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Research Article

Derivatives of Phytoestrogen Genistein: An Approach to Cancer Treatment

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ABSTRACT

Phytoestrogens, phenolic non-steroidal compounds, extracted from plants are structurally or functionally similar of mammalian estrogens. They possess hormonal activity and can bind to estrogen receptors. Phytoestrogens consist of isoflavones, lignans and coumestans, in which Isoflavones are abundantly found in legumes mainly in soybeans. Out of all soy isoflavones, genistein have most potent estrogenic activity. Menopausal symptoms, such as hot flashes, urogenital atrophy, incontinence, insomnia, heart problems and osteoporosis are dominates in the end of menstrual cycle as estrogen level begin to decline. Genistein can act like weak estrogens by binding to the estrogen receptor on cell membranes and as estrogen antagonists by preventing estrogens from binding to the receptors, depending on their concentration and other factors. In prevention of hormone dependent cancer, cardiovascular diseases by improving plasma lipid concentrations, osteoporosis and cognitive decline, Genistein shows positive effects.

Keywords: Phytoestrogen, Estrogen receptor, Genistein, Derivatives.

INTRODUCTION

Most common cancer in women is breast cancer and the primary cause of death in women falling in age group between 30 and 54(Agarwal et al, 2008)[1] (Datta et al, 2012)[2]. Hormone-related risk factors such as late onset of menopause, delayed age of first pregnancy and elevated estradiol concentrations in postmenopausal women are known to be associated with breast cancer (McPherson et al, 2000)[3]. Now a day's chemotherapy is the most common technology for prevention of cancer, in which various synthetic and naturally agents are used to block the development of cancer. Natural products are used in medicine have been accepted as important source of cancer chemoprevention drug discovery and development (Mathewa et al, 2010.)[4]. More than 80% people in developing countries are dependent on traditional medicines for their primary health benefits.The medicinal plants provide active chemopreventive molecules and their products have antioxidant activity helps in protecting cells from damage (Bhanot et al, 2011)[5]. There are many agents found in fruits, vegetables and whole grains that have been shown to exert anticancer effect. Legumes are major source of proteins and calories and in addition, these are excellent source of nutraceuticals constitutes such as fibre, polyphenols such as flavonoids, isoflavones and tannins. Phytoestrogens widely present in legumes such as soybeans that contain non-hormonal properties which may result in anticancer effects. Estrogens are a class of steroidal hormones produced in mammals responsible for many physiological activities which are important in homeostatic regulation of many cellular and biochemical events. In humans, the three most important estrogenic hormones are estradiol, estrone and estriol. 17β-estradiol is generally the most active of the three, and may be converted to the less potent estrone or estriol by estrogen-metabolizing enzymes to regulate hormone levels. When estrogen level begin to decline with a woman's age or end of menstrual cycle takes place, menopausal symptoms dominates. Both deficiencies and excess of estrogen are associated with many health issues. Administration of estrogen (ERT) to postmenopausal women is beneficial in reducing the risk associated with estrogen deficiency. Unfortunately, this form of therapy is associated with health risks such as the development of estrogen-dependent tumors (breast and uterus cancer).

Phytoestrogen

Phytoestrogen are used as an alternative to the ERT because of the chemical structure similarity with estradiol. These are a class of plant produced steroidal compounds that are structurally or functionally similar to mammalian estrogens and play important role in prevention of menopausal symptoms, cancers, osteoporosis and heart disease. These may have the same effects as estrogen or block estrogen's effects. Their structural similarities to the human estrogens allow them to bind to estrogen receptors. These behaves like estrogen (agonist) and estrogen blocker (antagonist) at low and high doses. The affinity of genistein is comparable to that of estradiol. The affinity of other isoflavones is very low than that of estradiol. The effects of isoflavones depends on the level of endogenous estradiol, isoflavones and estradiol compete for their binding on ERs. And the interaction of isoflavones with ERs leads to the activation of estrogen response elements located on the inner side of the nuclear membrane. In this

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way transcription processes are affected. They are hydrolysed before entering the the blood stream and making isoflavones more water soluble, thus improving their excretion (Kuiper et al. 1998)[6]. The major groups of phytoestrogens found in the human diet are Isoflavones, which are found in soy bean and soy-based products. Isoflavone is found naturally in the form of glucoside and aglycone.

MATERIAL AND METHODS

Breast cancer cell lines

These are the cell line isolated from the cancerous breast tissues of cancer patients. There are various types of breast cancer cell lines having different properties. Here we are taking MCF-7 and MDA-MB 231 breast cancer cell lines in to account due to the large number of literature available on these 2 cell lines. For the presence of functionally significant adenosine receptor subtypes, MCF-7 and MDA-MB-231, human breast cancer cell lines, were screened. MCF-7 cells did not contain adenosine receptors as judged by the lack of an effect of non-selective agonists on adenylyl cyclase activity or intracellular Ca^{2+} levels but posses estrogen receptors and help to identify antiestrogenic and antiaromatase activity including other mechanism. MDA-MB-231 cells showed both a stimulation of adenylyl cyclase and a PLC-dependent increase in intracellular Ca^{2+} in response to non-selective adenosine receptor agonists but do not have estrogen receptors and does not explain antiestrogenic and antiaromatase activity (De Vincenzoe et al,1998)[7].

MCF-7 cell line

Supplement for MCF-7 cell line is DMEM media + 10% FBS, 1% penicillin/streptomycin and 1X of fetal bovine serum and maintaining condition is 37°C temperature in humidified, concentrated CO2 (5%) atmosphere (Singletary et al., 2002)[8].

MTT Assay

MTT assay is the assay widely use for the evaluation of anticancer activity of the drug on different type of cancer cell line. It is the most reliable method for the evaluation of anticancer activity at preclinical stage.

Principle of assay

This is a colorimetric assay in which reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) MTT by mitochondrial succinate dehydrogenase is measured. The MTT passes into the mitochondria after entering the cells where it reduced to an insoluble (dark purple) colored formazan product. After that cells are solubilized in an organic solvent Isopropanol, to solubilize formazan. Then released, solubilized formazan reagent is measured by spectrophotometric Technique. Since only metabolically active cells reduce MTT, so the level of activity is a measure of the viability of the cells (Soni et al, 2013)[9]. Phytoestrogens are phenolic non-steroidal plant extracted compounds. These belongs to a hetrogenous group of herbal substances. They are called estrogen like molecules because of their structural similarity to estradiol. These are structurally or functionally mimic mammalian estrogens and possess significant estrogen agonist/antagonist activity depending on estrogen concentration to show potential benefits for human health (Ososki et al, 2003)[10].

In vitro studies

The antiproliferative effect of isoflavones have been observed by researchers at high concentrations not only in estrogen dependent cells that is MCF-7 cell line but also in estrogen independent cells such as MDA-MB-231 cell line. Growth inhibition of tumor cell by phytoestrogen may be mediated by different mechanisms. It is suggested that genistein may induce apoptosis in breast cancer cell lines and produce synergistic inhibitory effect when combined with cancer therapies. These have been shown to induce apoptosis in high-invasive MDA-MB-231 and the low invasive MCF-7 breast cancer cell lines at high concentration (Nikov et al, 2001)[11]. Genistein showed a biphasic effect, at high concentrations (25-100µM), it reduced the proliferation of MCF-7 cells and at low concentrations (1-10µM), it stimulated the cell proliferation (Wietrzyk et al, 2005)[12]. Similarly, daidzein inhibited the cell proliferation at higher concentrations (50-100µM) and stimulated the proliferation at low concentrations but the inhibition and proliferative effect was much weaker than that of genistein (Zhang et al, 2007)[13]. Genistein inhibits the growth of MDA-MB-231 breast cancer cells via regulating the expression of apoptosis related genes and through p53 independent pathway it induces apoptosis (Li et al, 1999)[14]. These may inhibit cell proliferation through G2/M cell cycle arrest and induce cell apoptosis in MDA-MB-231 cells (Li et al, 2008)[15].

Synthesis of Genistein derivatives

All the reactions and the purity of products were monitored using thin- layer chromatography, visualizing the spots under ultraviolet light and iodine chamber. Mass spectra were determined by TOF/ TOF ™ Series Explorer ™ 72027.

Synthesis of 4-(5,7-dihydroxy-4-oxo-4H-chromene-3-yl) phenyl benzoate (G3)

A suspension of genistein (1mg) and potassium carbonate in freshly distilled DMF (2ml) were stirred at room temperature under nitrogen gas for 2 hours. Freshly distilled BzCl (1ml) was added and the reaction mixture was stirred overnight and then poured into water and neutralized with HCl (10%). The precipitated product was filtered off and washed with water and dried.

Synthesis of 4-(8-chloro-5,7-dihydroxy-4-oxo-4H-chromene-3-yl) phenyl benzoate (G4)

To the 1gm of 4-(5,7-dihydroxy-4-oxo-4H-chromene-3-yl) phenyl benzoate, thionyl chloride (2ml) was added and kept on stirring at room temperature for 5 hours. And the precipitated product was collected.

Cytotoxic studies of genistein derivatives (cell line studies)

Preparation of DMEM media

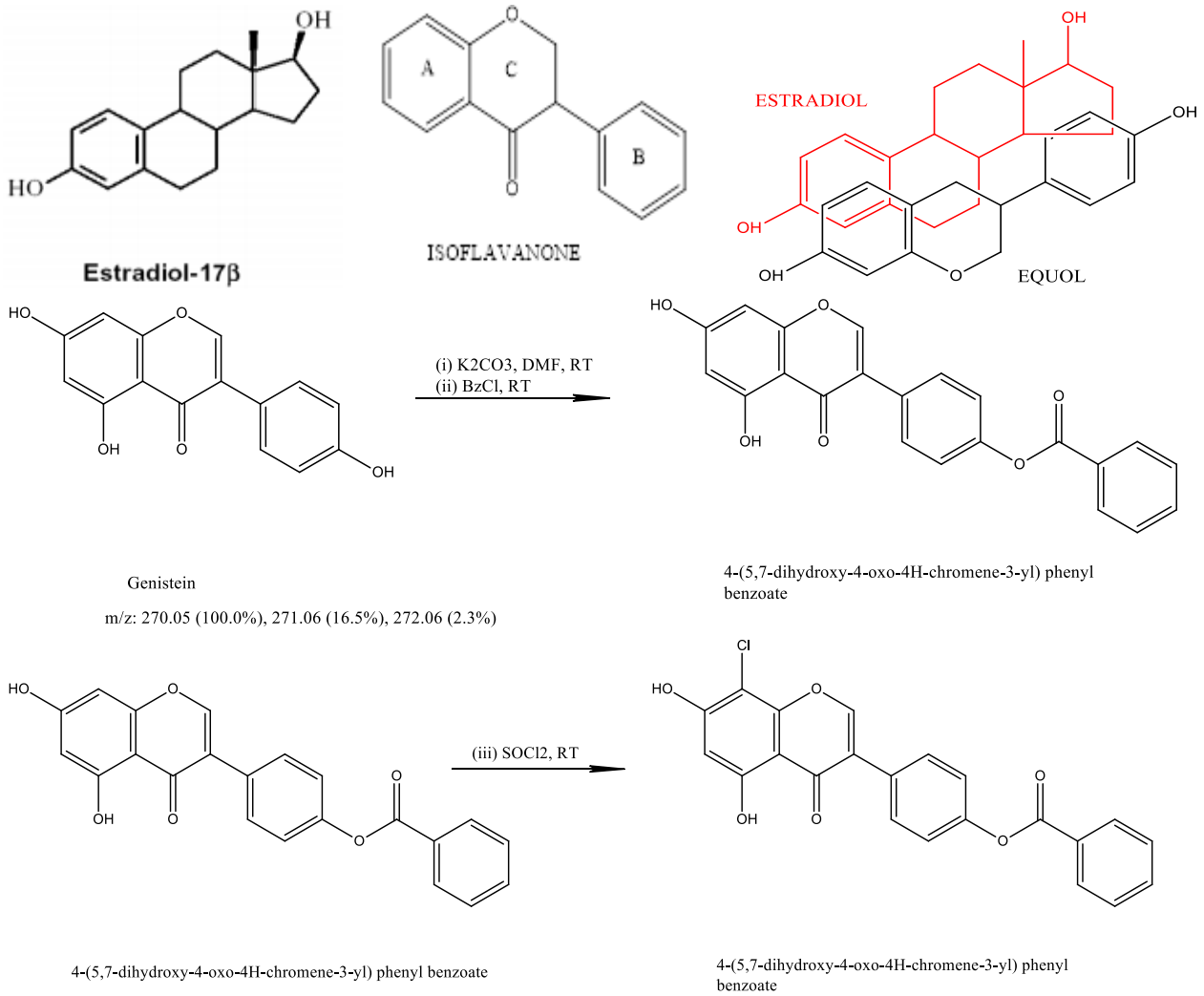
13.5 gm of sterile powdered DMEM along with 3.7 gm of $NaHCO_3$ was dissolved in 700 ml of autoclaved distilled water. The medium was dissolved thoroughly and the pH was adjusted to 7.2 by using 0.1M HCl and 0.1M NaOH. The final volume was adjusted to 1000 ml by using autoclaved distilled water. Then the media was filtered by Millipore disc filter (0.22 um). Filtered medium was kept

Table 1: Calculation of % cell inhibition from absorbance values.

S. No	Concentration (ug/ml)	Mean Absorbance	$[(A_c-A_t)/A_c]$ x 100	% Inhibition
1.	200	0.06	[(0.75-0.06/0.75)] x 100	92.2
2.	100	0.07	[(0.75-0.07/0.75)] x 100	90.9
3.	50	0.08	[(0.75-0.08/0.75)] x 100	89.4
4.	25	0.08	[(0.75-0.08/0.75)] x 100	88.7
5.	10	0.10	[(0.75-0.10/0.75)] x 100	87.0
6.	0.5	0.13	[(0.75-0.13/0.75)] x 100	83.1

Table 2: Calculation of % cell inhibition from absorbance values.

S. No	Concentration (ug/ml)	Mean Absorbance	$[(A_c-A_t)/A_c]$ x 100	% Inhibition
1.	200	0.06	[(0.75- 0.06/0.75)] x 100	92.2
2.	100	0.08	[(0.75-0.08/0.75)] x 100	88.8
3.	50	0.14	[(0.75-0.14/0.75)] x 100	81.6
4.	25	0.18	[(0.75-0.18/0.75)] x 100	75.4
5.	10	0.27	[(0.75-0.27/0.75)] x 100	64.5
6.	0.5	0.45	[(0.75-0.45/0.75)] x 100	41.4



overnight in CO_2 incubator to check the growth of any contamination.

Preparation of antibiotic solution (Pen strep)

1ml of 100x Pen Stop solution was pipetted out and dissolving it in 9 ml autoclaved distilled water and stored at -20° C.

Preparation of complete cell culture media

8.9 ml of DMEM media is added to the falcon tubes. 1 ml of fetal bovine serum is also added to the DMEM media. 0.1 ml of Antibiotic solution is added to the DMEM media completing the Cell culture media. 5Aliquots of 10 ml each of Serum were prepared to prevent regular freezing & thawing of it and were stored at -20°C.

Preparation of Phosphate Buffer Saline, 1x (PBS)

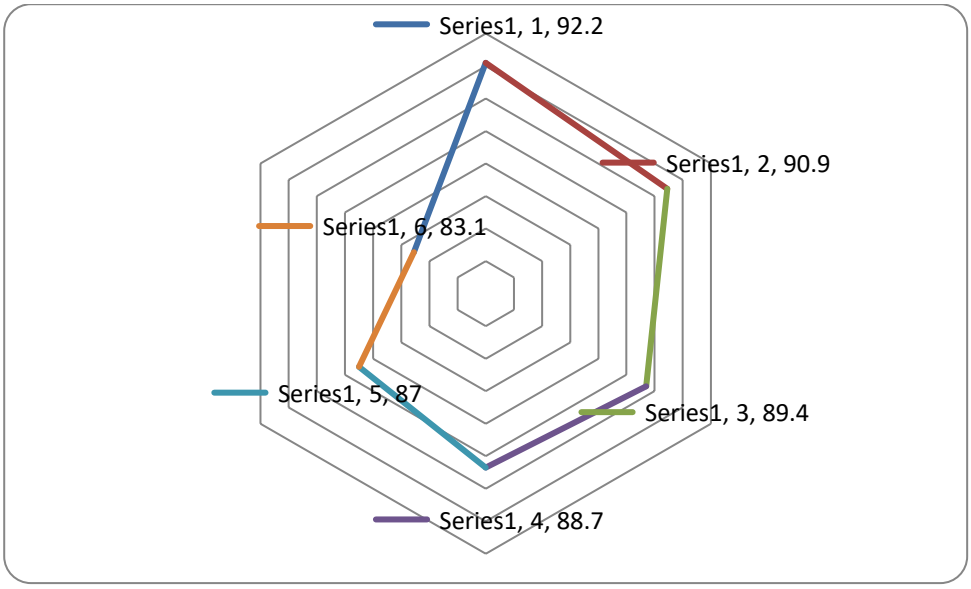


Figure 1: (% cell inhibition on MCF7 cell lines)

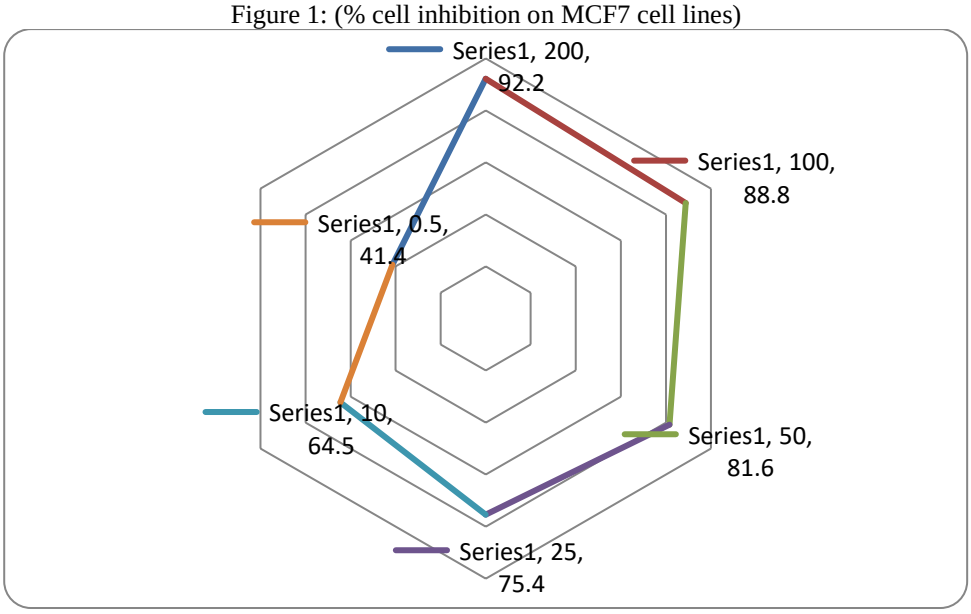


Figure 2 : (% cell inhibition on MCF7 cell lines)

Weigh 8 gm of Sodium Chloride (NaC1), 0.2 gm of potassium Chloride (KCl), 1.44 gm of Sodium hydrogen phosphate (Na_2HPO_4) and 0.24 gm of Potassium di-hydrogen phosphate (KH_2PO_4). All the salts were dissolved in distilled water. Then pH was adjusted to 7.4 using 0.1M HCl before making-up the final volume to 1000ml. Solution was sterilized by autoclaving.

Cell Counting

Cell counting is an important procedure which helps in the determination of the number cells present in the suspension. It is useful during processes like storage, revival and also for experiments like cell cytotoxicity assays.

The concentration of a cell suspension may be determined by placing the cells in an optically clear chamber under a microscope. The cell number within a defined area of known depth is counted and the cell concentration is derived from the count.

Cell counting by hemocytometer

The hemocytometer was cleaned with 80-90% ethanol. Cover slip was cleaned and was Press down in order to attach it properly to the slide. Cell culture was trypsinized and was resuspended in a fresh medium. The suspension was mixed thoroughly to disperse the cells equally and 1ml of suspension was pipette out into a vial. The cells were pipetting vigorously to disperse any clumps, from this 20 ul was collected. Suspension of cell was immediately transferred to the edge of the hemocytometer chamber, and was expelled out under the cover slip by capillary action. Overfilling the chamber was inhibited as this would change its volume. Cells lying in the central area were counted for both chambers. For routine sub culture, count between 100 and 300 cells per $mm^{2.}$

Analysis

Calculate the average of two counts and derive the concentration of sample by using the formula: C= n/v

Where, C- Cell concentration (cells/ml)

n -Number of cells counted, and

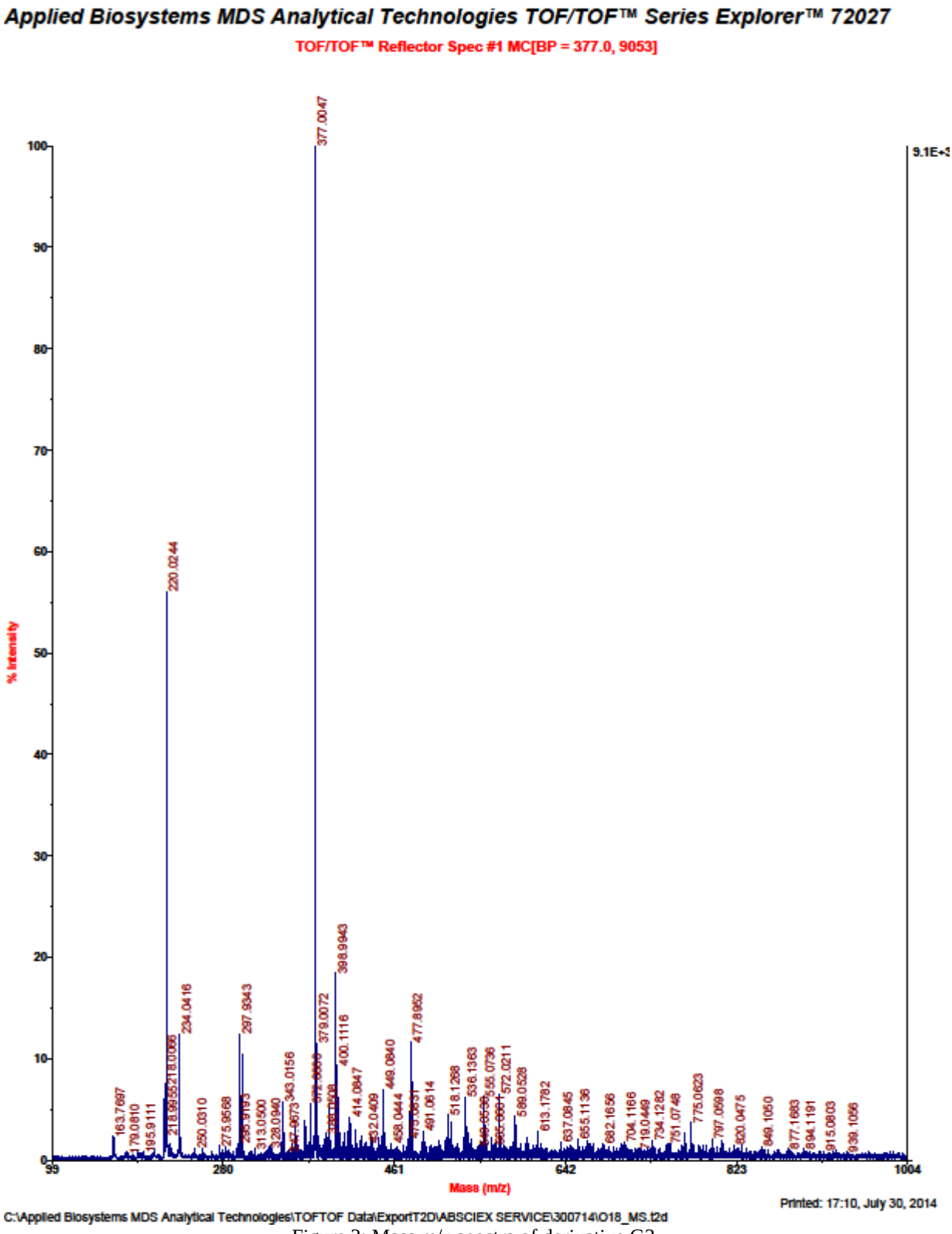


Figure 3: Mass m/z spectra of derivative G3.

v - Volume counted (ml)

For the improved Neubauer hemocytometer, the depth of the chamber is 0.1 mm and the central area $1mm^2$; therefore v is 0.1 mm^3 or 1 x 10^{-4} ml. The formula then becomes:

$c = n/10^{-4}$ or $c = n \times 10^4$

Observed cell concentration:

C= n/v

Cell proliferation assay

Measurement of cell viability and proliferation forms the basis of in vitro assays of a cell population. The MTT cell proliferation assay measures of cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis the reduction in cell viability.

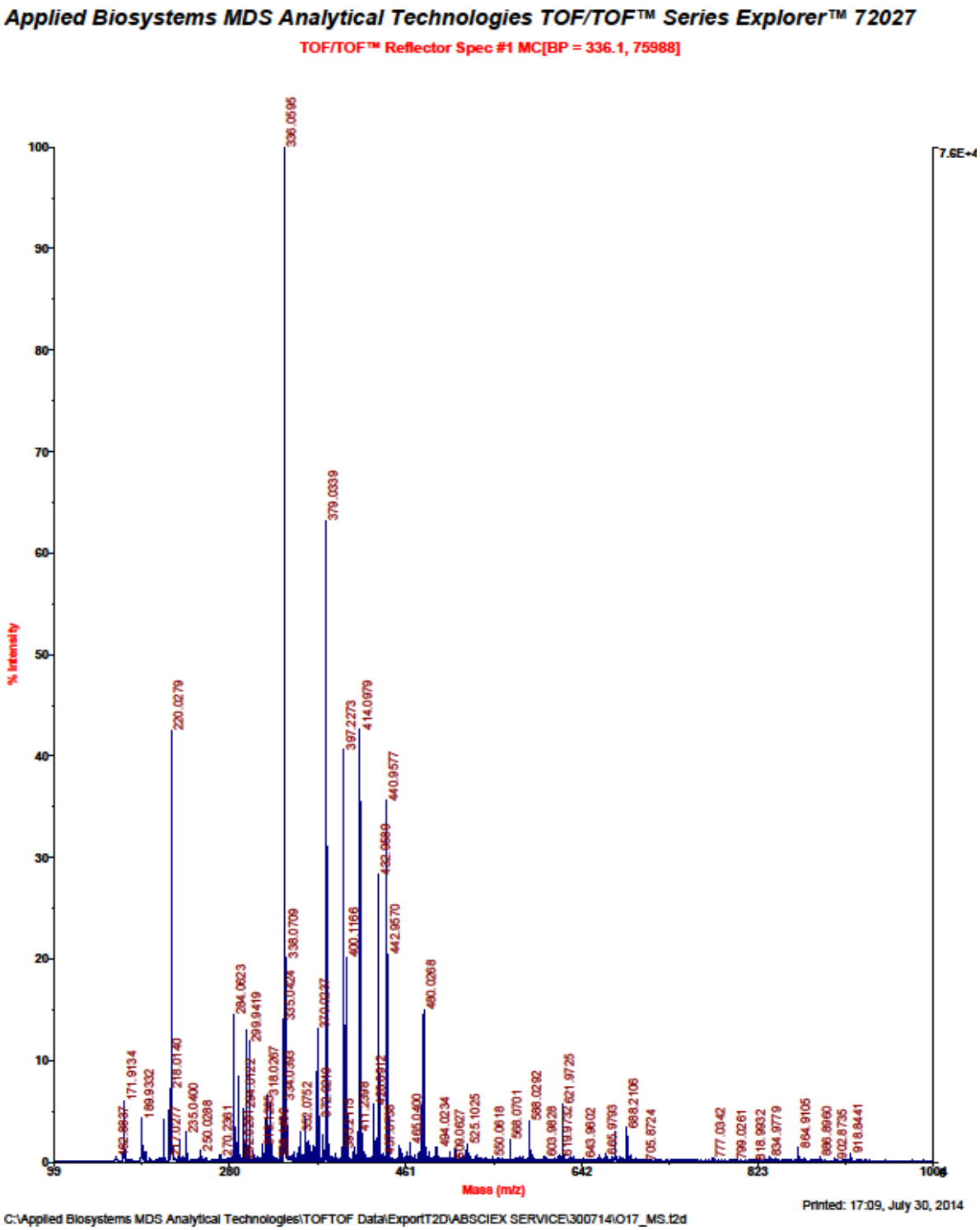


Figure 4: Mass m/zspectra of derivative G4.

Preparation of stock solution of test samples

Protocol for cell proliferation assay

Adhered cells were harvest from the culture by trypsinization followed centrifugation at 1500 rpm for 5 min. Cell count was done and was again re suspended at 1x105/ml in fresh medium. Cells were then placed plated in 96 well plate in triplicates. These were then incubated overnight in CO_2 incubator to get adhered. These were then treated with different concentration of standard drug and were marked as control cells. Cells were again incubated for 24 hrs. MTT reagent at a concentration of 10ul/ml was added to each well, including the control and was again placed in incubator for 4hrs. Carefully media was removed without disturbing the cells. 100 ul of DMSO (MTT solvent) was added in each well and was covered with aluminium foil. This was placed in dark for 4hrs. Absorbance was then measured at 492nm in ELISA plate reader.

Cell viability (Dye Exclusion assay)

Protocol

During the experiment with the cell revival procedure viability measurements were done to know the % of viable cells. Trypan blue was used 1-2 min before to measure cell viability. cell suspension and trypan blue were mixed in 1:1 ratio of 1-2 min before counting. Counting was done using hemocytometer placed under microscope. Dead cells under the microscope looked blue as these cells take up the dye through their damaged plasma membrane while the viable cells looked colourless behind dark background.

Analysis

Count total number of cells and total number of viable (unstained) cells. Percent viable cells as follows

%viable cells= (number of unstained cells/ total number of cells) x 100

Observation

% viable cells = (number of unstained cells / total no of cells) x 100

RESULT AND DISCUSSION

Mass spectroscocopic studies of Genistein derivatives

Mass spectroscocopic studies of G3

The expected mass of the compound was found out to be 374m/z and we got m+1 peak at 377 m/z as shown in Fig 3.

Mass spectroscocopic studies of Genistein G4

The expected mass of the compound was found out to be 408 m/z and we got m+1 peak at 411 m/z as shown in Fig 4.

MTT assay study (G3)

MTT assay study (G4)

CONCLUSION

Soybean are excellent source with a low content in saturated fat and a great amount of dietary fibre. Isoflavones are natural phenolic compounds. The structure similarity to estrogens allows these compounds to bind to estroegn receptors. It is beneficial to treat many disease related to estrogen deficiency in females. This work includes the extraction of isoflavones and isolation of genistein to check the yield, purity to produce the valuable compound in economic cost. The Mass analysis report of the synthesized genistein derivatives showed m+1 peak at 371 and 411 m/z. And these are expected to possess anticancer activity like genistein.

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