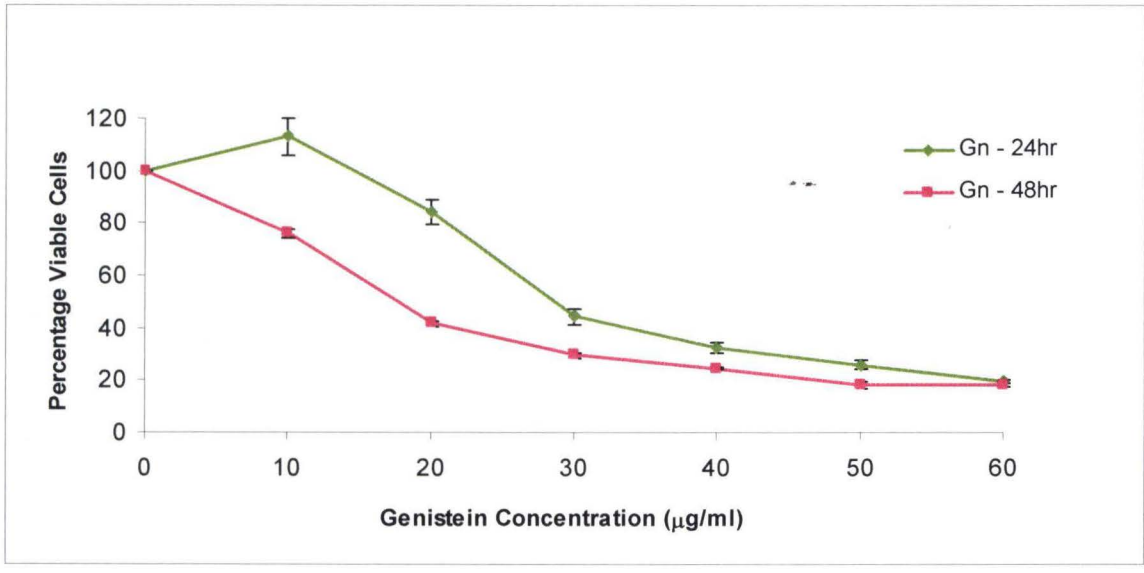

Figure 12 Growth and viability of MCF-7 cells was assessed using the MTT assay. The cells were treated with varying concentrations of (Gn_0 – Gn_{60}) for 24hr and 48hr. Percentage viability between the dosage levels were significantly different ($P<0.05$). Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

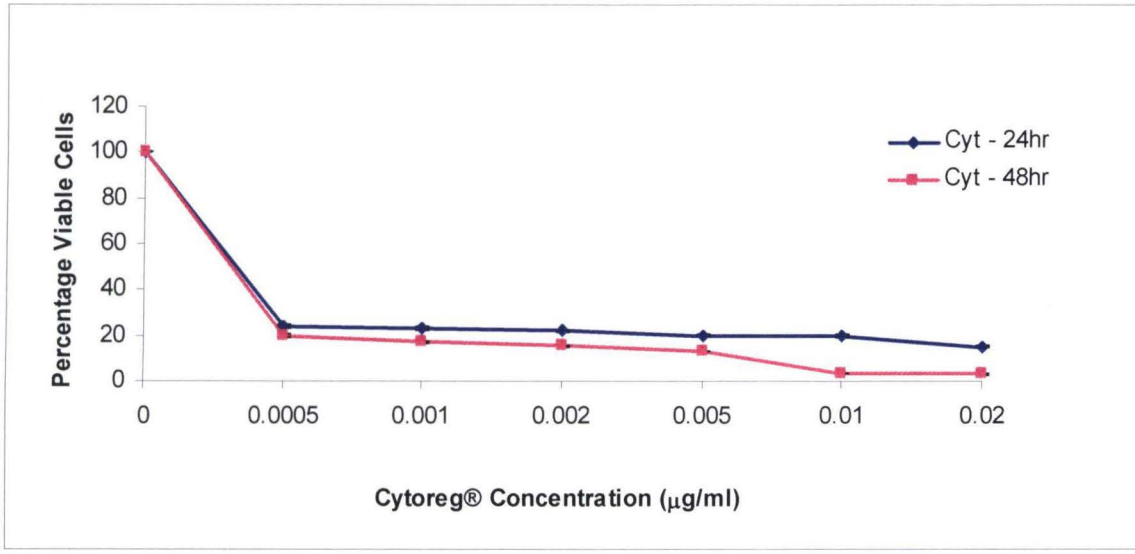

Figure 13 Growth and viability of MCF-7 cells was assessed using the MTT assay. The cells were treated with varying concentrations of Cytoreg® ($Cyt_{0.0005}$ – $Cyt_{0.02}$) for 24hr and 48hr. Percentage viability between the dosage levels were significantly different ($P<0.05$). Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

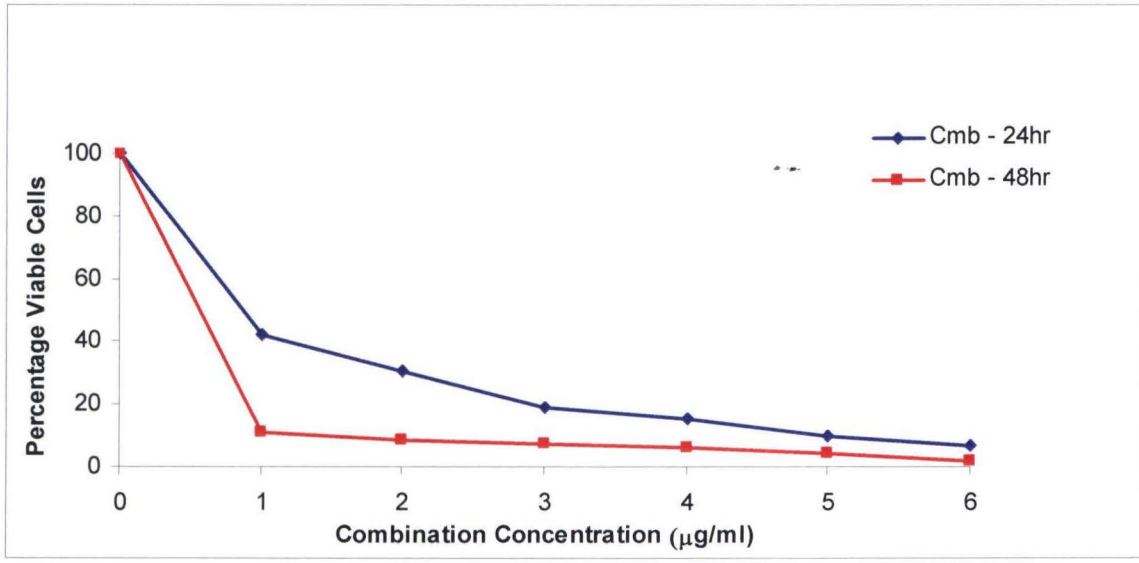

Figure 14 Growth and viability of MCF-7 cells was assessed using the MTT assay. The cells were treated with varying concentrations of genistein and Cytoreg® combined (Gn0/Cyt0.0005 – Gn60/Cyt0.02) respectively for 24hr and 48hr. Percentage viability between the dosage levels were significantly different($P<0.05$). Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

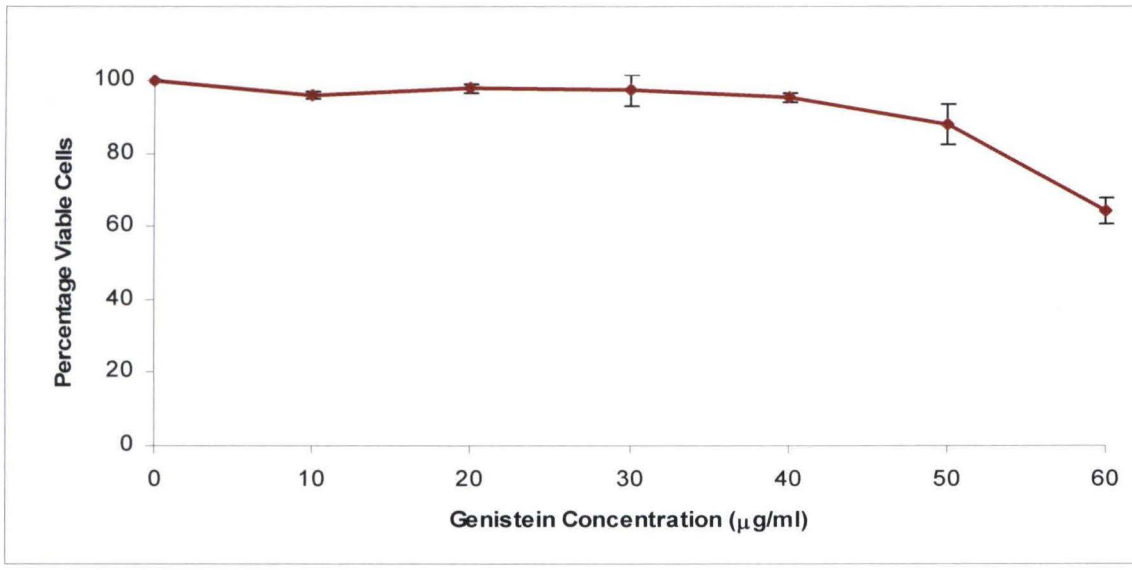

Figure 15 MTT assay was performed to assess the viability and chemosensitivity of normal cells. Cells were exposed to varying concentrations of genistein (Gn_0 – Gn_{60}) for 48hr, incubated at 37°C, 5% CO_2, and 89% humidity, as previously described. Similar results were observed for cytoreg® ($Cyt_{0.0005}$ – $Cyt_{0.02}$). Percentage viability between the dosage levels were significantly different ($P<0.05$). Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

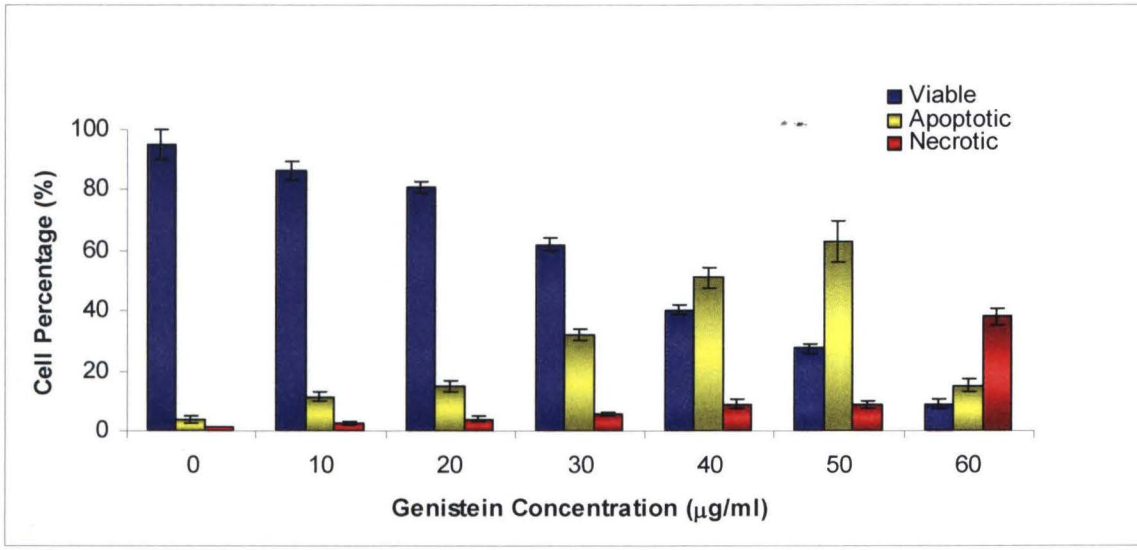

Figure 16 Apoptosis in MCF-7 cells were assessed using the EtBr/AcrO assay. The cells were exposed to varying concentrations of genistein (Gn_0 – Gn_{60}) for 24hr at 37°C, 5% CO_2 and 89% humidity. Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

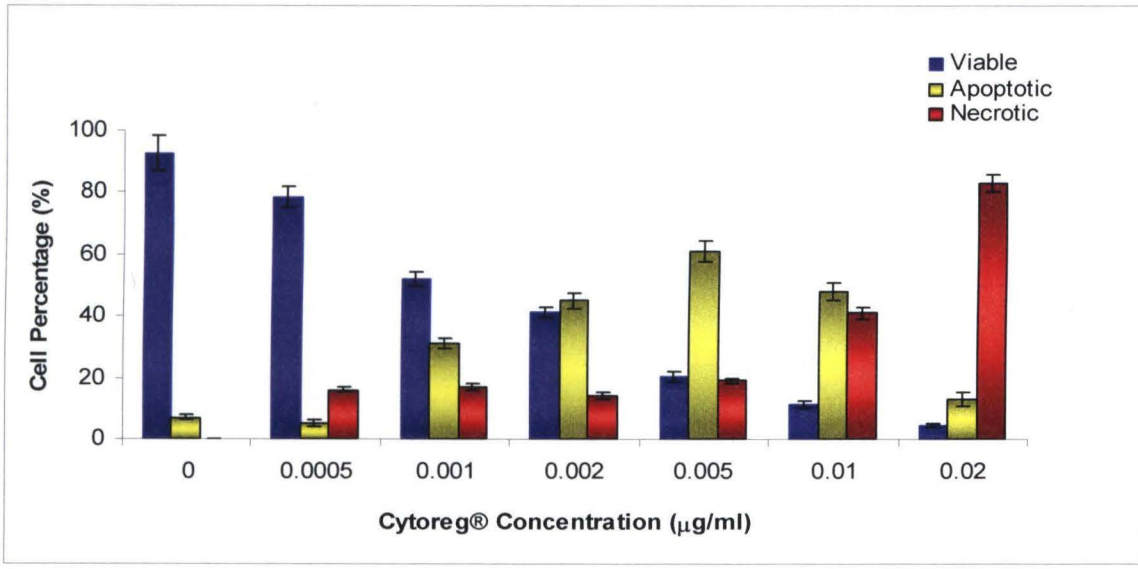

Figure 17 Apoptosis in MCF-7 cells were assessed using the EtBr/AcrO assay. The cells were exposed to varying concentrations of Cytoreg® (Cyt0.0005 – Cyt0.02) for 24hr at 370C, 5% CO2 and 89% humidity. Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

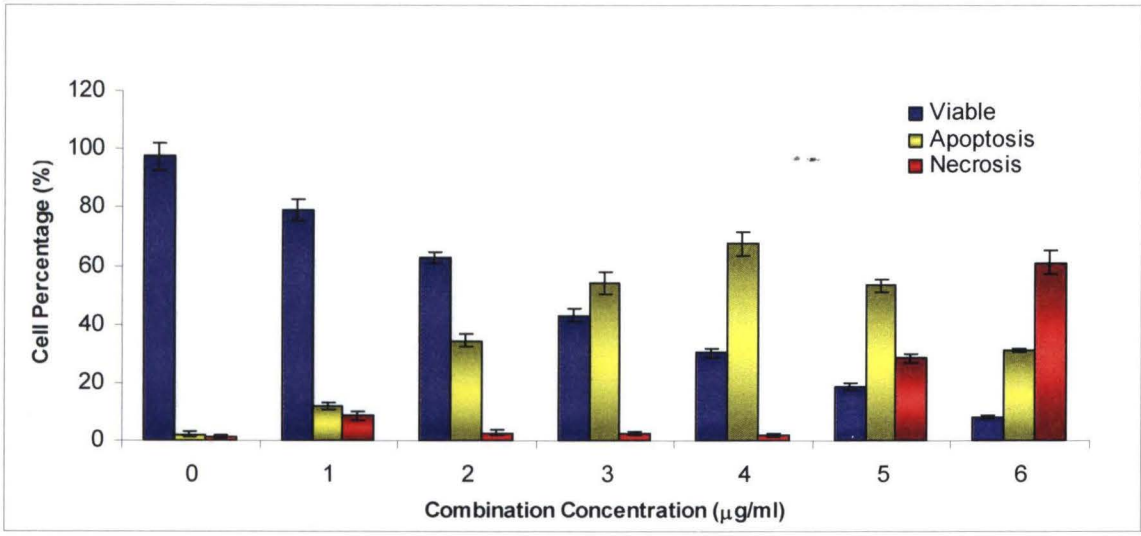

Figure 18 Apoptosis in MCF-7 cells were assessed using the EtBr/AcrO assay. The cells were exposed to varying concentrations of Cytoreg® (Cyt0.0005 – Cyt0.02) and genistein (Gn0 – Gn60) combined for 24hr at 370C, 5% CO2 and 89% humidity. Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

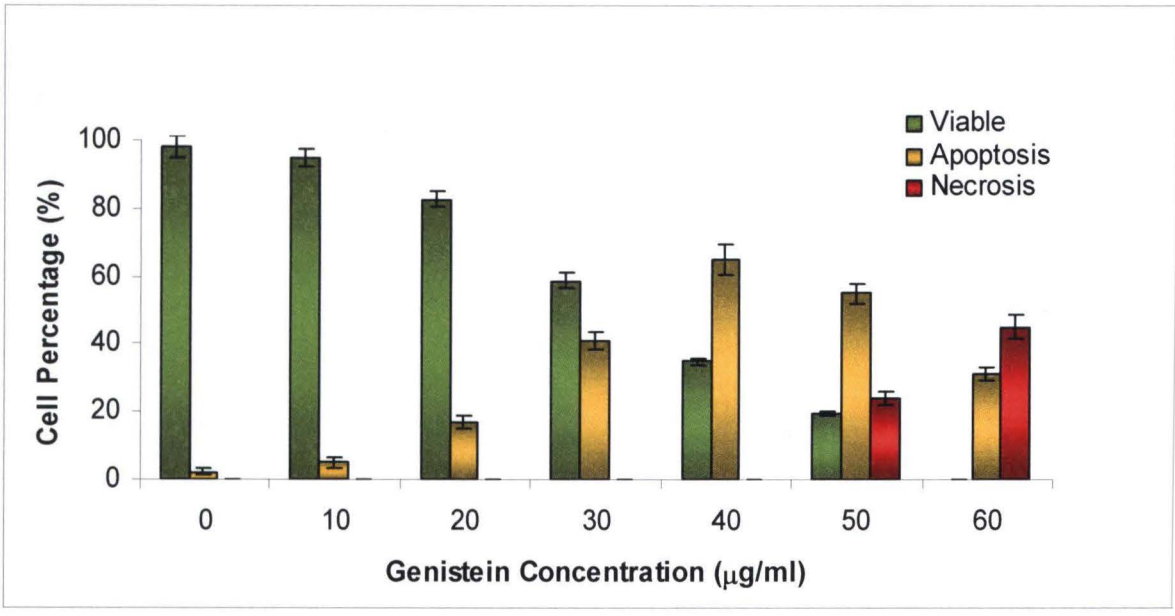

Figure 19 Apoptosis in MCF-7 cells were assessed using the EtBr-Rh123 assay. The cells were exposed to varying concentrations of genistein (Gn_0 – Gn_{60}) for 24hr at 37^0C, 5% CO_2 and 89% humidity. Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

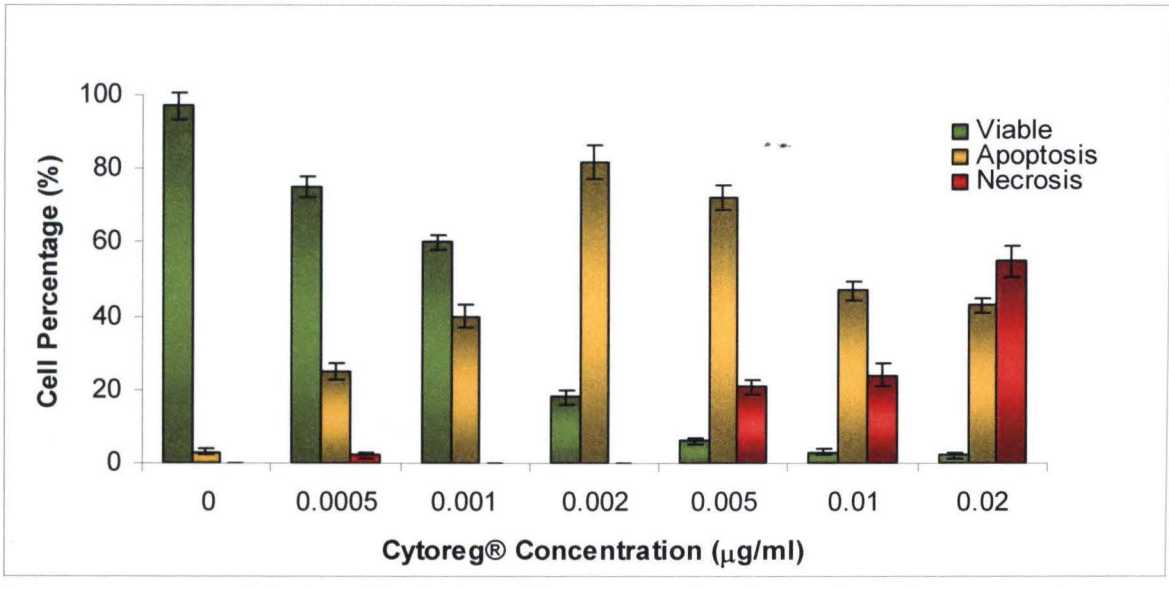

Figure 20 Apoptosis in MCF-7 cells were assessed using the EtBr-Rh123 assay. The cells were exposed to varying concentrations of Cytoreg® (Cyt0.0005 – Cyt0.02) for 24hr at 370C, 5% CO2 and 89% humidity. Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

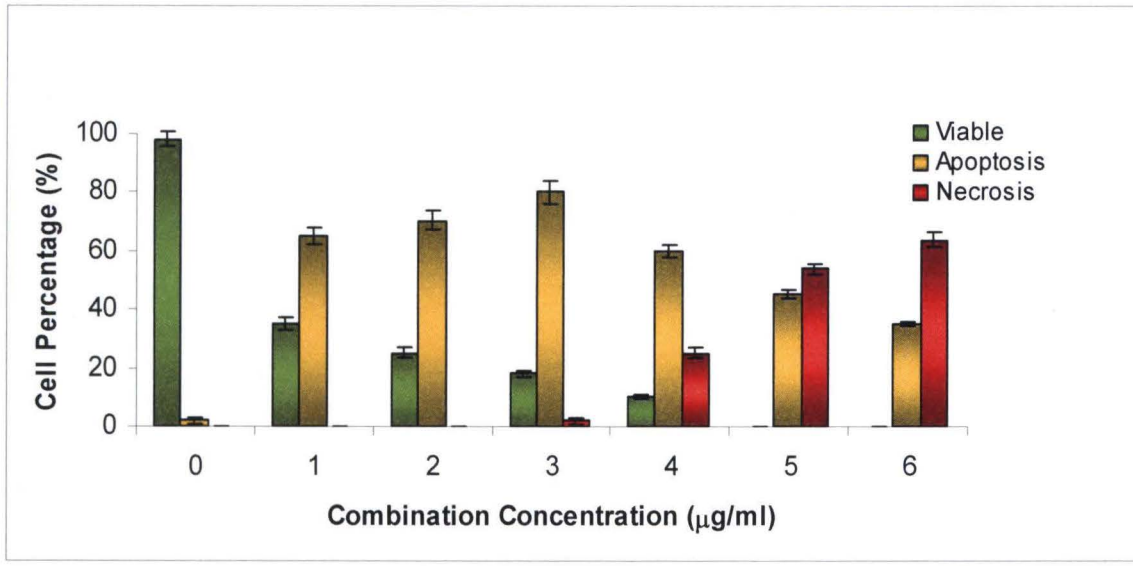

Figure 21 Apoptosis in MCF-7 cells were assessed using the EtBr-Rh123 assay. The cells were exposed to varying concentrations of genistein and Cytoreg® combined (Gn0/Cyt0.0005 – Gn60/Cyt0.02) respectively for 24hr. Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

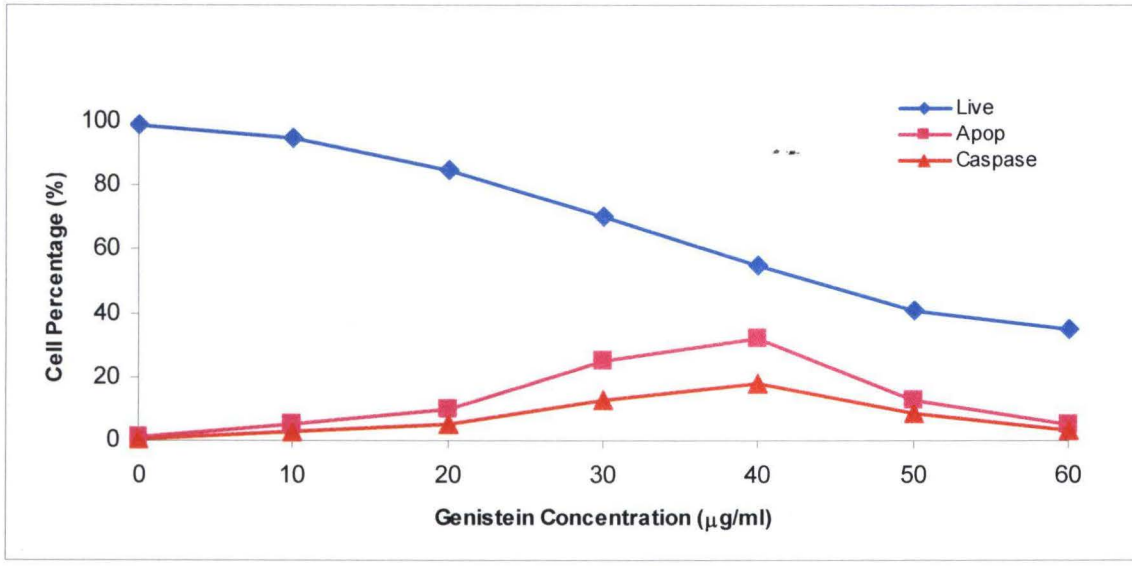

Figure 22 The correlation between apoptosis and caspase activation in MCF-7 cell was assessed using the FAM poly-caspase binding assay. The cells were exposed to varying concentrations of genistein (Gn0 – Gn60) for 48hr at 370C, 5% CO2 and 89% humidity, as previously described. The pattern of response to Cytoreg® was similar to that of genistein. Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

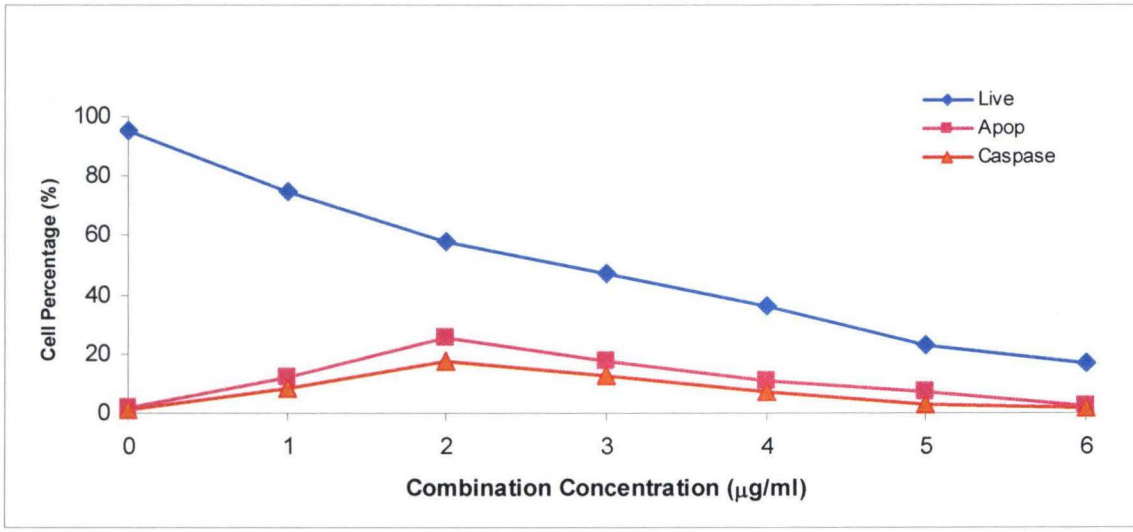

Figure 23 The correlation between apoptosis and caspase activation in MCF-7 cell was assessed using the FAM poly-caspase binding assay. The cells were exposed to varying concentrations of genistein-Cytoreg® combination for 48hr at 37^0C, 5% CO_2 and 89% humidity, as previously described. Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

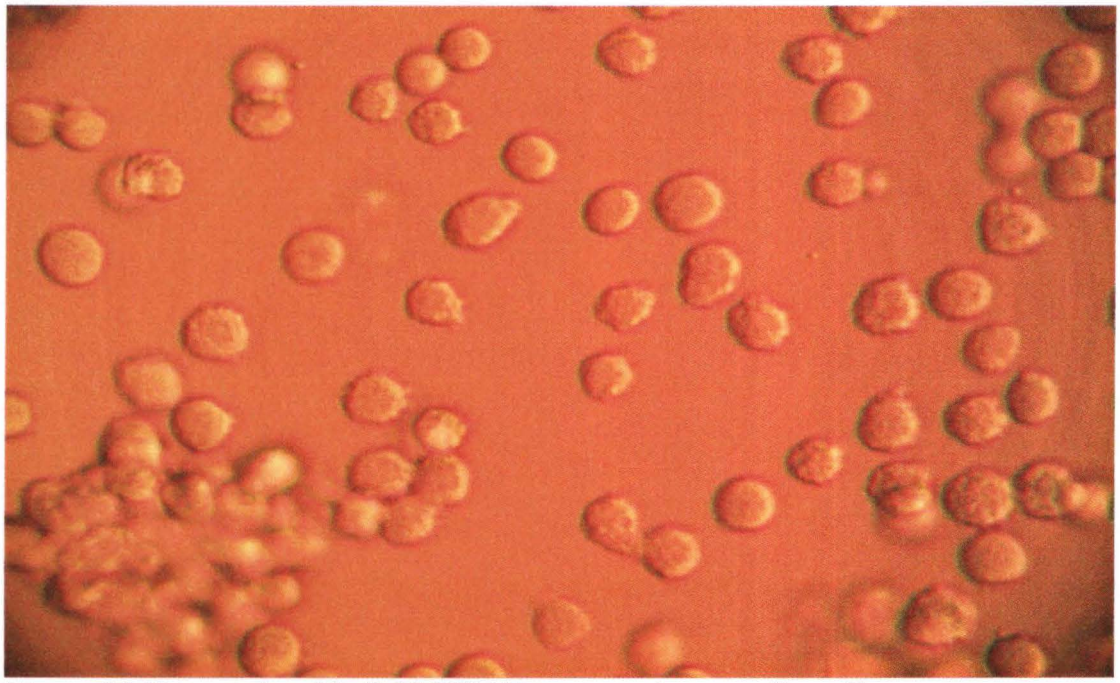

Figure 24 Untreated, epithelial MCF-7 breast carcinoma cells at 0hr.

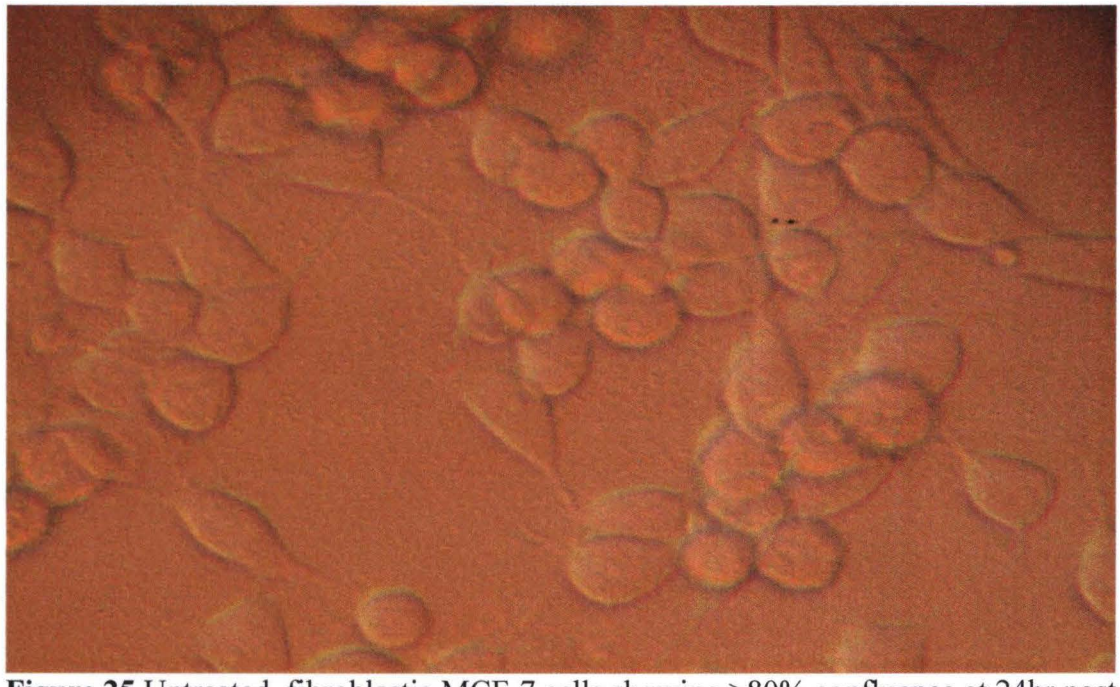

Figure 25 Untreated, fibroblastic MCF-7 cells showing >80% confluence at 24hr post incubation.

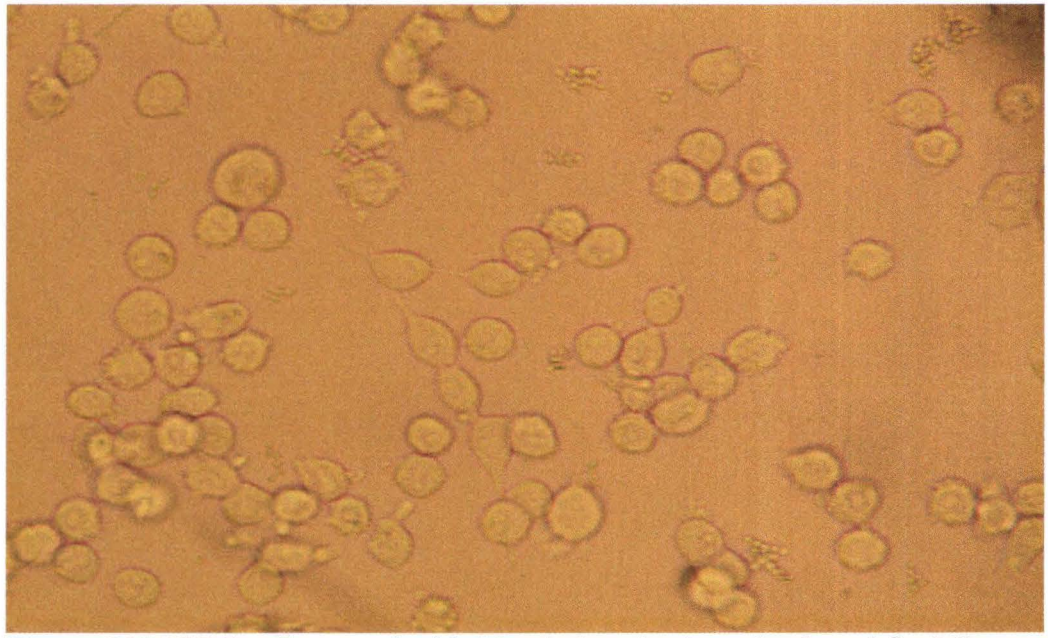

Figure 26 MCF-7 cells treated with 10µg/ml genistein and cultured for 24hr. Photographed with digital camera (Nikon 2000) under inverted microscope. Note the morphology of the cells-epithelial (circular, detached) and fibroblastic (attached).

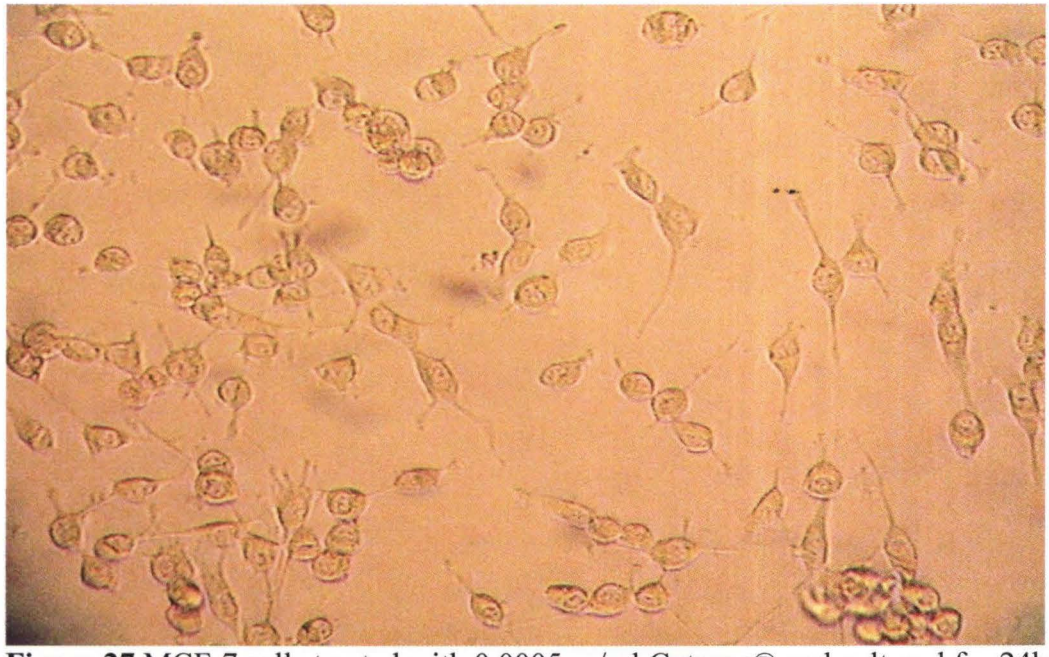

Figure 27 MCF-7 cells treated with 0.0005µg/ml Cytoreg® and cultured for 24hr. Photographed with digital camera (Nikon 2000) under inverted microscope. Note the initial changes in the morphology of the fibroblastic cells.

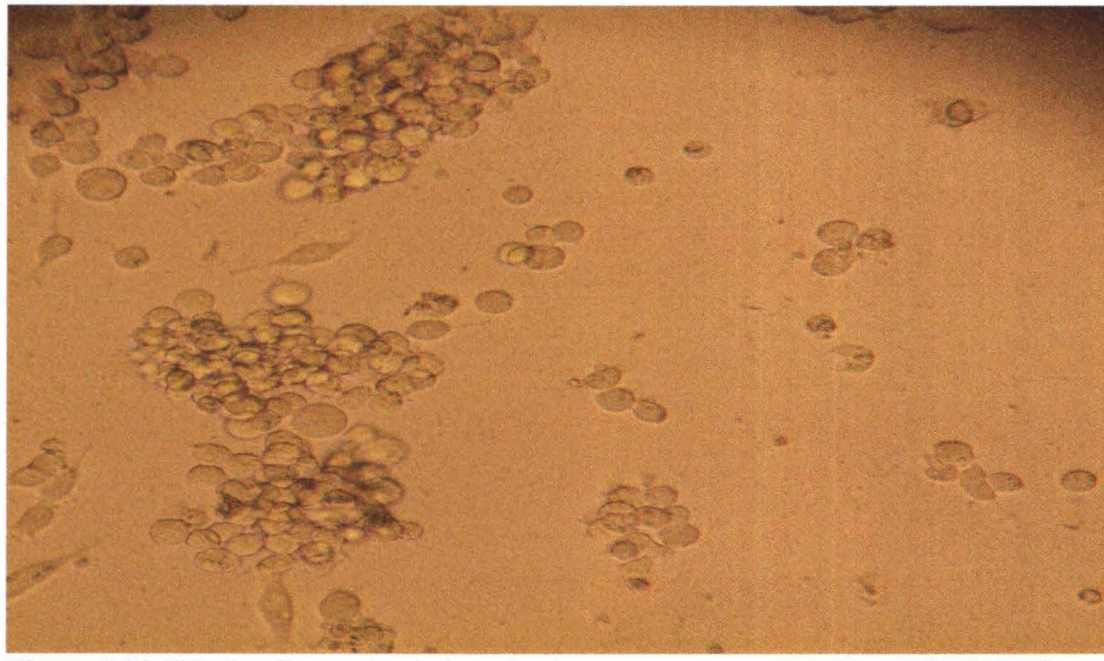

Figure 28 MCF-7 cells at 20ug/ml genistein treatment concentration. Displays clumping due to treatment; indicative of apoptosis MCF-7 cells treated with 20ug/ml genistein and cultured for 24hr. Photographed with digital camera (Nikon 2000) under inverted microscope. Note apoptotic cells – nuclei shrinkage and condensed chromatin.

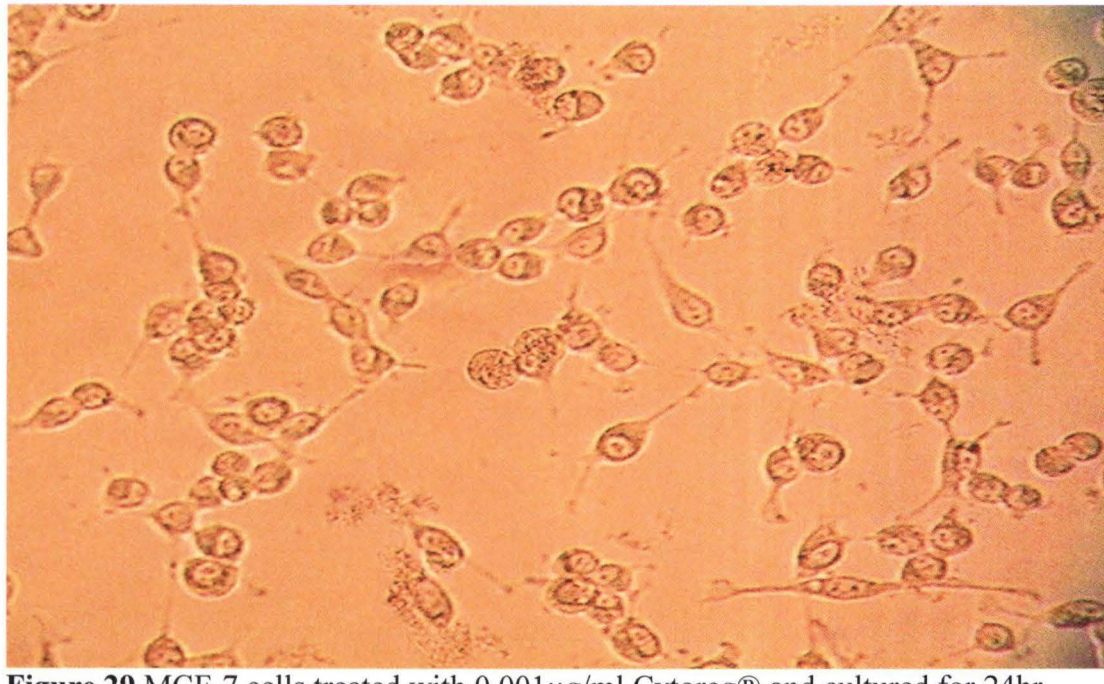

Figure 29 MCF-7 cells treated with 0.001µg/ml Cytoreg® and cultured for 24hr. Photographed with digital camera (Nikon 2000) under inverted microscope. Note the autophagy and initial apoptosis in the cells.

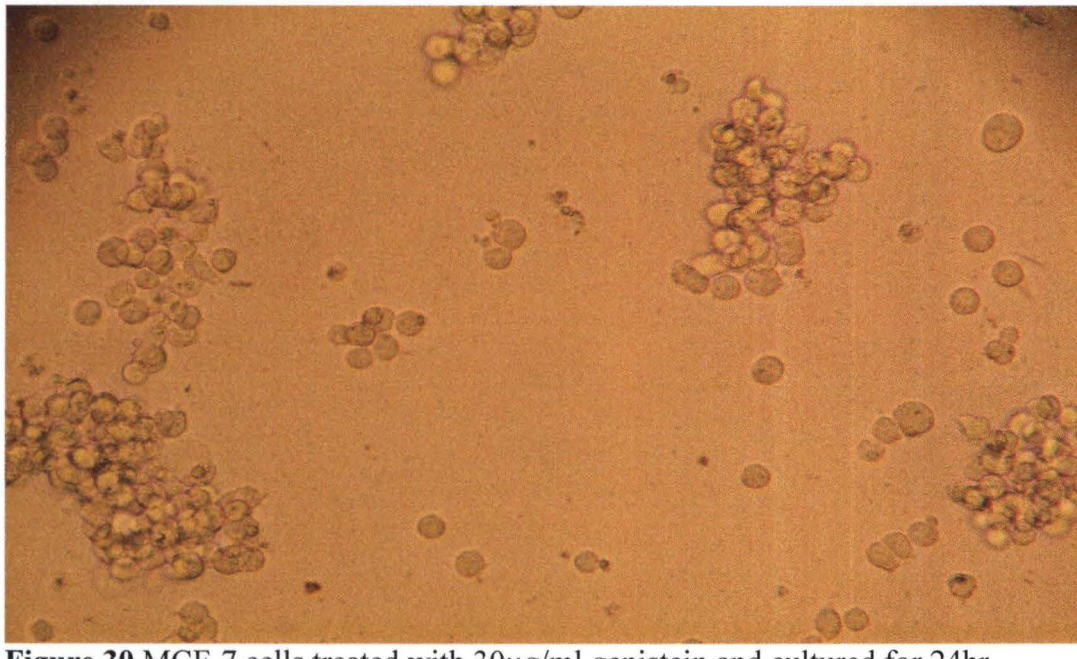

Figure 30 MCF-7 cells treated with 30µg/ml genistein and cultured for 24hr. Photographed with digital camera (Nikon 2000) under inverted microscope. Note apoptotic cells – nuclei shrinkage and condensed chromatin.

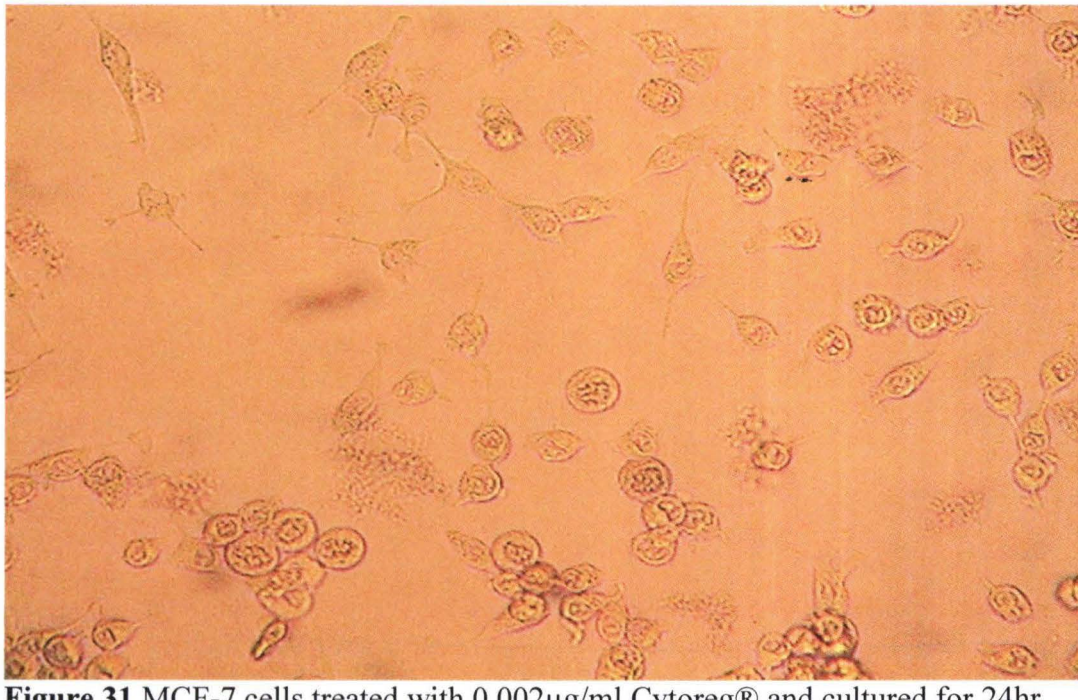

Figure 31 MCF-7 cells treated with 0.002µg/ml Cytoreg® and cultured for 24hr. Photographed with digital camera (Nikon 2000) under inverted microscope. Note the autophagy and initial apoptosis in the cells.

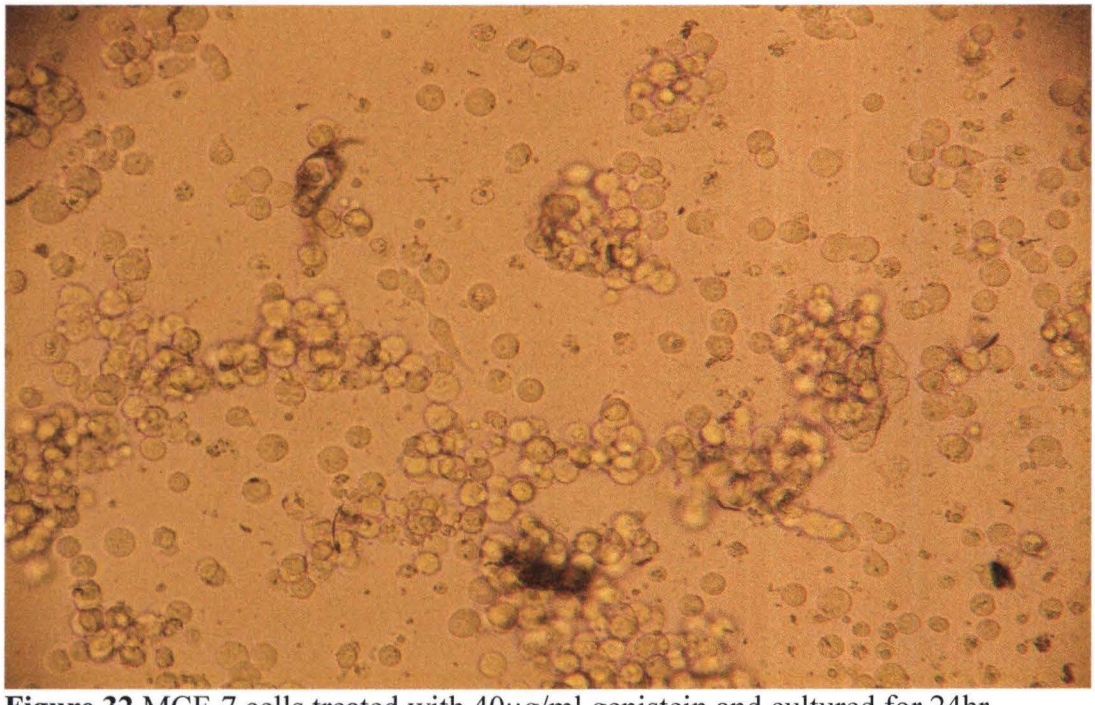

Figure 32 MCF-7 cells treated with 40µg/ml genistein and cultured for 24hr. Photographed with digital camera (Nikon 2000) under inverted microscope. Note apoptotic cells – nuclei shrinkage, cytoplasmic constriction and condensed chromatin.

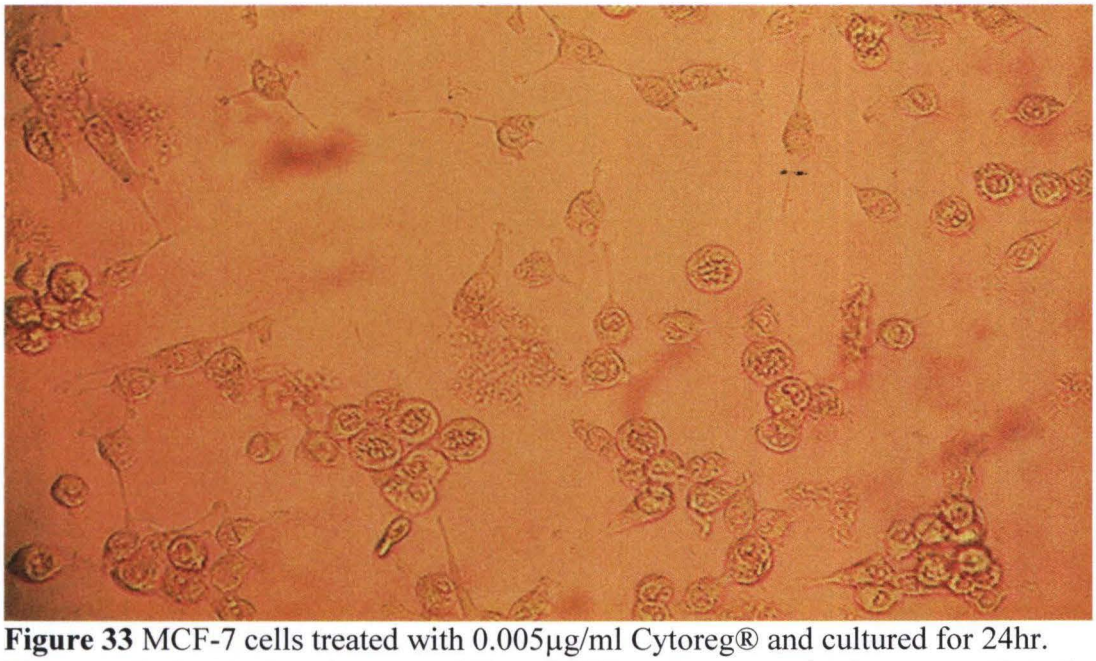

Figure 33 MCF-7 cells treated with 0.005µg/ml Cytoreg® and cultured for 24hr. Photographed with digital camera (Nikon 2000) under inverted microscope. Note the autophagy and initial apoptosis in the cells.

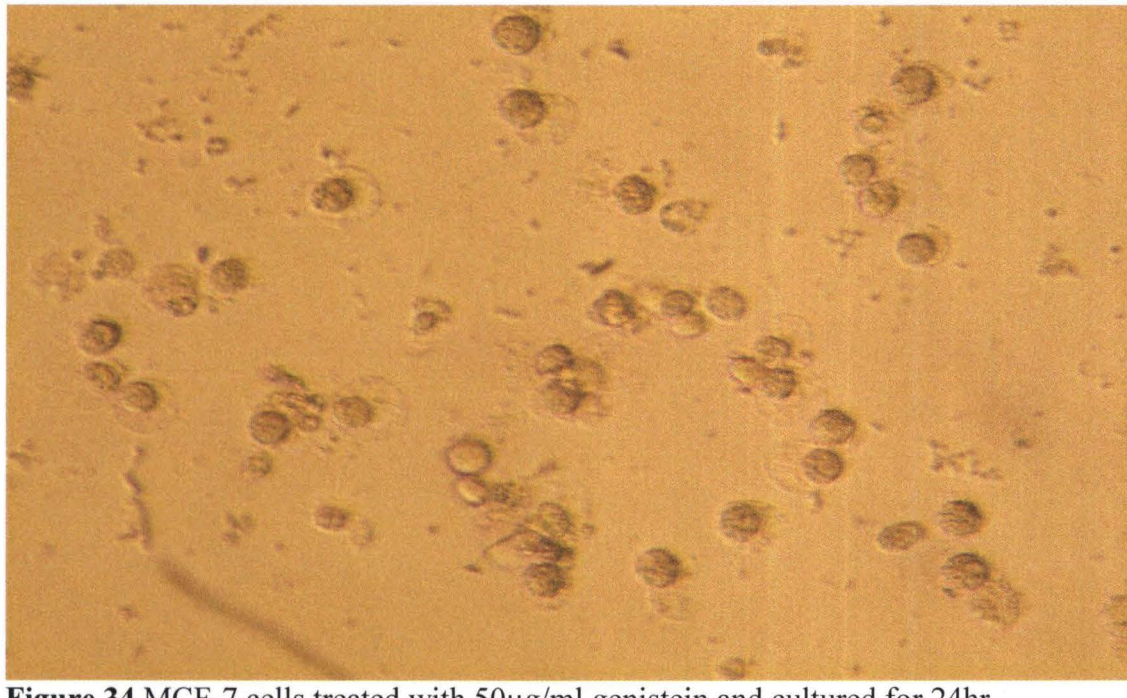

Figure 34 MCF-7 cells treated with 50µg/ml genistein and cultured for 24hr. Photographed with digital camera (Nikon 2000) under inverted microscope. Note apoptotic cells–nuclei shrinkage, condensed chromatin and formation of apoptotic bodies.

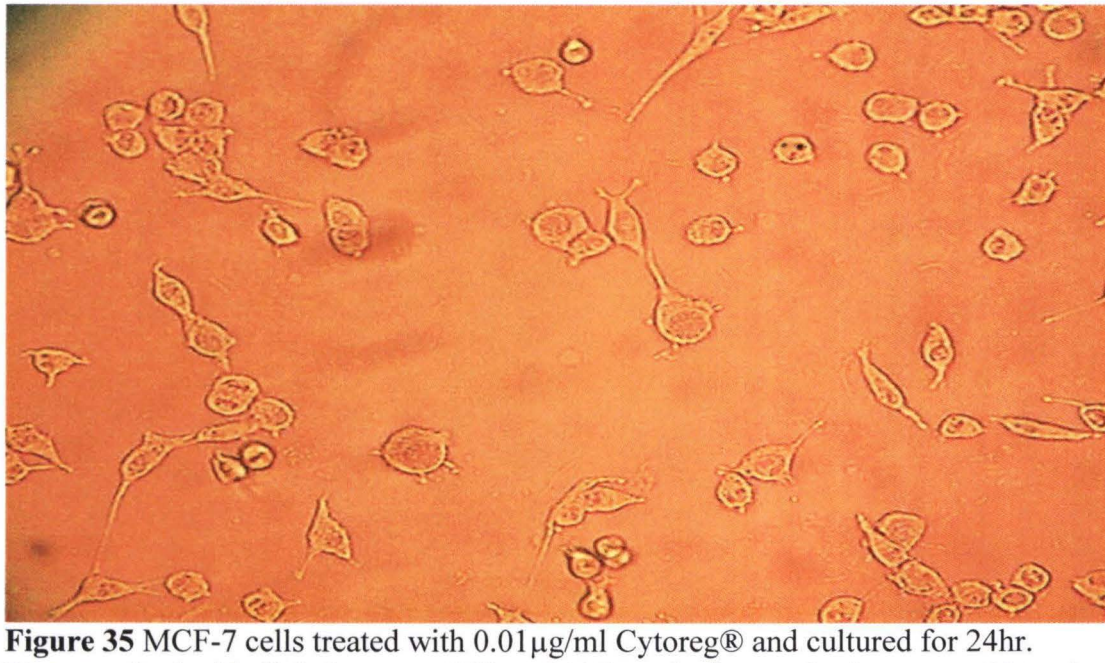

Figure 35 MCF-7 cells treated with 0.01µg/ml Cytoreg® and cultured for 24hr. Photographed with digital camera (Nikon 2000) under inverted microscope. Note the autophagy and initial apoptosis in the cells.

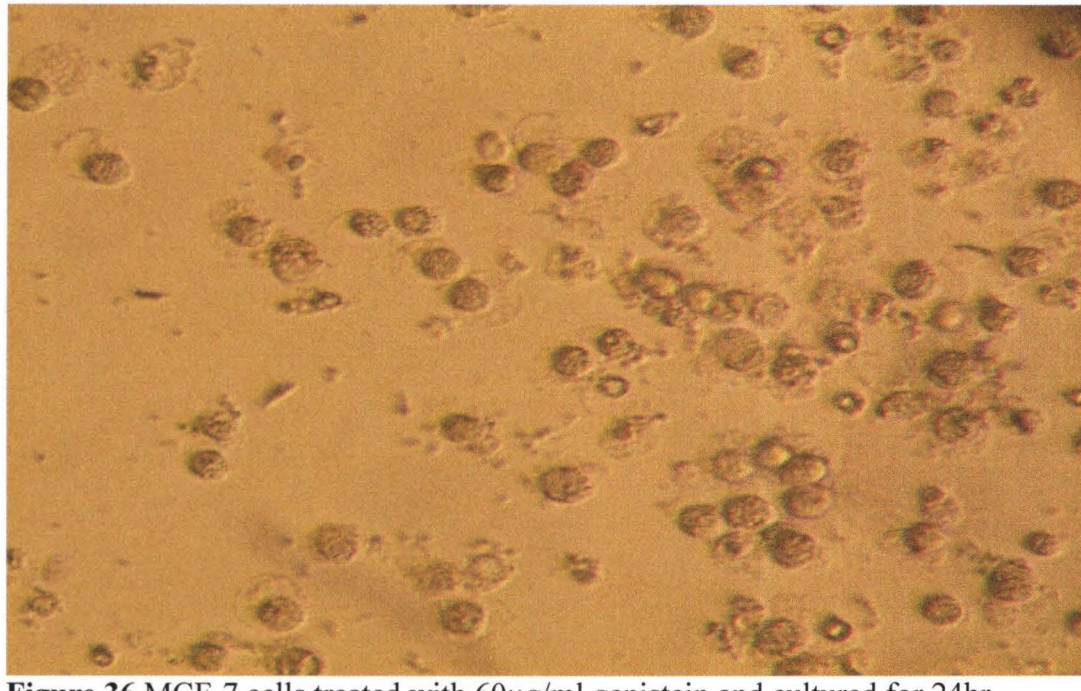

Figure 36 MCF-7 cells treated with 60μg/ml genistein and cultured for 24hr. Photographed with digital camera (Nikon 2000) under inverted microscope. Note apoptotic cells–nuclei shrinkage, condensed chromatin and formation of apoptotic bodies.

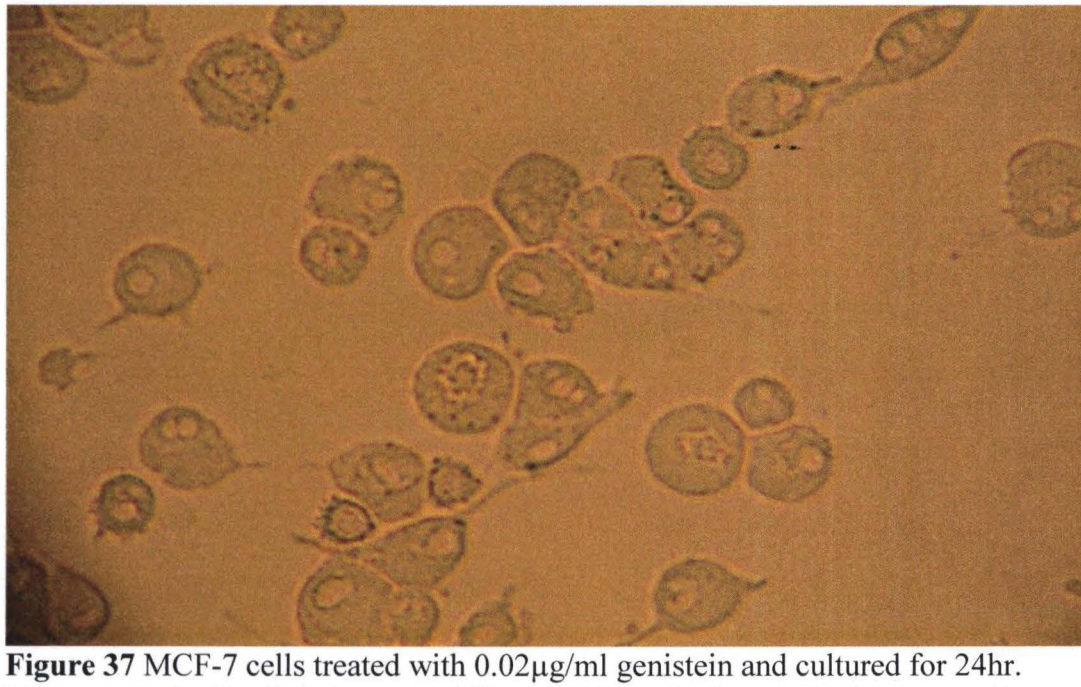

Figure 37 MCF-7 cells treated with 0.02μg/ml genistein and cultured for 24hr. Photographed with digital camera (Nikon 2000) under inverted microscope. Note autophagy and karyorrhexis in the cells.

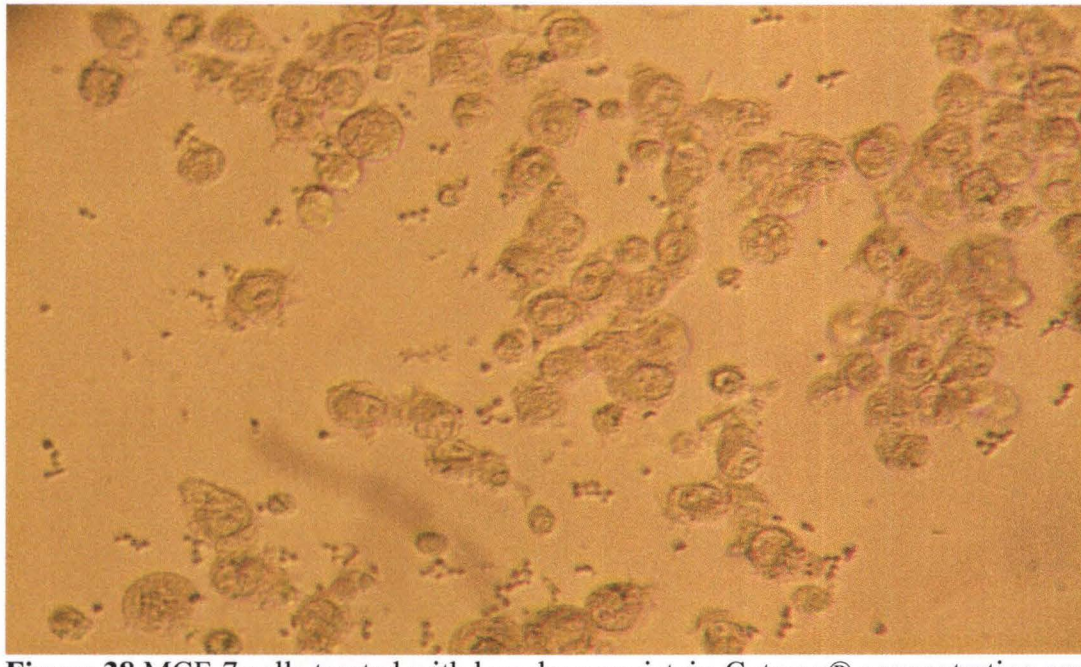

Figure 38 MCF-7 cells treated with low dose genistein-Cytoreg® concentration and cultured for 24hr. Photographed with digital camera (Nikon 2000) under inverted microscope. Note autophagy and apoptosis–nuclei shrinkage and condensed chromatin.

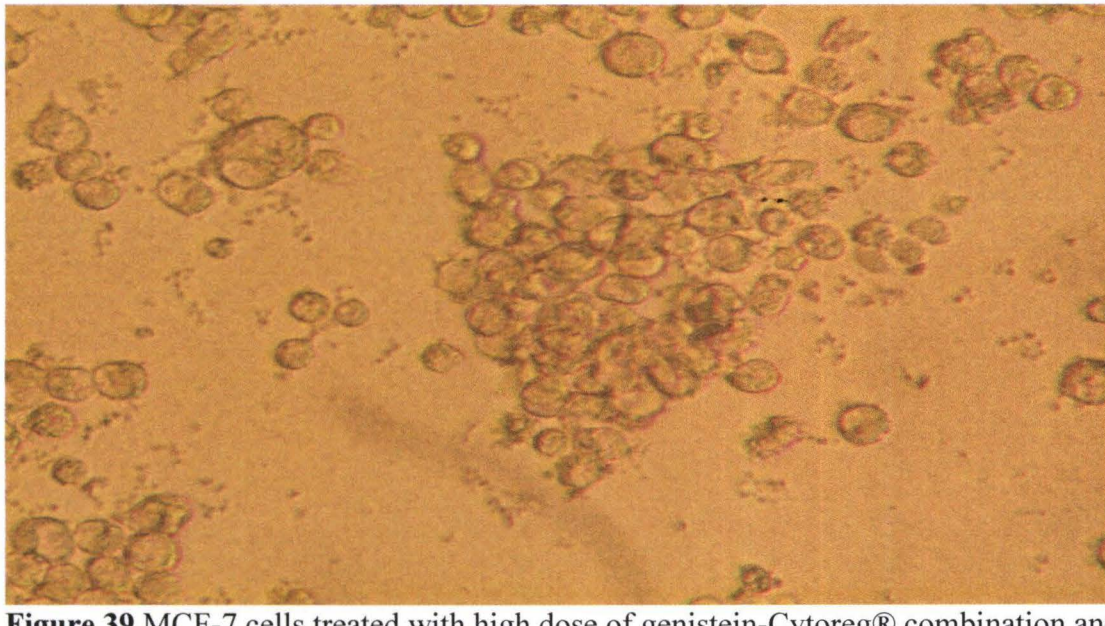

Figure 39 MCF-7 cells treated with high dose of genistein-Cytoreg® combination and cultured for 24hr. Photographed with digital camera (Nikon 2000) under inverted microscope. Note autophagy and apoptosis–nuclei shrinkage and condensed chromatin.