

EVALUATION OF ANTICANCER PROPERTY OF GOSSYPOL USING HeLa CELL LINE

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ABSTRACT

Cervical cancer is the fifth most common cancer in humans, the second most common cancer in women worldwide and the most common cancer causing death of women in the developing countries. Gossypol, a polyphenolic compound extracted from cotton plants, has been found to be an anticarcinogenic agent. The aims and objectives of our study were to evaluate the anticancer property of Gossypol, to find the IC_{50} value for Gossypol & to find the cell viability using MTT assay. The cells were incubated with different concentrations 1%, 0.75%, 0.5%, 0.25%, 0.125%, 0.062% and 0.031% for 24 and 48 hours. After gossypol treatment, the cytotoxic effects were measured with MTT tests. Finally, the IC_{50} lies on 0.125%.

Keywords - Anticarcinogenic, cell line ,Cervical cancer, Gossypol, HPV, IC_{50} , MTT, Trypsinization.

I. INTRODUCTION

1.1 Tissue Culture

Culturing of tissues in a favorable artificial environment is called tissue culture. Tissue culture is divided into two types: (i) primary cultures and (ii) secondary cultures. Primary cell culture is the maintenance of growth of cells dissociated from the parental tissue. The (I) Primary cell culture could be of two types depending upon the kind of cells in culture (Jacoby *et al.*, 1979). They are Adherent cells and Suspension cells. Adherent Cells require attachment for growth are said to be anchorage dependent cells and Suspension Cells which do not require attachment for growth are anchorage independent cells/suspension cells. For example is lymphocyte. (ii) Secondary cell cultures: When a primary culture is subcultured, it is known as secondary culture or cell line. A cell line or cell strain may be finite or continuous depending upon whether it has limited culture life span or it is immortal in culture. On the basis of the life span of culture, the cell lines are categorized into two types. One is Finite cell lines and another one is infinite cell line. The Cell lines which have a limited life span and go through a limited number of cell generations (usually 20-80 population doublings) are known as finite cell lines. The Cell lines which are transformed under laboratory conditions or *in vitro* culture conditions give rise to continuous cell lines. They grow either in a monolayer or in suspension. The growth rate is rapid and doubling time can be 12-24 hours (Capes et al, 1993).

1.2 HeLa Cells

A HeLa cell is a cell type in an immortal cell line used in scientific research. It is one of the oldest and most commonly used human cell lines. The line was derived from cervical cancer cells taken on February 8, 1951, from Henrietta Lacks, a patient who eventually died of her cancer on October 4, 1951 (Walboomers 1999). The cell line was found to be remarkably durable and prolific as illustrated by its contamination of many other cell

lines used in research (Rahbari et al., 2009). HeLa cells have been reported to contain human papilloma virus 18 (HPV-18) sequences (Singh 2005). Culture conditions for HeLa cells are air is 95%, carbon dioxide (CO₂) is 5% and temperature is 37°C (Batts 2010). The Advantage of HeLa Cells is developed standardized methods for culturing cells. It is used to develop methods for accurately determining the number of chromosomes in cells beneficial for cancer research. Used to study effects of radiation and used to test safety of cosmetics and pharmaceuticals replacing lab animals.

1.3 Gossypol

Gossypol is a natural phenol derived from the cotton plant (genus *Gossypium*). Gossypol is a phenolic aldehyde that permeates cells and acts as an inhibitor for several dehydrogenase enzymes. It is a yellow pigment. Gossypol is a non-volatile yellow pigment first isolated in 1889 from the seeds, roots, and stems of cotton plants of the genus *Gossypium* (family: Malvaceae) the Scientific Name(s): *Gossypium* spp. Family: Malvaceae (mallow), Common Name(s): Gossypol, AT-101, ApoG2. *Gossypium* are shrubs that grow to a height of 3 m, have broad lobed leaves, and contain seeds in a capsule or “boll” of fibers. These fibers are harvested and woven in the textile industry. The seeds of *Gossypium* species vary widely in gossypol content, with levels ranging from 0.13% to 6.6%. Gossypol was first identified as an antifertility agent as a result of epidemiologic studies conducted in China during the 1950s. Investigators had been puzzled by the extremely low birth rates in a particular geographic region. Further investigation revealed that the antifertility component was gossypol. Gossypol can have various chemical structures Shown in Fig(1). Gossypol's main function in the cotton plant is to act as an enzyme inhibitor (Wang *et al.*, 2009).

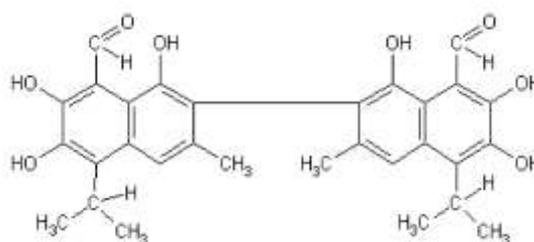


Figure 1: Chemical Structure of Gossypol

Gossypol reduces mitotic index and decreases the rate of DNA synthesis to some extent in all types of cell tested, including tumor cells. Many studies reported that protein synthesis can also be reduced in various cell lines, while others have identified various ways in which gossypol can arrest cell growth by inhibiting enzymes involved in DNA replication. Several studies showed that gossypol promotes apoptosis in tumor cells (Adams *et al.*, 1960). Gossypol also uncouples mitochondrial oxidative phosphorylation; chelates iron, copper, aluminum and zinc; manifests both pro-oxidant and antioxidant characteristics; alters membrane potential, fluidity and permeability; binds to tubulin and inhibits microtubule assembly; and disrupts gap junctions and cell-cell communication before causing appreciable cytotoxicity (Berardi 1969). Oncology testing is a Gossypol is also under investigation as a possible chemotherapy drug. It is currently believed that gossypol in itself will not kill cancerous cells; however, it changes the chemistry within the cancer cell and makes it more susceptible to traditional chemotherapy drugs. Phased trials have been done on resistant prostate and lung cancer.

II. REVIEW OF LITERATURE

Esra *et al.*, 2006 [1] Gossypol, a polyphenolic compound extracted from cotton plants, has been found to be an anticarcinogenic agent. The aim of our study was to investigate whether gossypol induced cell death on ME-180 cervix cancer cells, and whether it was a potent inhibitor of some antioxidant enzymes, like catalase, glutathione reductase and glutathione-S-transferase. The cells were incubated with four different doses (5, 10, 15 and 20 μ M) for 24, 48 and 72 hours. After gossypol treatment, the cytotoxic effects were measured with MTT tests. Using DNA agarose gel electrophoresis, cellular internucleosomal DNA fragmentation of the cells treated with gossypol and untreated was examined. Consequently, gossypol caused different fragmentation on tumour cells due to apoptosis. Gossypol was found to be a potent inhibitor of catalase, glutathione reductase and glutathione-S-transferase.

Yi Wen *et al.*, 2006 [2] Gossypol, a natural compound present in cottonseeds, displays antiproliferative and pro-apoptotic effects against various cancer cells. The (-)-gossypol enantiomer is a more potent inhibitor of cancer cell growth. Here, the molecular mechanisms of apoptosis induced by (-)-gossypol were studied in human prostate cancer cells. The effects of (-)-gossypol on the expression of apoptotic-regulated gene markers in both death receptor- and mitochondria-mediated apoptotic pathways, such as the Bcl-2 family and caspase, etc., were detected by RT-PCR and Western blot analysis. (-)-gossypol also activated caspases-3, -8 and -9 and increased PARP [poly (ADP-ribose) polymerase] cleavage. By using caspase inhibitors, (-)-gossypol caused apoptosis via the caspase-dependent pathways.

Jiahua *et al.*, 2004 [3] Racemic gossypol, a naturally occurring polyphenolic yellow pigment present in cottonseed products, inhibits in vitro proliferation of Dunning prostate cancer cells (human prostate cancer cells derived from a bone marrow metastasis (PC3)), MCF-7 and primary cultured human prostate cells. The results show that (\pm)-GP caused reductions in DNA synthesis and prolonged the DTs in PC3 cells. RT-PCR and ELISA results show that (\pm)-GP elevate the mRNA expression and protein secretion of transforming growth factor beta1 in PC3 cells. Consistent with these findings, (\pm)-GP has been shown to decrease the cyclin D1 mRNA expression and protein expression in PC3 cells. These results indicated that the inhibitory effects of (\pm)-GP on the proliferation of human prostate cancer PC3 cells are associated with induction of TGF, β 1, which in turn influences the expression of the cell cycle-regulatory protein, cyclin D1, in prostate cancer cells.

Shawky 2007[4] The anti-proliferative activity and mitochondrial toxicity of gossypol in endometrioma cells was maintained in short-term cultures. Three endometrioma cell lines from patients were treated with 25 or 50 nmol/L gossypol for up to 12 days. The effect of gossypol on the cell growth was recorded. A phosphorescence oxygen analyzer was used to determine the effects of gossypol on mitochondrial oxygen consumption of six endometrioma cell lines from patients. Cellular gossypol accumulations in three endometrioma cell lines from patients were measured by high-pressure liquid chromatography. Proliferation of the endometrioma cells was inhibited by 25 and 50 nmol/L gossypol. Respiration of the endometrioma cells was inhibited by 10 μ mol/L gossypol. Cellular gossypol was detected in the endometrioma cell lines that were treated for 24hrs with 10 and 0.3 μ mol/L gossypol.

Maryam Mehrpour¹ *et al.*, 2009 [5] Gossypol is the Bcl-2 family proteins regulate commitment to apoptosis primarily through their capacity to control the permeability of the mitochondrial outer membrane permeabilization (MOMP) which triggers the release of multiple apoptogenic factors into the cytosol and thereby apoptosis. Various Bcl-2 family members affected this key event of the apoptotic cascade in different

ways, determining their pro- or anti-apoptotic status. The Bcl-2-type proteins inhibit MOMP, thereby preserving cell viability. In contrast, Bax-type proteins and the diverse group of BH3-only proteins facilitate MOMP and thus promoted cell death. Recently, several drugs that act as BH3 mimetics have been identified, including Gossypol. Their review revisits the properties of the gossypol family, their use as anticancer agents for cancer therapy.

Suresh *et al.*, 2010 [6] Prostate cancer (PCa) continues to represent a burgeoning medical problem in the United States. Recent studies suggested that gossypol, a bioactive phytochemical produced by cotton plants, is a promising agent against prostate cancer. Their current studies were undertaken to examine the chemotherapeutic efficacy of gossypol on human prostate cancer cell lines and prostate tumor initiating cells. Gossypol reduced viability of three prostate cancer cell lines with an IC₅₀ between 3–5 µM. These studies demonstrate for the first time that gossypol treatment induces DNA damage and activates p53. Collectively, this data supports the use of gossypol as a novel agent for PCa.

Foong Ying Wong *et al.*, 2012 [7] Over expression of Bcl-2 has been associated with gemcitabine resistance. The aim of this study is to determine whether Gossypol can overcome gemcitabine resistance in cell lines with high level of Bcl-2 expression in combination drug therapy. Their study demonstrated that in 10 cell lines derived from different cancers, high Bcl-2 baseline expression was observed in cell lines that were resistant to gemcitabine. Furthermore, synergistic effect of combination therapy was observed in gemcitabine-resistant cell lines with high Bcl-2 expression, but not in gemcitabine-sensitive cell lines regardless of Bcl-2 expression. Gossypol treatment resulted in the decrease of anti-apoptotic genes such as Bcl-2 and Bcl-xl and an upregulation of the pro-apoptotic gene.

III. METHODOLOGY

3.1 Thawing of Cryopreserved Cells

Thawing of cells is defined as the transfer of cryo cells to room temperature. Thawing of cells is of two types depending on the cryoprotectant. They are rapid thawing and slow thawing. In most of the cases, rapid thawing is used.

3.1.1 Procedure

The cryovial containing the frozen cells from liquid nitrogen is taken and immediately placed into a 37°C water bath. The cells were thawed out quickly (1 minute) by gently swirling the vial in the 37°C water bath until there is just a small bit of ice left in the vial. The vial was transferred into a laminar flow hood. Before opening, the outside of the vial was wiped with 70% ethanol. The desired amount of pre-warmed saline appropriate for the cell line is transferred drop wise into the centrifuge tube containing the thawed cells. Cell suspension is centrifuged for 5–10 minutes. After the centrifugation, pellets were collected. Then the cell pellet was re-suspended in complete growth medium, and transferred into the appropriate culture vessel and into the recommended culture environment.

3.2 Cell Counting Using a Haemocytometer

It is often necessary to count cells, for example, when plating cells for transfection experiments. One method for counting cells is to use a haemocytometer shown in fig 2. A haemocytometer contains 2 chambers. Cell concentration is determined by counting the number of cells within a defined area of known depth (volume).

3.2.1 Procedure

The cell suspension was mixed with trypan blue in the ratio 1:1. Cell suspension was loaded in the hemocytometer and the cells were counted under a microscope. The total number of cells presented in the suspension was calculated using the formula below:

$$\text{Average cell count} \times \text{dilution factor} \times 10^4 \times \text{final volume}$$

The average cell count was calculated by taking an average of cells in the four corner chambers or the opposite corner chambers. 10^4 is the total volume of all the four chambers.

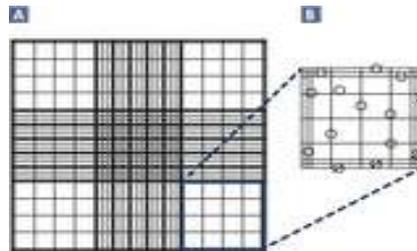


Figure 2: Cell Count Using Haemocytometer

3.3 Viability Staining

Trypan blue staining provides a method for distinguishing between viable (i.e., capable of growth) and nonviable cells in a culture. This staining method is based on “dye exclusion”: cells with intact membranes exclude the dye and are considered viable.

3.3.1 Procedure

Harvest the cells by trypsinization (adherent cell cultures) and resuspend the cells in an appropriate volume of medium to give a cell density of at least 10^6 cells/ml. Add 10 μ l of trypan blue to 10 μ l the cell suspension. Mix thoroughly, and allow standing for 1–2 min.

$$\% \text{ viability of cells} = (\text{No. of viable cells} / \text{Total No. of cells}) \times 100$$

Then, count the stained and unstained cells using a hemocytometer. Blue-stained cells are nonviable and unstained cells are viable.

3.4 Trypsinization

Trypsinization shown in Fig 3 and 4 before and after is a technique that uses the proteolytic enzyme trypsin to detach adherent cells from the surface of a cell culture vessel. This procedure is performed whenever the cells need to be harvested (e.g., for passaging, counting, or for nucleic acid isolation).

3.4.1 Procedure

Medium was discarded from the dish and washed with saline. 3mL of trypsin/EDTA solution was added. 2-3 minutes were allowed for trypsin to work.



Figure 3: Before Trypsinization



Figure 4: After Trypsinization

Reaction of trypsin was terminated with DMEM medium containing FBS, when ~ 50% of cells were floating, the suspension was collected in a centrifuge tube. Centrifuged at 1500rpm for 10-15 minutes. Supernatant was discarded and the pellet was re-suspended in the medium.

3.5 MTT Assay

Day 1: Dish was taken from the incubator and the medium was discarded. Wash with saline to remove the trace amount of globular protein and other compounds in the dish. The saline was discarded and trypsin was added for 2 to 3 mins. Media was added to inhibit the activity of trypsin and centrifuged for 10 to 15 mins at 1500 rpm and pellet was formed. The pellet should be dissolved with media. From this, we took 10 µl of sample and 10 µl of dye (trypan blue) and counted the cells using haemocytometer. The cells were prepared in 96 well microtiter plates containing a final volume of 100 µl / well. They were incubated for 24 hrs.

Day 2: The cells were observed using an inverted microscope. The test compounds and controls were prepared, added in 96-well micro titer plates containing a final volume of 100 µl/well. The drug concentrations were 0.031%, 0.062%, 0.125%, 0.25%, 0.5%, 0.75%, 1% by serial dilution method. 96 well plates were taken from the incubator and the medium was discarded. The controls and concentration of drugs were also added in the 96 well micro titer plates. It was incubated for 24 and 48 hrs.

Day 3: 50 µl MTT Solution was added per well. It was incubated 3 to 4 hours at 37°C in a CO₂ incubator and formazan crystals were formed. 100 µl of DMSO solution was added to each well to dissolve formazan crystals. It was mixed to ensure complete solubilization. It was incubated for 1 hour. Absorbance was recorded at 545 nm using ELISA reader.

$$\% \text{ Vehicle Control} = \{ \text{Vehicle control} / \text{Media control} \} \times 100$$

$$\% \text{ Cell Viability} = \{ \text{Concentration} / \text{Media control} \} \times 100$$

IV. RESULTS & DISCUSSION

4.1 Observations of Crystal Formation

The cells were observed using a microscope. Cell death increases when the concentration decreases. Then, the crystals were formed based on the concentration of drug (Gossypol). Shown in figure 5.

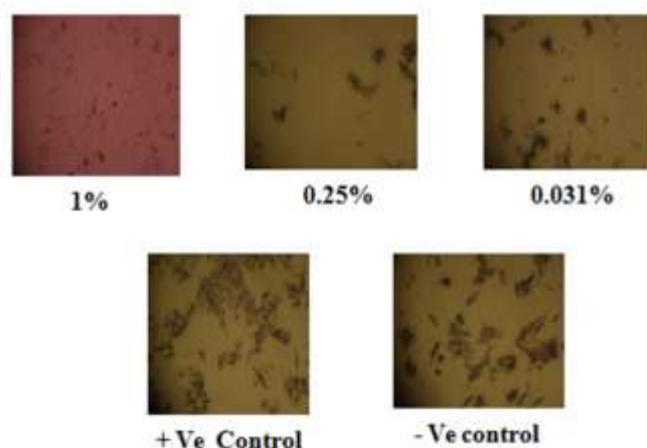


Figure 5: Crystal Formation at Different Concentration

Since 1970, though studies have concentrated on the contraceptive effects of gossypol, recently more interest has been emphasized on its antitumoral effects. It has been known that Gossypol has antiproliferative effects on different cancer cells. Gossypol generally exhibits many biological activities by disturbing cellular energy metabolism. Gossypol was shown to be the most potent inhibitor of tumor cells using MTT assay. Gossypols have been found to be active against a wide variety of types cancer, and have been shown to suppress multidrug-resistant cells and metastatic tumors. In several clinical trials, gossypols have been shown to meet toxicity criteria and are well tolerated in patients with metastatic adrenal cancer, malignant gliomas, and refractory metastatic breast cancer. Gossypol was reported to arrest cell cycle at the G_0/G_1 phase (Van poznak 2001). Gossypol has also been demonstrated to inhibit the proliferation of cancer cells by inhibiting nuclear enzymes, such as DNA polymerase α and topoisomerase II, suppressing DNA synthesis, reducing protein kinase C activity (Hu *et al.*, 1993).

Concentration in %	Cell viability
0.031	100% \pm 0%
0.062	94.39% \pm 0%
0.125	71.54% \pm 0%
0.25	46.26% \pm 0%
0.5	43.1% \pm 0%
0.75	24.3% \pm 0%
1	24.27% \pm 0%

Figure 6: Action of Gossypol at 24 hrs

In this study, Gossypol has been assessed in different concentrations and its effect on HeLa cell line for 24 and 48 hours. Shown in Fig.7. In this context, the death of cells increased when concentration is decreased. The cytotoxic effect of gossypol and fragmentation of nucleus has exhibited parallelization. The most cytotoxic effect of gossypol on the cells has been detected at the end of 72 hours after treating with gossypol. The fragment of the DNA indicates that gossypol induces apoptosis and some of these cells were in necrotic pathway.

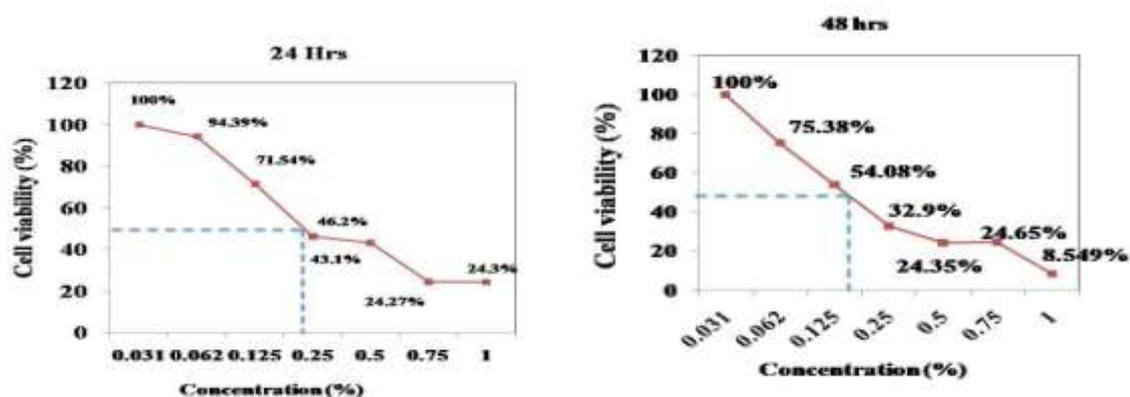


Figure 7: Different Concentrations of Gossypol and its Effect on HeLa Cell Line for 24 and 48 Hours

In the present study, it has shown that gossypol inhibits the activity of these enzymes in dose and this activity was increased till 48 hours of post treatment. As the HeLa cell line is originated from human cervix cancer cells, the results of the present study will provide this valuable information for oncology and clinicians for the treatment of cervical cancer. It has been concluded that the evaluation of anticancer property of gossypol using HeLa cell line was determined using MTT assay in our present study. The results of MTT assay has demonstrated that gossypol can be efficiently used in anticancer therapy (Benz *et al.*, 1987). So, the results of present study and Benz *et al.*, 1987 study are in accordance.

Concentration in %	Cell viability
0.031	100% ± 0%
0.062	75.38% ± 0%
0.125	54.08% ± 0%
0.25	32.9% ± 0%
0.5	24.35% ± 0%
0.75	24.65% ± 0%
1	8.549% ± 0%

Figure 8: Action of Gossypol at 48 hrs

V. CONCLUSION

Cervical cancer occurs when abnormal cells on the cervix grow out of control. The cervix is the lower part of the uterus that opens into the vagina. Cervical cancer can often be successfully treated when it is found early. It is usually found at a very early stage through a Pap test. Gossypol is a polyphenolic compound which is extracted from cotton seed. Gossypol has many biological properties to inhibit the activity of tumor cells. In this experiment, the evaluation of anticancer property of gossypol using HeLa cell line was determined.

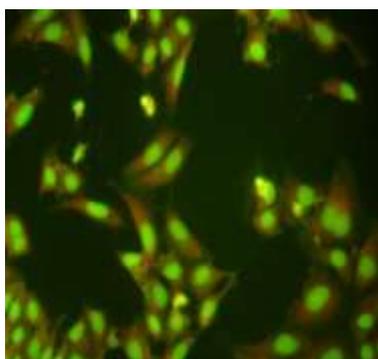


Figure 9: IC₅₀

Now a days, plant products are very efficient to cure many diseases and this work also showed similar results. Gossypol has been assessed in different concentrations and its effect on HeLa cell line for 24 and 48 hours was measured. The IC₅₀ lies between 0.25% to 0.125%. Shown in Fig.9. Acridine orange staining was performed to confirm the apoptosis of the cells. Gossypol had not undergone apoptosis or there was no apoptotic pathway.

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