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Research Article

Diluted *Lycopodium* Induced Cell Death and Clinical Improvement in Hepatocellular Carcinoma

Debasmita Chatterjee[1], Banhishikha Singh[1], Ashoke Kumar Pradhan[2], Krishnendu Paira[1] and Satadal Das[1*]

[1]*Tissue Culture Unit, Department of Biotechnology, Heritage Institute of Technology, Kolkata, West Bengal, India*
[2]*Institute of HYDT Research & Education, Kolkata, West Bengal, India*

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ABSTRACT

Hepatocellular carcinoma (HCC) is a common cancer with high incidence rate, and 5-year survival rate in HCC is less than 20%. Thus, in search of newer anticancer agents effective in HCC, we have explored possible usefulness of an alternative medicine *Lycopodium* against the human liver cancer cell line, HepG2 along with its clinical efficacy. The HepG2 cell line was challenged with *Lycopodium 6C* (diluted *Lycopodium* <1pg /mL available as alternative medicine) along with vehicle alcohol control in 24 hours. The cytopathic effect and viability test with methylene blue stain were observed. The cells were harvested for total RNA extraction, and gene expression levels of targeted cytokines -Interferon gamma (IFN γ); Interleukins - IL-6, IL-8, IL-10, IL-1β, Transforming Growth Factor- TGF-β1, TGF-β3 and Tumor Necrosis Factor alpha (TNF-α) by RT-PCR were studied. DNA fragmentation assay and cell viability assay by MTT method were also tested. After ethical permission we applied this medicine as adjunct therapy to observe any beneficial role of the medicine. Statistically significant changes of IL-10, IL-1β and TGF-β3 were observed after challenge with *Lycopodium 6C*. The IL-10 gene expression in malignant cells was significantly reduced with *Lycopodium 6C*; however, the expression is more with vehicle alcohol compared to normal control set. Thus, the medicine could decrease the excessive IL-10 gene expression to a moderate level. IL-1β and TGF-β3 gene up-regulation by the vehicle alcohol were also mitigated by the medicine *Lycopodium 6C*. Mild DNA fragmentation was also seen in cancer cells after challenge with the medicine. Two cases suffering from hepatocellular carcinoma showed much clinical improvement after therapy with this medicine. *Lycopodium 6C* may act as a supporting alternative medication for treating HCC.

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Introduction

Cancer is a deadly disease that has already taken the entire world in its grip, and so the problem requires a multifaceted approach for its treatment [1]. Among the varied forms of cancer, hepatocellular carcinoma (HCC) is very common due to its high frequency and incidence rate. A chronic inflammatory environment induces the development of HCC which usually represent the chronic active hepatitis following viral infection or due to substance abuse (alcohol) [1, 2]. Surgical resection and/or transplantation are considered to be the two most efficient treatment methodologies for liver cancer, but unfortunately not all patients could avail these treatment procedures [3]. Among the other modes of treatment, homeopathy is considered to be an alternative treatment approach with no such significant side effects and also lessens the pain associated with the disease [4]. The homeopathic clinicians prescribes both mother tinctures and *potentized* preparations for the treatment purpose depending on the symptomatic manifestations of the patient and have claimed that both mother tincture and *potentized* preparations are useful against the disease condition [4]. Researchers have also revealed that the homeopathic medicine *Lycopodium* could reduce the pathogenesis associated with the tumor like growth, and in turn could also retard the growth of the tumor itself. Moreover, the homeopathic preparation was evident to enhance the life span of the tumor carrying animals [4, 5].

Correspondence to: Satadal Das, Tissue Culture Unit, Department of Biotechnology, Heritage Institute of Technology, Kolkata, West Bengal, India; ORCID: 0000-0002-9843-3466; E-mail: satadal.das@heritageit.edu, drsatdas@hotmail.com

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In our study we have chosen *Lycopodium 6C* against the HCC cell line HepG2. *Lycopodium* is also known as “clubmosses”, “creeping cedars” and “ground pines” and the plant belongs to family “Lycopodiaceae” [6]. The medicine showed varied uses in homeopathy such as for constipation, chronic lung and bronchial disorders, fever, aneurysms and also against cancer [6]. It can also be useful against gastric inflammation and chronic kidney disease. Several researchers have also claimed about its hepato-protective, anti-oxidant, immune modulatory, neuroprotective, antimicrobial, analgesic, and anti-cancer properties [6]. Thus in the present study we have explored the anti-cancer activity of the homeopathic preparation “*Lycopodium 6C*” against the human liver cancer cell line (HepG2).

Materials And Methods

I Procurement of Cell line

The HepG2 was procured from National Centre for Cell Science, Pune, India. The cells was transported in a T25 cm^2 flask within growth medium, Minimum essential medium (Eagle) with 2mM L-glutamine and Earle’s Balanced Salt Solution (BSS) that was adjusted to contain 1.5g/L sodium bicarbonate, 0.1mM non-essential amino acids and 1.0mM sodium pyruvate, 90% concentrated fetal bovine serum of final concentration of 10%.

II Procurement of Chemicals

The medicine *Lycopodium 6C* was purchased from “HAPCO, India” - an India government recognized homeopathic medicine producing company”. It was a diluted and *potentized* alcoholic plant extract material prepared by homeopathic pharmacopoeia guidelines. The Dulbecco’s Modified Eagle Medium (DMEM) (1X) along with Glutamax was procured from Gibco, ThermoFischer, USA. The medium was supplemented with F-12 (1X) nutrient mixture Ham + L-glutamine (Gibco, ThermoFischer, USA) for the better growth of the HepG2 cell line. The Fetal bovine serum (FBS) and the antibiotic –antimycotic solution namely Penicillin/Streptomycin/Amphotericin B Solution (100X), Phosphate buffer saline (PBS, 1X) of pH 7.4, Trypsin enzyme (0.05 X) were all purchased from Gibco, Thermo Fischer, USA. The MTT assay kit EZ Count was purchased from Himedia, India. The molecular biology chemicals such as the RNA isoplus were purchased from Takara, the cDNA synthesis reverse transcriptase kit and the iTaq Sybr green supermixture for RT-PCR were purchased from Bio-Rad, USA.

III Cell Culture

The HepG2 cells were allowed to reach 80% confluency in a T25 cm^2 flask for 48 hours with DMEM+F12 supplemented with antibiotic solution, and 10% FBS media for 48 – 72 hours [7]. The media change was given at a regular interval of 48 hours after washing the cellular debris with PBS (1X). The cells were splitted using Trypsin solution for 10 minutes and then the flask was agitated manually, so that the adherent cells leave the base of the flask. Immediately after 10 minutes, the trypsin was inactivated with DMEM media supplemented with FBS (10%). The cells were then centrifuged at 1200 rpm for 12 minutes. The supernatant medium was decanted and fresh media was added of required volume to carry out the experiment in a 6wells plate [6, 7].

1 mL of cells containing media was added to each well of the plate and kept in 5% carbon dioxide incubator in a humidified environment for the next 24 hours. The next day the wells were washed with 1X PBS and again fresh media was added. The cells were allowed to reach a confluency of 10^5 - 10^6 before the inoculation of the inoculums [6, 7].

IV Inoculation of Medicine

The cells were challenged with 100 µL of medicine, *Lycopodium 6C* to three wells each (triplicate sets), Alcohol vehicle in another three wells and the rest three wells served as control without any inoculums. The plates were rotated clockwise and anti-clockwise for mixing of the inoculums. Immediately after inoculation pictures of cell line were taken (0 hour after inoculation).

V Modified Methylene Blue Assay

Modified methylene blue assay can be used for a wide range of cell counting process. This method is advantageous compared to other staining method because the stain can be applied directly upon the adherent cell lines that are growing in any size culture plates [8]. The methylene blue solution is prepared with Phosphate buffer saline solution (1X, PBS), 1.25% glutaraldehyde, and 0.06% methylene blue. The stain was added of volume 1 ml to each well of the 6 well culture plate, and it was incubated at 37ºC for one hour. Then the methylene blue solution was thoroughly rinsed with PBS solution and then pictures were taken under inverted microscope to differentiate among the live and dead cells. The cells were observed under inverted microscope under 20X, and 40X to record the cytopathic effect [8].

VI Gene Expression Analysis of Cytokines

The cells of each well were harvested with 1ml RNA isoplus and the total RNA was extracted following the instructions of the manufacturer. The total RNA was estimated using A260/280 ratio and the cDNA were synthesized using the reverse transcriptase kit (Bio-Rad, USA). Then 2µL of the cDNA was utilized for SyBR green RT PCR assay (Bio-Rad, USA) in CFX-96 model of RT PCR, Bio-Rad, USA. The gene expression analysis was conducted of the following genes namely, Interferon (IFN) gamma, Interleukins- IL-6, IL-8, IL-10, IL-1β, Transforming growth factors (TGF) – β1 and β3, and Tumor necrosis factor alpha (TNF α) against the housekeeping gene, β actin [9, 10].

VII MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) Assay for Cell Viability

The HepG2 cells viability was measured using the MTT assay kit EZ Count (Himedia Pvt. Ltd., India). The assay was done keeping the required controls such as medium control (without HepG2 cells), Cell line control (Media + HepG2 Cells), and the vehicle control (Medium containing the vehicle solvent or the experimental drug). The volume of experimental drug inoculated upon the cell line was 100µL and the culture plate was incubated at 37ºC for one hour in carbon dioxide and humidified atmosphere. Following incubation, 10µL of MTT reagent

was added to the experimental sets and control sets. Thereafter, the plate was incubated in the above-mentioned condition for the next 4 hours. The plate was observed at regular intervals under the inverted microscope as it would develop formazan crystals. After the formation of crystals, 100μL of the solubilization solution was added to all the wells, and the plate was shaken by hand for about 5 minutes, and then the plate was kept within the incubator for overnight incubation. The crystals would dissolve within the overnight incubation, and the coloration would develop. Then the absorbance was taken at 570 nm by an automated 96 well plate ELISA reader (Roboniks, India). The data was graphically recorded [11].

VIII DNA Fragmentation Assay

The phenomenon of DNA fragmentation and condensation of nucleus contents are considered to be the activities to be taking place during late apoptosis. Endonuclease which is located at the margins of nucleus can break the chromatin DNA into short fragments during the apoptosis. Here in our experiment, the cells of each well were trypsinized and cell scrapper was used to collect maximum number of cells from each wells. Then the cells were washed with PBS twice and proteinase K was added to each tube and incubated at 56ºC for 1 hour at water-bath [12]. Then the whole genomic DNA was isolated following the standard protocol of phenol-choloroform extraction. After extraction the isolated DNA was dissolved in 60μL of elution buffer and the purity of the DNA was checked at A260/A280 ratio using UV-Vis Spectrophotometer (Agilent, Singapore). Absorbance of 1.7 was considered to be of pure isolated DNA.

Then the whole genomic DNA of all the samples were loaded within 1% agarose gel for comparison of the intact DNA band of control with the medicine and alcohol control counterparts. Then the gel was stained using ethidium bromide for band visualization and photography using UV-Vis trans-illuminator [12].

IX Application in Patients Suffering from Hepatocellular Carcinoma

After getting ethical permission we could apply this medicine in two cases suffering from hepatocellular carcinoma as adjunct therapy with consent taken from the patients.

X Statistical Analysis

The data was analysed for statistical significance using one-way analysis of variance (ANOVA) using statistical software, GraphPad Prism 9.3.1.

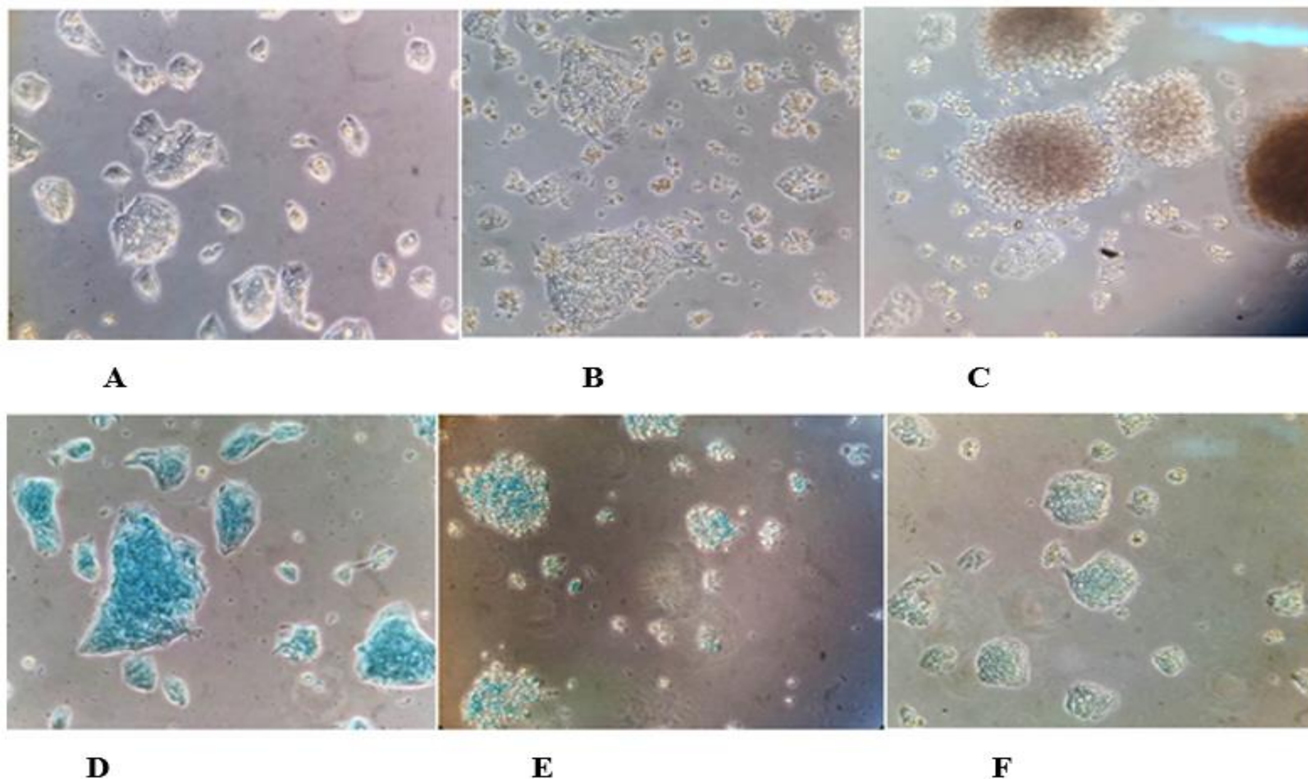

Figure 1: Cell cytopatheic effect before and after methylene blue assay **-** In the control set the HepG2 cells were in usual shape and size along with intact outline. The inoculums were added when confluency was around 60% to 80%. In the medicine set, the cells were round in shape indicating apoptosis. Maximum cells were dead and were uplifted from the base (detachment of cells) of the culture plate after 24 hours of the inoculation. In case of alcohol (vehicle control set), the cells also showed apoptosis, i.e., rounding of cells and cell detachment from base of the culture plate. The size of the cells decreased considerably; however, some cells were still alive in decreased size and were attached to the base of the culture plates. **A)** Represents control HepG2 cell line (after 24 hours). **B)** Cell line after 24 hours of drug inoculation, *Lycopodium 6C*. **C)** Cell line after 24 hours of inoculation of vehicle control (alcohol). **D**) Control HepG2 cells after 24 hours after methylene blue staining. **E)** Cell line after 24 hours of drug inoculation, *Lycopodium 6C* after methylene blue staining. **F)** cells after 24 hours of inoculation with vehicle control (alcohol) after methylene blue staining.

Results

I Cell Morphology and Cytopathic Effect

In the control set the HepG2 cells were in usual shape and size along with intact outline. The inoculums were added when cells were around 80% confluent. In the medicine set, the cells became small round in shape indicating possibility of apoptosis. Maximum cells were dead and were uplifted from the base (detachment of cells) of the culture plate after 24 hours of the inoculation. In case of alcohol (vehicle control set), the cells also showed apoptosis, i.e., rounding of cells and cell detachment from base of the culture plate. The size of the cells decreased considerably; however, some cells were still alive in decreased size, and were attached to the base of the culture plates. After methylene blue staining, the control cells were stained with intact cellular morphology, usual size and intact margins, whereas in case of alcohol (vehicle control) the cells were decreased in size with membrane blebs. However, few cells had retained the stain indicating that the cells were still alive. In the medicine set, the cells were all rounded in shape and were floating within the media, indicating that all the cells have undergone apoptosis or necrosis (Figures 1A-1F).

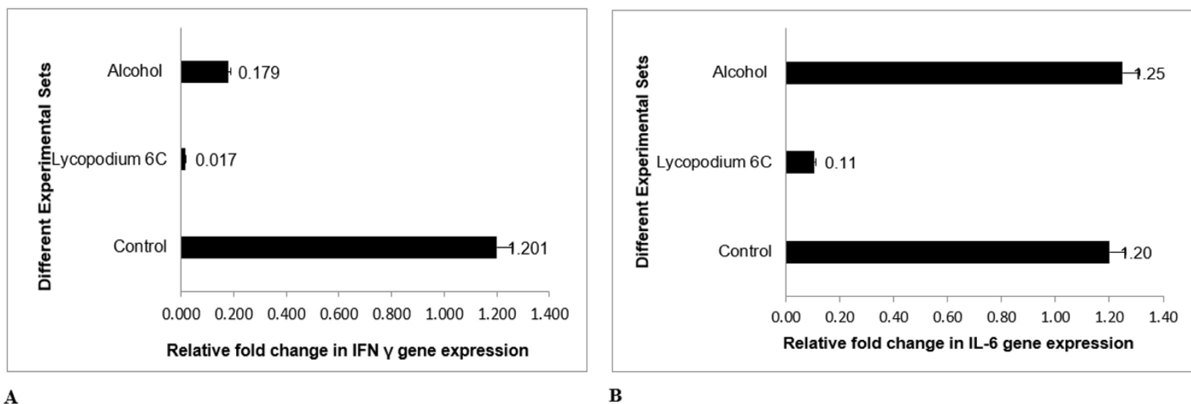

Figure 2: Differential expressions of IFN γ and IL-6 genes in different experimental sets – the bar graphs represents the mean value ± SEM of three independent experimental findings **A)** IFN γ was found decreased in the medicine set in comparison to the alcohol and normal control. **B)** In case of cytokine IL -6, the expression of this particular gene was found to be mildly high in the alcohol and normal control, but on the contrary the gene expression of IL-6 was decreased by the *Lycopodium 6C* set. The change is not statistically significant.

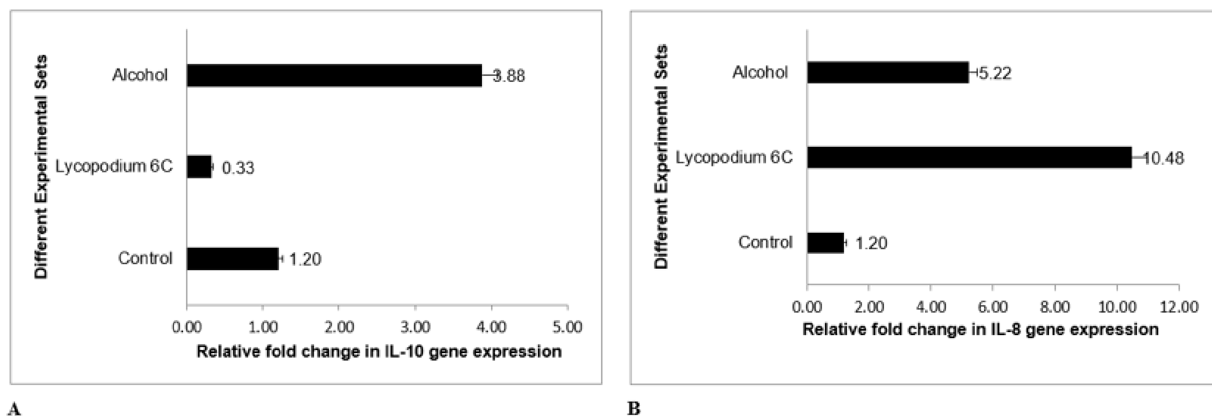

Figure 3: Differential expressions of IL-10 and IL-8 genes in different experimental sets – the bar graphs represents the mean value ± SEM of three independent experimental findings **A)** the observation was noted for the IL -10 where the medicine diminished the gene expression in that set, however, the gene expression of this particular cytokine is high within the alcohol control with respect to the normal control set. **B)** However, in case of the pro-inflammatory cytokine, IL -8 the values were enhanced by the medicine, *Lycopodium 6C* set and alcohol control in comparison to its normal control.

II Differential Expressions of Cytokine Genes Using RT PCR Technique

The differential gene expressions of the selected cytokines were represented in the bar diagrams (Figures 2-5). IFN γ was found decreased in the medicine set in comparison to the alcohol and normal control. In case of cytokine IL -6, the expression of this particular gene was found to be mildly high in the alcohol and normal control, but on the contrary the gene expression of IL-6 was decreased by the *Lycopodium 6C* set. The same observation was noted for the IL -10 where the medicine diminished the gene expression in that set, however, the gene expression of this particular cytokine was high within the alcohol control, with respect to the normal control set. However, in case of the pro-inflammatory cytokine, IL -8 the values were enhanced by the medicine, *Lycopodium 6C* set and alcohol control in comparison to its normal control. IL-1 β which is considered to be a key mediator in the

inflammation process was found to be high in the alcohol control set when compared to normal control, and the value was much reduced by the medicine in that set. TGF, which exhibits anti-tumerogenic effect in the initial stage by induction of cytostatis and apoptosis was found to be decreased in the alcohol control and medicine set with respect to control. However, TGF β3 gene expression showed marked increase in the alcohol control and it was reduced in the medicine set whereas in the normal control set, the value was 1.20. The last parameter was Tumor necrosis factor α (TNF α), was found to be increased in the alcohol set and reduced in the medicine set when compared to control. MTT assay revealed that more number of HepG2 cells were alive when compared to alcohol vehicle control at the selected drug doses of 10 to 120 µL (Figure 6). The DNA fragmentation assay also revealed mild fragmentation of DNA (Figure 7).

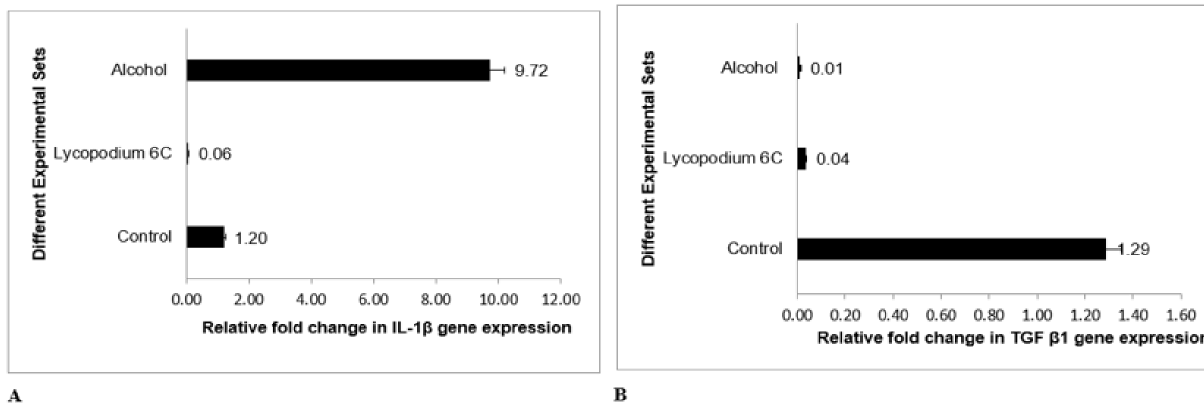

Figure 4: Differential expressions of IL-1β and TGF-β1 genes in different experimental sets – the bar graphs represents the mean value ± SEM of three independent experimental findings. **A)** IL-1 β which is considered to be a key mediator in the inflammation process was found to be high in the alcohol control set when compared to normal control and the value was much reduced by the medicine in that set. **B)** TGF, which exhibits anti-tumerogenic effect in the initial stage by induction of cytostatis and apoptosis was found to be decreased in the alcohol control and medicine set with respect to control.

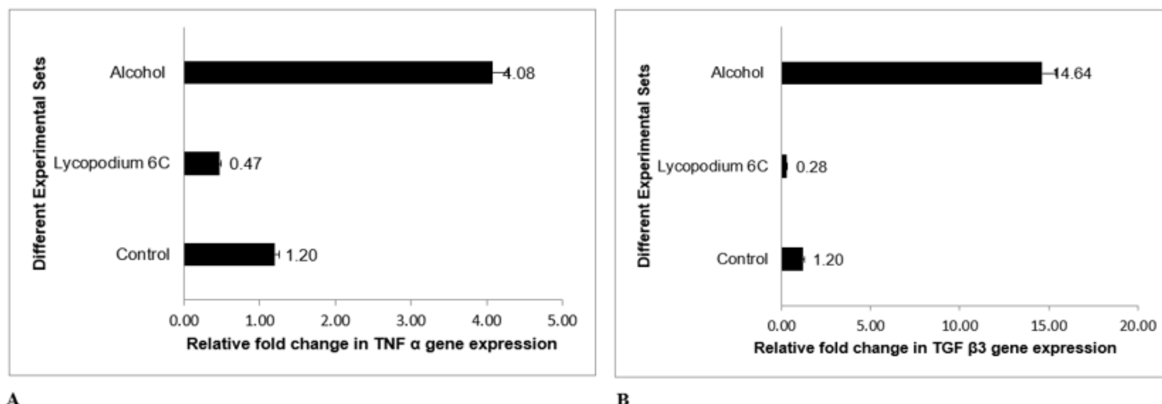

Figure 5: Differential expressions of TNF-α and TGF-β3 genes in different experimental sets – the bar graphs represents the mean value ± SEM of three independent experimental findings **A)** Tumor necrosis factor α (TNF α), was found to be increased in the alcohol set and reduced in the medicine set when compared to control. **B)** However, TGF β3 gene expression showed marked increase in the alcohol control and it was reduced in the medicine set whereas in the normal control set, the value was 1.20.

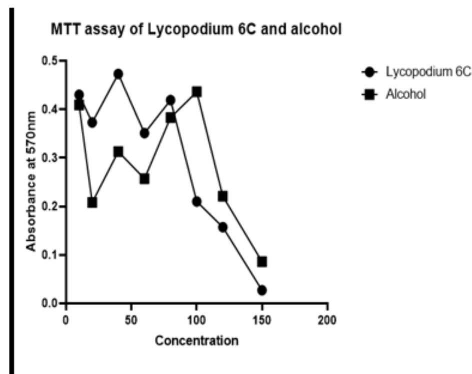

Figure 6: MTT assay curve of *Lycopodium 6C* and alcohol - MTT assay revealed that more number of HepG2 cells were alive when compared to alcohol vehicle control at the selected drug doses of 10 to 120 µL.

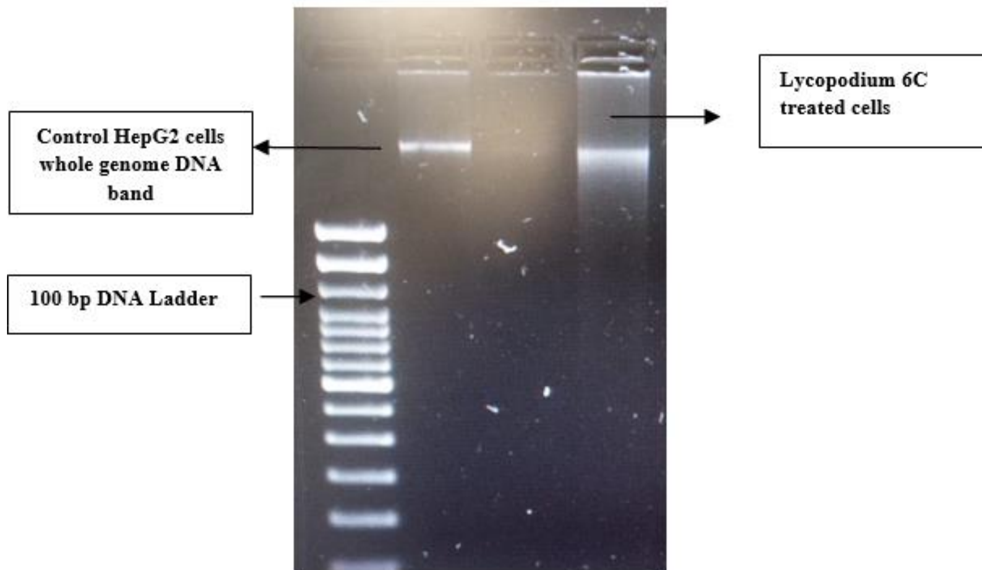

Figure 7: DNA Fragmentation Assay **-**The DNA fragmentation assay also revealed mild fragmentation of DNA.

III Clinical Study Results

Both the cases showed significant clinical improvement after treatment with the medicine. Details of those two patients are given in the (Table 1). Both the cases were males and they were hepatitis B positive with markedly increased AFP. Pain, nausea, vomiting, anorexia, tenderness of abdomen, and weakness relieved 1-2 months after administration of the medicine.

IV Statistical Analysis

The data showed statistical significance for the cytokine parameters, IL-10, IL-1β and TGF-β3 with P-value less than equal to 0.05. The other set values are mentioned in the (Table 2).

Table 1: Different parameters of the two cases those were treated with *Lycopodium*.

Name	Age (Yr)	Sex	Report / Findings										Remark (s)
			FNAC	USG	Hb (G/dL)	Bilirubin (mg/mL)	SGPT (U/mL)	HBsAg	CEA (ng/mL)	AFP (ng/mL)	CA 19.9 (U/mL)	CA 72.4 (U/mL)	
NB	37	M	HCC	Ascites, Mild hepato-splenomegaly, mesenteric lymphadenitis	13.8	0.7	62	+	<0.5	13895.4	24.31	0.31	Clinical improvement for 2 months after giving two doses of the medicine. Pain, nausea, vomiting, anorexia relieved.
HGR	68	M	HCC	Hepato-splenomegaly, SOL in right lobe, portal vein thrombosis, periportal lymphadenopathy	11.7	1.06	28	+	3.4	340	68.04	0.77	Clinical improvement for 1 month after giving one dose of the medicine. Pain, nausea, vomiting, tenderness of abdomen, severe weakness relieved.

Table 2: Statistical Analysis ANOVA summary table of the gene expression analysis values.

SL. No.	Set Analysed (A-C)	F value	P-value	R squared value
1	IFN γ	2.769	2.083 (NS)	0.6486
2	IL-6	1.666	0.3261 (NS)	0.5263
3	IL-8	0.8445	0.5118 (NS)	0.3602
4	IL-10	17.52	0.0221 (S)*	0.9212
5	IL-1β	72.61	0.0029 (S)*	0.9798
6	TGF-β1	2.425	0.2363 (NS)	0.6178
7	TGF-β3	73.41	0.0028 (S)*	0.9798
8	TNF-α	2.674	0.2155 (NS)	0.6406

*Significant at 0.05 level.
s: significant; ns: non-significant.

Discussion

It is well documented that IFN γ plays a significant role in the host defense procedure but it is still unclear that in what mechanisms does HCC evades or blocks the signal transduction mechanism of immune supervision of IFN- γ [13]. It was observed within a clinical data that the expression of IFN- γ receptors on the surface of cells was induced or stimulated among 27 non –cancerous liver tissue samples. On the contrary, in case of non-stimulated IFN- γ receptors, the size of the tumor was large (statistically significant, P = 0.032) along with higher serum alpha-fetoprotein (AFP) level (P value = 0.001) [13]. However, in another research study, IFN- γ has a significant role in the induction of anti-tumerogenic response [14]. The anti-tumerogenic activity of IFN- γ is based on the following functions – anti-tumerous, pro-apoptotic and cytostatic activities and due to these activities, it has a significant role in adjuvant immune-therapy against varied forms of cancers. However, the researchers have confirmed that the resulting concentration of IFN- γ in the micro-environment of the tumor determines its anticancer role.

In our data, we could observe that gene expression of IFN- γ has decreased up to 0.02 fold with respect to the housekeeping gene, β-actin [14]. The role of IL-6 is multifunctional that demonstrates a varied types of activities in different pathological conditions [15]. The main activities of this cytokine can be studied in the environment of liver, where it is mainly produced. Group researchers studied its clinical prognostic role in case of HCC and found a significant positive correlation of IL-6 serum concentration and tumor size among the HCC subjects. The study concluded that IL-6 could help in progression of HCC by acting as an autocrine tumoral growth factor and in turn reducing immune supervision [15]. Our data indicated that *Lycopodium 6C* could control the gene expression of IL-6 thus limiting its role in tumor progression. Another group of researcher also studied the mean serum level of cytokine IL-10 among the HCC subjects via ELISA method and concluded that the level of IL-10 is quite high and it can act as a biomarker along with AFP and IL-6 for the patient [16]. It was also mentioned by other researchers that high level of IL-10 correlates with worse prognosis of patients with negative survival rate suffering from varied forms of cancer [17]. In our data, the value of IL-10 was found to be reduced in the *Lycopodium 6C* set, however, the value was higher in the alcohol set when compared to normal control set. Thus, the medicine could control the high gene expression level of IL-10 which is directly correlated with worsening of the pathophysiological condition.

The cytokine IL-8 was also found to be high among liver cancer tissues and it was evident clinically that there is metastasis with elevated frequency of portal vein, venous and bile duct invasions [18]. The researchers also confirmed that IL-8 serves as an angiogenesis factor in case of HCC and plays a significant role in the metastasis and invasion of HCC [18]. However, in experimental findings we found there is up-regulation of expression of IL-8 gene in the medicine set when compared with respect to alcohol and control sets. Several authors have conferred the activities of pro and anti-tumerogenic activity to cytokine IL-1 and its family [19]. We could see an up-regulation of IL-1β in the alcohol set but the expression level has been controlled by the medicine *Lycopodium 6C*. Transforming growth factor beta is evident to play role to inhibit the growth of tumor in the early stage of liver cancer with the induction of cytostasis and apoptosis, however, can promote malignant cases in their advanced stages [20]. The gene expression study revealed that the value of TGF beta 3 is high in the alcohol set which got ameliorated in the medicine set. The last and the most important factor is tumor necrosis factor alpha (TNF α) which is evident from research study to promote the growth of tumor along with poor prognosis of HCC [21]. Our study revealed that alcohol increased the level of gene expression of TNF α which got reduced with the application of medicine, *Lycopodium 6C* when compared to control.

Thus, all the cytokine expression changes studied in this experiment appears beneficial to the patient with liver cancer except IL-8 gene expression. However, raised 10.48-fold cytokine gene expression *in vitro* is not so significant, and it is usually counteracted by other cytokines which may lead to a negative action on the cancer cells leading to a possible remission of cancer *in vivo* as a whole. Our preliminary observation with two cases of hepatocellular carcinoma showed symptomatic improvements after administration of this medicine.

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Funding

None.

Conflicts of Interest

None.

Author Contributions

Author DC has carried out the experimental work and written the draft manuscript. Author BS has assisted DC in the experimental work. Author SD has planned the entire experiment, analysed the findings and corrected the final version of manuscript. AKP studied two clinical cases and KP helped in the experiment.

Data Availability

All the data gathered during this experiment are included within this article and additional files attached.

Ethical Approval and Consent

Not applicable.

Abbreviation

HCC: Hepato-Cellular Carcinoma
HepG2: Human liver cancer cell line
IFN γ: Interferon Gamma
IL-6: Interleukin – 6
IL-8: Interleukin – 8
IL-10: Interleukin – 10
IL-1β: Interleukin – 1β
TGF-β1: Transforming Growth Factor- β1
TGF-β3: Transforming Growth Factor- β3
TNF-α: Tumor Necrosis Factor Alpha
RT-PCR: Real Time Polymerase Chain Reaction
DNA: Deoxy Ribonucleic Acid
MTT: 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
Earle's BSS: Earle's Balanced Salt Solution
DMEM: Dulbecco's Modified Eagle Medium
RNA: Ribonucleic Acid
cDNA: complementary DNA
FBS: Fetal Bovine Serum
PBS: Phosphate Buffer Saline

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