

# Effect of the herbal formulation HF1 on the expression of PD-L1 in PC3 cells

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## SUMMARY

Cancer is a disease in which abnormal cells divide in an unregulated and uncontrolled fashion, leading to the formation of tumors. One of the mechanisms supporting cancer cell survival is immune evasion. Cancer cells evade the immune system by producing PD-L1, a ligand that is normally produced by non-malignant cells and that interacts with the PD-1 receptor on T cells; this interaction between the PD-L1 ligand and the PD-1 receptor acts like an “off switch” for the production of large amounts of T cells. Though this interaction prevents T cells from attacking normal cells, it also helps cancer cells hide from the immune system. In this *in vitro* study, we aimed to determine whether treatment with a proprietary herbal formulation (HF1; under patent by Sri Raghavendra Biotechnologies Pvt Ltd, Bangalore) affects CD274 gene expression in the prostate cancer cell line, PC3.

We hypothesized that the gene expression of CD274 gene (though we have used protein name, PD-L1 to refer the gene) will be reduced in PC3 cells (a prostate cancer cell line, able to express PD-L1 upon induction) treated with HF1 when compared to PC3 cells that have been induced to express PD-L1. We found that HF1 treatment resulted in a 4-fold decrease in PD-L1 expression when compared to control ( $p < 0.001$ ). Results shows that HF1 and other antioxidants may decrease PD-L1 expression and thus could be useful to develop as a novel cancer therapy.

## INTRODUCTION

Tumors are the result of specific genetic mutations that allow cells to grow and spread abnormally (1). There is an immune response to these cancerous cells by both T and B cells. For instance, tumor-infiltrating lymphocytes (TILS) produced in tumor microenvironment, especially CD8+ T cells, have been shown to play a critical role in controlling tumor progression in hepatocellular carcinoma (2).

Programmed death ligand 1 (PD-L1) expression is a mechanism used by tumor cells to evade immunological action. PD-L1 is a 40 kDa, type 1 trans-membrane protein. The protein plays a significant role in suppressing immune system during inflammatory conditions such as hepatitis (3). PD-L1 works by binding to PD-1, its receptor, which is found on activated T cells and B cells. PD-1 has two potential

ligands, PD-L1 and PD-L2. PD-L1 is expressed in many hematopoietic cells and some parenchymal cells, such as pancreatic islet cells and vascular endothelial cells, while PD-L2 expression is linked to macrophages and dendritic cells (DCs) (4).

Once PD-L1 binds to the receptor, the T-cell delivers a signal that inhibits the production of interleukin-2 (IL-2) and cell proliferation. IL-2 is a cytokine that is involved in the process of differentiating T cells into effector T cells and memory T cells, thus helping the body fight infections (5).

In normal tissue, feedback between transcription factors like STAT3 and NF- $\kappa$ B restricts the immune response to protect host tissue and limit inflammation (5, 6). Unlike normal cells, cancer cells exploit this mechanism by upregulating local PD-L1 expression caused by lack of feedback control between transcription factors (7). Furthermore, a phenomenon called T-cell exhaustion occurs, which is characterized by stepwise and progressive loss of T-cell functions (8). The regulation of PD-L1 expression has been connected to various factors. For example, upon interferon gamma (IFN $\gamma$ ) stimulation, PD-L1 is expressed on the surface of T cells, natural killer (NK) cells, macrophages, myeloid DCs, B cells, epithelial cells, and vascular endothelial cells (9).

As a result, novel cancer treatments, such as Nivolumab (Bristol-Myers Squibb), use monoclonal antibodies as an approach to block the interaction between PD-1 and PD-L1 or PD-L2. Another significant area of cancer treatment research is the use of antioxidants like polyphenols to alter cancer growth; however, the effects of antioxidants on immune evasion mechanisms in cancer cells remain poorly understood.

PC3 is a human prostate cancer cell line used in prostate cancer research and drug development. PC3 cells are useful in investigating biochemical changes in advanced

Herbal substance	Anticancer effect
Epigallocatechin-3-gallate (EGCG)	Arrests specific prostate cell lines at the G0-G1 phase (cell cycle). Inhibit metalloproteinase in vitro. These family of enzymes help in tumor invasion by extracellular matrix (ECM) degradation.(10)
Curcumin	Suppressive effect on hepatic fibrogenesis (fiber formation) and carcinogenesis (tumor formation).(10)

**Table 1: Clinical use of herbal medicine exhibiting anticancer activity.**

prostate cancer cells and in assessing their response to chemotherapeutic agents (10).

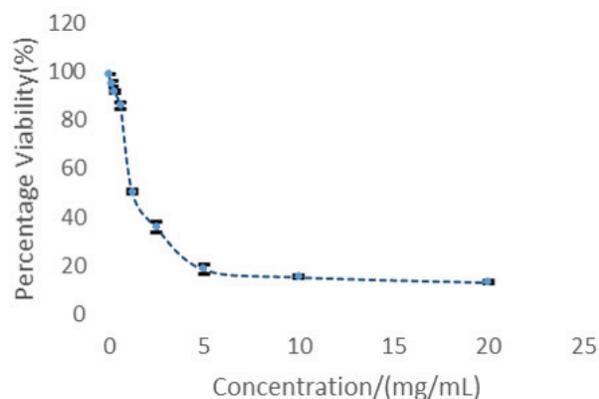
In this investigation, we aimed to determine the effects of HF1, a proprietary herbal formulation, consisting of mainly green tea extract and turmeric, on the expression of PD1 in PC3 cells. Studies have shown that the herbal components in HF1 demonstrate anticancer activity (Table 1). The polyphenols present in HF1 form an important dietary component and both India and China produce high quality tea, such as Assam tea (11). Green tea and curcumin are significant part of Indian agriculture (12), and therefore makes it a viable option to utilize their anticancer properties and make it accessible to the population as a nutraceutical. One reason for the potential anticancer properties of HF1 is that its components can be oxidized to generate reactive oxygen species (ROS) in cell culture medium and cause cell death (13). It has been demonstrated that administration of EGCG at 0.02%-0.32% in drinking water dose-dependently inhibited small intestinal tumorigenesis in mice (14). In this study, the extent to which HF1 acts as an immune checkpoint inhibitor (15) will be tested *in vitro*. The principle behind immune checkpoint inhibition is to ensure that cancer cells cannot produce PDL1 as they could normally.

## RESULTS

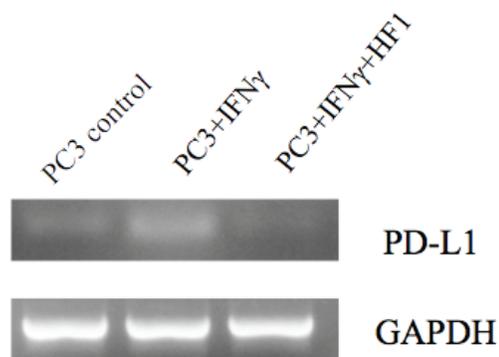
IC<sub>50</sub> value is the dose at which 50% cells would be viable. This dose is important to be known as it provides the toxic dose of a compound. Here, we examined the effect of HF1 on PC3 cells and obtained an IC<sub>50</sub> value of HF1 using MTT assay to carry out further experiments.

Results shows that 50% cells were viable at 1.27 ± 0.03 mg/mL concentration of HF1 (Figure 1). Thus, to prevent cytotoxic effect, HF1 was used at the concentration of half the IC<sub>50</sub> (1.27 ± 0.03 mg/mL), 0.6 mg/mL.

The levels of PDL1 was studied in various groups by using PCR technique. We examined the effect of HF1 on IFN $\gamma$ -induced PD-L1 expression in PC3 cells. Treatment with IFN $\gamma$  (10 ng/mL) increased PD-L1 mRNA levels. However,



**Figure 1: Cytotoxicity effect of HF1 on PC3 cells.** A plot of percent viability (%) vs. concentration (mg/mL) of HF1. The obtained IC<sub>50</sub> was 1.2 mg/mL. Error bars represent standard deviation across three independent sets of experiments.



**Figure 2: Gel electrophoresis showing expression of PD-L1 and GAPDH (n=3).** PC3 control (untreated cells), PC3 cells treated with IFN $\gamma$ (10 ng/mL) for 24 hours (PC3+IFN $\gamma$ ), PC3 cells treated with IFN $\gamma$ (10 ng/mL) and HF1 (0.6 mg/mL) (PC3+IFN $\gamma$ +HF1) for 24h.

treatment with IFN $\gamma$  and HF1 extract (0.6 mg/mL) lead to a decrease in the levels of PD-L1 mRNA compared to IFN $\gamma$  treatment alone (Figures 2 & 3, Table 2). This shows that antioxidants could be used as a novel therapy in cancer treatment.

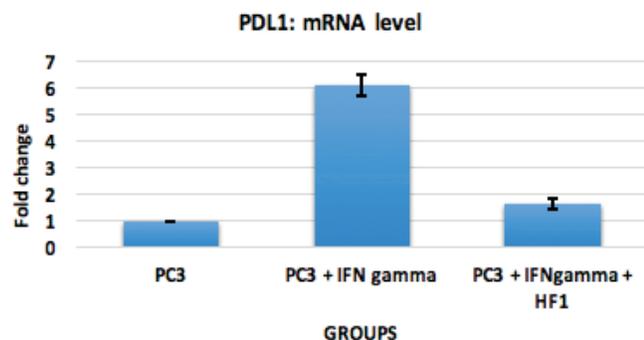
mRNA was measured using Quiagen kit. The mRNA expression level of HF1-treated group showed less than one-third of the expression level of the PC3 cells treated with IFN $\gamma$  (this group represents the state of PD-L1 overexpression found in some cancer cells) (Figure 3). The mRNA level in the PC3 cells treated with both IFN $\gamma$  and HF1 was compared to the PC3 untreated control cells, which served as the baseline of comparison in this experiment.

Expression levels were quantified based on the gel using ImageJ software and were normalized it to GAPDH levels. The PC3+IFN $\gamma$  group had a fold difference of 6.10 ± 0.40 when normalized against the PC3 control group. In comparison, the PC3+IFN $\gamma$ +HF1 showed a fold decrease of 1.63 ± 0.18 ( $p < 0.001$ ), which is strikingly close to the baseline expression of PD-L1 considered as in PC3 control group.

To replicate the overexpression found in cancerous cells, the cytokine IFN $\gamma$  was added to induce ligand expression (18).

## DISCUSSION

In this study, we investigated whether HF1, which contains green tea extract and curcumin, modulates PD-L1 levels. We measured PD-L1 mRNA levels in PC3 cells without treatment, with IFN $\gamma$  alone (10 ng/mL), or with a combination of IFN $\gamma$  and HF1 (at 0.6 mg/mL, based on the IC<sub>50</sub> value of 1.2 mg/mL). We chose to study an herbal compound because various studies highlight their antioxidant and anti-cancerous properties (16). For example, the sub-group of polyphenols called galliccatechins (a prominent component of green tea) exerts apoptosis-inducing properties, showing anti-cancer potential. The mechanism of apoptosis induction is quite diverse across different anti-cancerous compounds and is under much study.



**Figure 3: Inhibition of IFN $\gamma$ -induced PD-L1 by HF1.** Inhibition of IFN $\gamma$ -induced PD-L1 by HF1. PC3 alone was considered as negative control, PC3+IFN $\gamma$  group was treated with IFN $\gamma$  (10 ng/mL) and PC3+HF1+IFN $\gamma$  was treated with both HF1 (0.6 mg/mL) and IFN $\gamma$  (10 ng/mL). The groups were compared with control.

The PD-1 protein is found on immune cells like T-cells. It functions as an "off switch" that ensures that T cells aren't attacking normal body cells. This protein recognizes PD-L1, a protein found on normal and cancerous cells. This interaction prevents the T-cell attack on the PD-L1-expressing cells. Some cancer cells have large amounts of PD-L1, which helps them hide from immune attack. Hence, lower expression signifies that the tumors may no longer be able to effectively defend themselves from T cells attack through the PD-1/PD-L1 interaction.

Plant and plant-derived product study is a revolutionary field, as these are simple, safer, eco-friendly, low-cost, fast, and less toxic when compared to conventional treatment methods (17). In addition, active phytochemicals are selective in their functions and act specifically on tumor cells without affecting normal cells (18).

Studies on PD-L1 highlight that the JAK/STAT pathway is associated with regulation (19). Herbal compounds have, in fact, been known to activate and inhibit such cell signaling pathways like MAPK/ERK, making them a prime candidate for PD-L1 regulation, as mentioned previously in reference 17.

The purpose of this investigation was to investigate the effect of an herbal formulation on the expression of the PD-L1 ligand. The hypothesis was that upon addition of a given concentration of the formulation, the expression of PD-L1 would decrease. We also predict (but have not tested) that this will decrease tumor growth and its lifespan as there will

Group	Fold difference of PDL1
PC3	1.00±0.00
PC3+ IFN $\gamma$	6.30±0.40
PC3+ IFN $\gamma$ +HF1	1.63±0.18
The fold difference obtained from three independent experiments conducted for each group of the experiment, with a calculated average and standard error mean (SEM).	

**Table 2. Fold difference of PDL1 between groups.** The fold difference obtained from three independent experiments conducted for each group of the experiment, with a calculated average and standard error mean (SEM).

be a higher concentration of T cells caused by reduction of PD-L1 on cancer cells.

These results suggest that use of herbal formulations like HF1 can help improve the efficacy of chemotherapy drugs and help by overcoming T cell sweating by down regulating PDL1 expression, and resulting in better efficacy of the treatment. This compound could be tested in a clinical trial where the effect of formulation could be studied on cancer patients, with respect to PD-L1 levels. Drug characteristics like absorption, distribution, metabolism, and excretion properties can be deduced and its effects on other cancerous cells that express this ligand need to be studied.

## METHODS

### Cell culture

Cryovials containing the PC3 cells were purchased from NCCS (National Centre for Cell Science), Pune. These vials had a passage number of seven. After rapid thawing, cells were cultured in Dulbecco's Modified Eagle Media (DMEM)/F-12 with 10 % serum (Invitrogen). After centrifugation (1500 rpm, 10 minutes), cells were counted using a hemocytometer, cultured and maintained at 37°C with 5% CO<sub>2</sub>.

At 70-80% confluency, the cells were harvested using 0.25% trypsin-EDTA solution (Invitrogen) and sub-cultured at the density of 0.3 million cells and/or used for MTT assays and PCR.

### HF1 Preparation

Hot water decoction was prepared using HF1 powder at the concentration of 0.2 g/mL and was syringe filtered (0.22  $\mu$ m).

### MTT Assay

PC3 cells were plated in a 96-well plate at the density of 3000 cells per well and were incubated at 37°C with 5% CO<sub>2</sub> levels for 24 hours.

Using serial dilution, a stock of 20 mg/mL of HF1-water extract was diluted to form 8 concentrations ranging from 20 mg/mL to 0.15 mg/mL using DMEM containing 10% serum. Triplicates for each concentration along with appropriate controls (positive control, vehicle control, and media control) were added to respective wells and incubated for 24 hours. Cells were incubated for 4 hours with MTT dye (Sigma)

Gene of Interest	GAPDH
Number of Samples	9
Number of Cycles	30
Denaturation Temperature	94°C
Annealing Temperature	66°C
Extension Temperature	72°C
Description	10 minutes at 95°C; 30 cycles: 94°C for 30 sec, 66°C for 30 sec, 72°C for 45 sec; 72°C for 10 min.

**Table 3. PCR information for GAPDH**

Gene of Interest	PD-L1
Number of Samples	9
Number of Cycles	30
Denaturation Temperature	94°C
Annealing Temperature	53°C
Extension Temperature	72°C
Description	10 minutes at 95°C; 30 cycles: 94°C for 30 sec, 53°C for 30 sec, 72°C for 45 sec; 72°C for 10 min.

**Table 4: PCR information for PD-L1**

and DMSO was used to dissolve Formazan crystals. The absorbance of the purple solution was detected at 545 nm using a spectrophotometer (UV10).

#### Expression and modulation of PD-L1 gene

Cells were counted and cultured in a 6-well plate at a density of 0.3 million cells in 3 mL of media per well. After 24 hours incubation, IFN $\gamma$  (10 ng/mL) and HF1 (0.6 mg/mL) were added to respective wells, forming three groups: PC3 control (without treatment), PC3+IFN $\gamma$ , PC3+IFN $\gamma$ +HF1. Volumes of IFN $\gamma$  and HF1 used were less than 10%, lower than the toxic dose.

#### mRNA isolation and cDNA preparation

After another 24 hours of incubation, the cells were trypsinized, centrifuged, and then the mRNA was isolated (Qiagen RNA isolation kit). mRNA was quantified and purity was determined using spectrophotometer at two wavelengths: 260 nm and 280 nm.

#### cDNA preparation

DEPC-treated tubes were used to prepare cDNA using Genie RT PCR kit. The standard protocol included addition of Oligo-dT, RNasin, DTT, dNTPs, MULV reverse transcriptase, and assay buffer to the mRNA samples.

The samples were incubated at 37°C for an hour, centrifuged at 12000 rpm (usually for a short spin of 15 seconds), and stored at -80°C for further use.

#### Semi-quantitative PCR

After calculating the appropriate cDNA volumes (to ensure a concentration of 200 ng cDNA/50  $\mu$ L PCR reaction), PCR reactions were set up with 12.5 $\mu$ L of the Jumpstart mix (Sigma), 1 nM of GAPDH forward and reverse primer (Eurofins) and the remaining volume of molecular grade water (Tables 3 & 4).

#### Gel Electrophoresis

5  $\mu$ L of each PCR product was mixed with 2  $\mu$ L of 6X gel loading dye (Sigma) before adding to the wells in a 3% agarose gel. 100 bp DNA ladder was also loaded. The gel was run for one and a half hours at 50 V.

Each gel image was exported and analyzed using ImageJ. LUT was inverted so that the bands were black

and the background was subtracted. The bands of interest were isolated using the rectangle tool. The band intensity peaks were plotted and the peaks were separated using the line tool. The area under each peak was measured for the housekeeping gene GAPDH and PD-L1 gene. PD-L1 mRNA level was normalized with the respective GAPDH level in each group.

We measured the fold change for normalized PD-L1 gene expression across the experimental groups: control, PC3+IFN $\gamma$ , and PC3+IFN $\gamma$ +HF1.

This fold difference can also be seen through the images produced through the ImageJ software. As mentioned earlier, the measure of area under the curve for each group is correlated with expression.

#### Data analysis

To check experimental reliability, three independent sets of the same experiment were performed.

The PD-L1 mRNA levels were normalized with the GAPDH levels by taking the PD-L1 to GAPDH ratio for each corresponding sample. These normalized values were then compared to the PC3 control values by taking the ratio of the levels of each group with PC3 control group values.

$$\text{arithmetic mean} = \frac{\sum_{n=1}^k x_n}{k}$$

An average value was calculated across the three trials for each group. The standard arithmetic average formula is used here (20).

The error bars represent the standard error of the mean (SEM). It indicates the spread that the mean of a sample of the values would have if you kept taking samples.

SEM is calculated by taking the standard deviation and dividing it by the square root of the sample size (21).

$$\begin{aligned} \text{standard deviation } \sigma &= \sqrt{\frac{\sum_{i=1}^k (x_i - x_{avg})^2}{n - 1}} \\ \text{standard error} &= \frac{\sigma}{\sqrt{n}} \end{aligned}$$

Using the GraphPad software, OneWay ANOVA was used to compare the means of the three groups. The output was a  $p$ -value of 0.001 was obtained. This shows that the difference between the means are statistically significant as conventionally a  $p$ -value < 0.05 shows high statistical significance.

**Received:** June 25, 2019

**Accepted:** November 6, 2019

**Published:** November 15, 2019

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