Comparison of anti-cancer efficiency of some homeopathic medicines on liver carcinoma cell line HepG2

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Abstract
In India, the prevalence of cancer is continuously rising and among different cancers, males account for about 3.9% of liver carcinoma cases. Statistical analysis revealed position of liver carcinoma within the top 10 cancers among males. Depending on the stage at which the cancer is progressing, there are three possible treatments for hepatic carcinoma: surgical resection which includes hepatectomy and liver transplantation, radiotherapy and ablation. Treatment for metastases is extremely difficult because of the sophisticated surgery required due to the liver's numerous blood vessels and bile duct. Homoeopathy is a notable adjunct treatment method due to its efficaciousness in enhancing the quality of life in cancer patients. In this work, we examined the anti-cancer efficacy of six homeopathic medicines—Calcarea carbonica, Arsenic iodatum, Mercurius vivus, Calcarea phosphorica, Calcarea iodata and Carduus marianus on hepatic carcinoma (HepG2) cell line.

The six medications listed were applied to cancer and normal cell lines, together with vehicle control. Tumor necrosis factor α, Interferon γ, Interleukins IL-6, 8, 10, 1β, transforming growth factor β1, were among the targeted cytokines analyzed. In addition to these, cell cytopatheic effect and viability assays were also done following standard protocols. The medications that were chosen based on the aforementioned factors, showed effective anti-cancer action against the HepG2 cell line. Among six medicines tested for anticancer activities against liver cancer cells, this in vitro investigation demonstrated the potential anti-cancer properties of the medicine Calcarea iodata.

Keywords: Homeopathic medicines**,** *in vitro* study, HepG2 cell line, HEK 293 cell line, cytopathic effect, cytokines expression, cell viability assay.

Introduction

Homeopathy is a therapeutic approach utilizing preparations of substances from natural sources like plants, minerals, animal products[22]. About 200 years ago, Samuel Hahnemann created the discipline. According to Hahnemann's theory (also known as the "like cures like" concept), a treatment can be used to alleviate a symptom in ultradiluted doses[14]. Several observational studies consistently demonstrate the benefits of homeopathic treatment for patients[25]. Others maintain that a therapy has "passed the test of time" as it has been in use for 200 years[14].

Since cancer is a fatal illness that has already claimed thousands of the lives throughout the globe, solving the issue will require a complex strategy. Hepatocellular carcinoma (HCC) is the one of the most prevalent types of cancers because of its high incidence rate[6]. Approximately 76% of the PubMed-indexed publications containing hepatic cancer cell lines are used to study hepatocellular carcinoma authored by many researchers using the HepG2 cell line as a model[21]. This cell line is now being utilized to study cytokines expressions as a model hepatocyte cell line for research in different metabolic pathways[21]. There are extremely few documented case studies of homeopathy in the field of cancer[24].

To find out if homeopathy could lessen the severity of adverse effects from radiation therapy, study conducted by Kulkarni et al[11] was a randomized controlled trial. In that research study, eighty-two patients with varied types of malignancies were randomly assigned to one of three parallel arms: Cobaltum C30, Causticum C30, or Placebo (where the "C" stands for centesimal homeopathic potency). An 18-point radiation reaction profile was used to examine patients once a week and it was observed that the reaction profile in both medicine groups was less in comparison to placebo[11]. Oberbaum et al[16,17] investigated the efficacy of Traumeel S oral rinse (TRS, New York, NY, U.S.A.) X in treating chemotherapy-induced stomatitis following autologous or allogeneic stem-cell transplantation. Thirty patients were randomly assigned to receive either the Traumeel S oral rinse or a placebo rinse.

Medicines like Calendula 2X, Arnica 2X, Millefolium 3X, Symphytum 6X, Chamomilla 3X, Belladonna 2X - 0.1 mL, Bellis perennis 2X - 0.05 mL, Aconitum 2X - 0.06 mL, Hypericum 2X - 0.03 mL, Echinacea purpurea 2X - 0.025 mL, Echinacea angustifolia 2X, Mercurius sol. 6X - 0.05 g, Hamamelis 1X - 0.01 mL and Hepar sulfuris 6X - 0.1 g are all present within Traumeel S oral rinse. The "X" denotes decimal homeopathic potency. Both parameters such as the time to deterioration and the reduction in the severity or duration (or both) of stomatitis showed significant differences favoring the Traumeel S group[16,17]. The World Health Organization (WHO)[26] stated that homoeopathy is the second most popular medical system in the world, with annual costs exceeding $1 billion[26].

Banerji et al[3] study reported regarding the four best cancer case series featured by National Cancer Institute, USA. Two cases were of non small cell lung carcinoma and other two were of squamous cell osesophageal carcinoma. The four cases showed that there was complete recovery and no recurrence of the condition after every six months was observed within a total treatment period of 4 years. Our findings[2,6,21] suggested that the cellular morphology in the medicine sets of Bryonia alba (6C), Lycopodium (6C) and ultra-diluted Arsenic trioxide (6C) was minimized and became round shaped, suggesting that the cells might have undergone apoptosis.

In an *in vitro* model HepG2 cell line, the maximum number of cells was dead and had detached from the base (cell detachment) of the culture plate after 24 hours of the medicine inoculation[2,6,21]. The CPE changes were also accompanied with several cytokines gene expression changes that highlighted the anti-cancer efficacy of the few studied medicines against hepatic carcinoma[2,6,21].

Material and Methods

Procurement of cell lines and chemicals: Hepatic carcinoma (HepG2) and normal human embryonic kidney (HEK 293) cell lines were purchased from National Centre for Cell Science (NCCS), Pune, India. The sixteen short tandem repeat (STR) loci were used to authenticate the cell lines and these loci were amplified using the commercially available AmpFISTR Identifier Plus PCR Amplification kit from Applied Biosystems. The Applied Bio-system® 3500 genetic analyzer was used to process the cell line sample. Applied Biosystems' Gene Mapper® ID-X v1.5 program was used to analyze the data. Appropriate positive and negative controls were employed for the confirmation. Hoechst staining and the Mycoplasma PCR technique were used for the testing and the results showed that there was no Mycoplasma contamination[2,6,21].

The medicines namely Calcarea carbonica, Arsenic iodatum, Mercurius vivus, Calcarea phosphorica, Calcarea iodata, Carduus marianus of potency 6C (the "C" stands for centesimal potency) were purchased from an Indian Government recognized homeopathic medicine manufacturing company HAPCO, India. The HepG2 cell line was cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM) (1X) along with Glutamax (Gibco, ThermoFischer, USA). F-12 (1X) nutrient mixture Ham + L-glutamine was purchased from Gibco, ThermoFischer, USA as a supplement for better growth of the HepG2 cell line. 10% of Fetal bovine serum (FBS) (Gibco, ThermoFischer, USA) and the antibiotic –antimycotic solution namely Penicillin/Streptomycin/Amphotericin B Solution (100X) (Gibco, ThermoFischer, USA) were also added to the medium. The other chemicals used to culture the cell lines were Trypsin enzyme (0.05 X), Phosphate buffer saline (PBS, 1X) of pH 7.4 procured from Gibco, ThermoFischer, USA. The cell cytotoxicity assay was done with MTT assay kit EZ Count (Himedia India Pvt. Ltd.) Molecular biology reagents such as RNA isoplus (Takara Bio USA), cDNA synthesis kit (reverse transcriptase kit, Bio-Rad, USA) and the iTaq Sybr green super-mixture for RT-PCR (Bio-Rad, USA) were also purchased.

Cell culture and Maintenance: In a T25 cm^2 flask, the HepG2 cells were cultured for 48 hours in DMEM with 10% FBS and F12 (supplement) treated with Pen-Strep antibiotic solution, till they reached 80% confluency. Following 48 hours, the cell debris was washed with PBS (1X). For the detachment of the cells from the T25 cm^2 flask base, the cells were treated with trypsin solution for ten minutes and the adhering cells were manually shaken off the flask's base. Immediately, trypsin was inactivated using DMEM medium supplemented with 10% FBS right after ten minutes. After that, the cells were centrifuged for 12 minutes at 1200 rpm.

To conduct the experiment on a 12-well plate, the supernatant medium was decanted and fresh media of the necessary volume were added. Each well of the plate was filled with 1 mL of media containing cells, which were then incubated for the following 24 hours in a humidified atmosphere with 5% carbon dioxide. The following day, 1X PBS was used to wash the wells and once more, new media charged. Before the medicines were inoculated, the cells were allowed to acquire a confluency of 10^5–10^6[21].

Cyto-toxicity Study and Medicine dosage inoculation: The cytotoxicity studies were done using the MTT assay kit EZ Count (Himedia India Pvt. Ltd.) following the detailed manufacturer's protocol. The dosages that were analyzed for inoculation were 10, 20, 30, 40, 50, 80, 100 and 150µL. After MTT assay, 100 µL of the medicines (determined from MTT assay) was inoculated within the 12 well cell culture plates. The confluency of the cell lines within each well of 12- well culture plate was maintained at 10^5. Following the inoculation of medicines, the cells were allowed to incubate at 37ºC for 24 hours at 5% of carbon-dioxide level (Fig. 1)[21].

Cytopatheic Effect Study (CPE) and Cell viability study with Methylene Blue Assay: Each well of the plate was observed under 40X magnification of inverted microscope and the CPE was noted. To study the cell viability, methylene blue assay was done following our pre-standardized protocol. According to the protocol, after one hour of incubation with the staining dye, the cells were washed with PBS and again observed under the 40X magnification of inverted microscope. Cellular morphology and viability were noted down for each experimental set done in triplicate for statistical validation[6,21].

Molecular Biology Assays: The cells were harvested with RNA isoplus (Takara Bio USA). The extracted RNA was kept at 4ºC for overnight drying. The next day, the dried RNA was dissolved within 60µL of nuclease free water at 56ºC for 10 minutes, then the A260/280 ratio was determined to assay the purity (RNA purity ranges in between O.D ratio of 1.8 – 2).

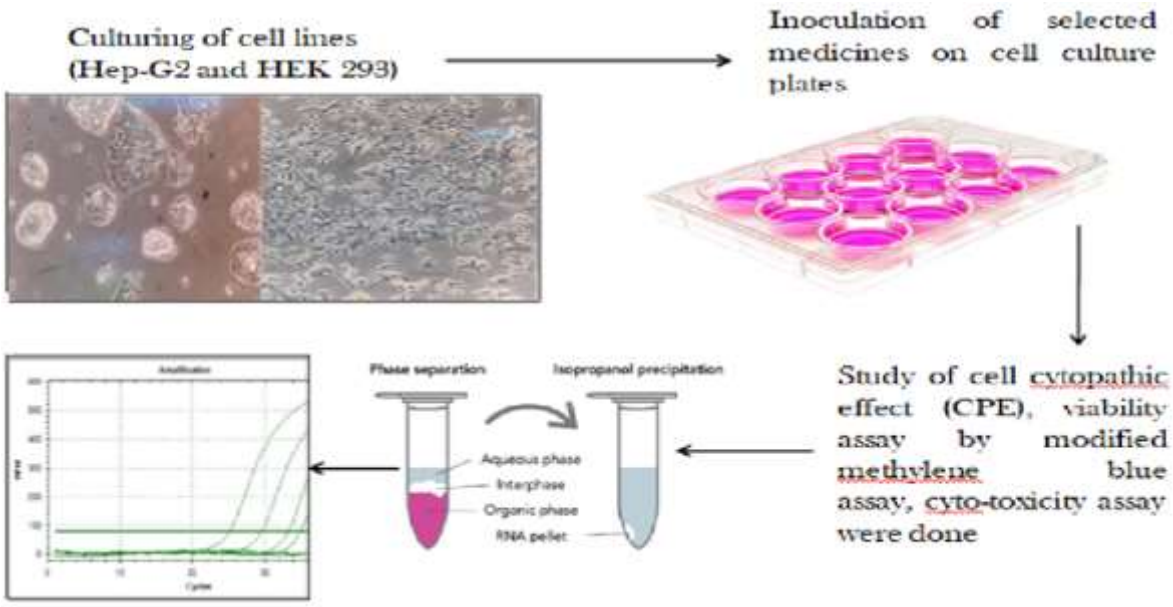

Fig. 1: Flow chart of the entire experimental process

Thereafter, cDNA was synthesized with the pure RNA using iscript reverse transcriptase kit (Bio-Rad, USA) and it was stored at -20ºC for further assays. RT-PCR study of the targeted genes for the gene expression assay for the cytokines: Interferon gamma (IFN γ), Interleukins – IL-6, IL-8, IL-10, IL-1β, Transforming Growth Factor β1 (TGF-β1) and Tumor Necrosis Factor (TNF-α) was done against the housekeeping gene β-actin. The gene expression calculation was done following the relative fold change in gene expression by the 2^ (-ΔΔct) formulae (Fig. 1).

Ethical Permission: The study has been conducted upon *in vitro* cell lines, therefore ethical permission was not required for the study.

Results

MTT Assay Analysis: The MTT assay was carried out upon hepatic carcinoma (Hep-G2) and normal human embryonic kidney (HEK 293) cell lines. Through the charting of the normalized absorbance values versus the number of cells, the halves of maximum inhibition of cell proliferation (IC50) of the medicines were found. Dosage of 100µL of the medicines was found to be effective against Hep-G2 cell line and showed no cyto-toxicity upon the normal HEK 293 cell line. Therefore, 100 µL doses of medicines were considered for the other cell line assays.

Cytopatheic Effect Study (CPE): The figures 2a-2g represent the cytopathic effect or the effect on cell morphology on HepG2 and HEK 293 cell lines after 24 hours of medicine application. Normally large vacuoles are typical during cell growth and clumps are common. The cells were between 70 and 80 percent confluent when the medicines were introduced. After being treated with the medicines: Arsenic iodatum, Calcarea carbonica, Carduus marianus, Calcarea iodata, Calcarea phosphoric and Mercurius vivus, the HepG2 cells began to exhibit blebs, changed the shape and became spherical, all of which were signs of apoptosis and cell death. In addition, the cells separated from the bottom surface within 24-h following the medicine application. Non-apoptotic with some apoptotic cells were the main findings in the alcohol control set.

In the alcohol (vehicle) control set, the cell sizes were diminished and membrane blebs were seen. However, the HepG2 cells treated with alcohol control retained the normal cellular morphology to some extent compared to the medicines-treated cells. The HEK 293 indicated that they were viable with intact cellular morphology. The cells in the HEK 293 control set were with proper cellular margination and typically exhibited dendritic extensions. However, the HEK 293 cells decreased in size and the shape was mildly altered indicating mild cyto-toxic effect upon the normal cell lines (Fig. 2).

Cytokine Gene Expression Study: All the medicines suppressed the production of IFN-γ. The induction of IL-6 by the medicines Carduus marinus, Arsenic iodatum, Calcarea phosphoric and Mercurius vivus may have a worsening effect on the condition of hepatic cancer and the prognosis of patients with HCC. All the medicines showed down-regulation of the gene expression of IL-10 and it was remarkably suppressed by Calcarea iodata, thus indicating towards the anti-cancer activity of the medicines. The anti-tumerogenic action of IL-1β counterbalanced the potential increase in IL-8 (angiogenesis factor) production caused by these four medications.

The anti-tumerogenic properties of IL-1β counterbalanced the increased production of IL-8 (angiogenesis factor) caused by the medicines Carduus marinus, Arsenic iodatum, Calcarea phosphorica and Mercurius vivus. Calcarea iodata exhibited a marginal up-regulation of TGF-β1 but it is much less when compared to control HepG2 cells.

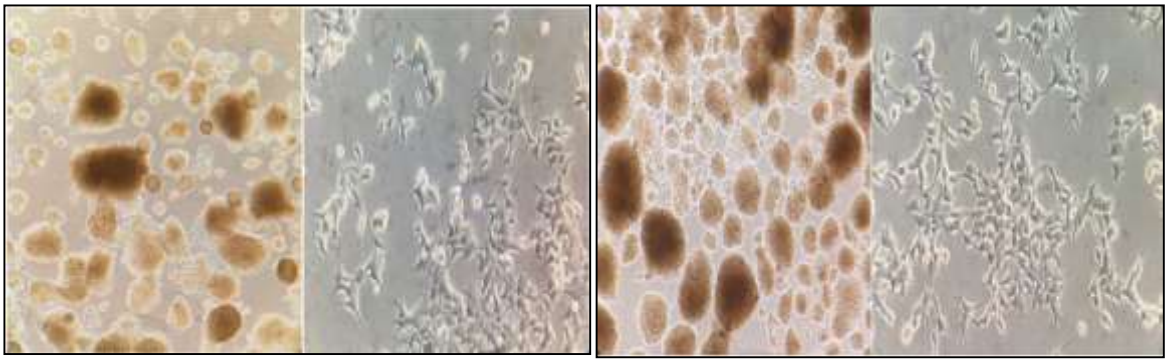

Fig. 2: (a) CPE of Arsenic iodatum on Hep-G2 and HEK 293 cells;
(b) CPE of Calcarea carbonica on Hep-G2 and HEK 293 cells

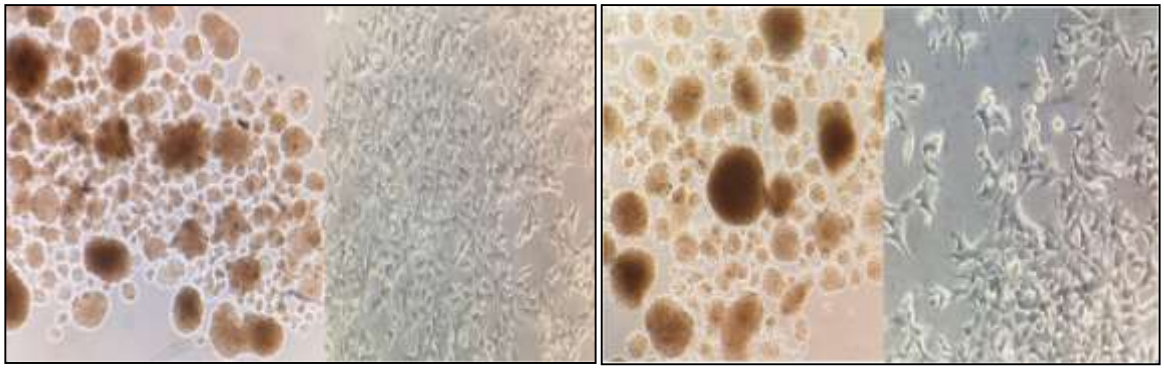

Fig. 2: (c) CPE of Carduus marianus on Hep-G2 and HEK 293 cells;
(d) CPE of Calcarea iodata on Hep-G2 and HEK 293 cells

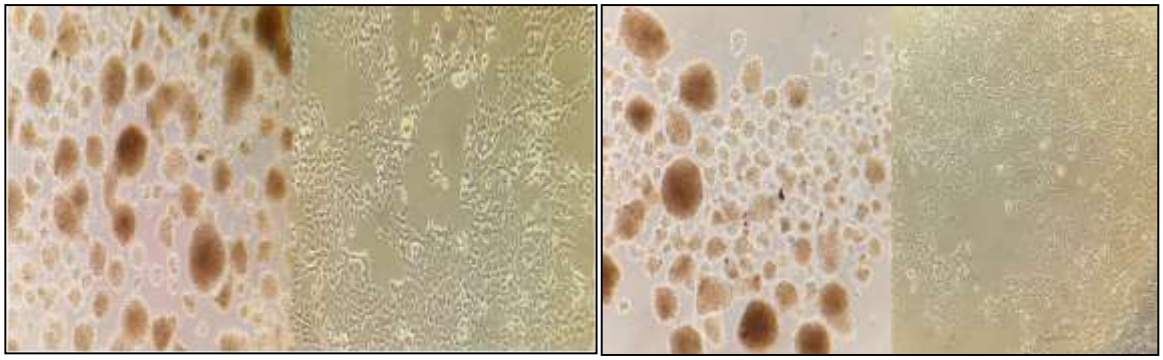

Fig. 2 (e) CPE of Calcarea phosphorica on Hep-G2 and HEK 293 cells;
(f) CPE of Mercurius vivus on Hep-G2 and HEK 293 cells

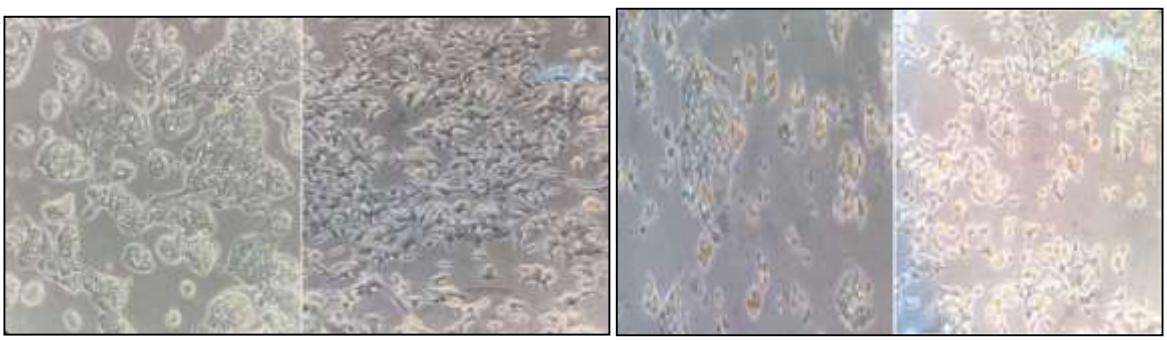

Fig. 2: (g) CPE of alcohol treatment on Hep-G2 and HEK 293 cells

The figures 2a-2g represent the cytopathic effect or the effect on cell morphology on HepG2 and HEK 293 cell lines after 24 hours of medicine application. Normally large vacuoles are typical during cell growth and clumps are common. The cells were between 70 and 80 percent confluent when the medicines were introduced. After being treated with the medicines: Arsenic iodatum, Calcarea carbonica, Carduus marianus, Calcarea iodata, Calcarea

phosphoric and Mercurius vivus, the HepG2 cells began to exhibit blebs, changed shape and became spherical, all of which were signs of apoptosis and cell death. In addition, the cells separated from the bottom surface within 24-h following the medicine application. Non-apoptotic with some apoptotic cells were the main findings in the alcohol control set. In the alcohol (vehicle) control set, the cell sizes were diminished and membrane blebs were seen. However, the HepG2 cells treated with alcohol control retained the normal cellular morphology to some extent compared to the medicines-treated cells. The HEK 293 indicated that they were viable with intact cellular morphology. The cells in the HEK 293 control set were with proper cellular margination and typically exhibited dendritic extensions. However, in the Calcarea phosphorica and Mercurius vivus medicine treated cells, the HEK 293 cells decreased in size and the shape was mildly altered indicating mild cyto-toxic effect upon the normal cell lines.

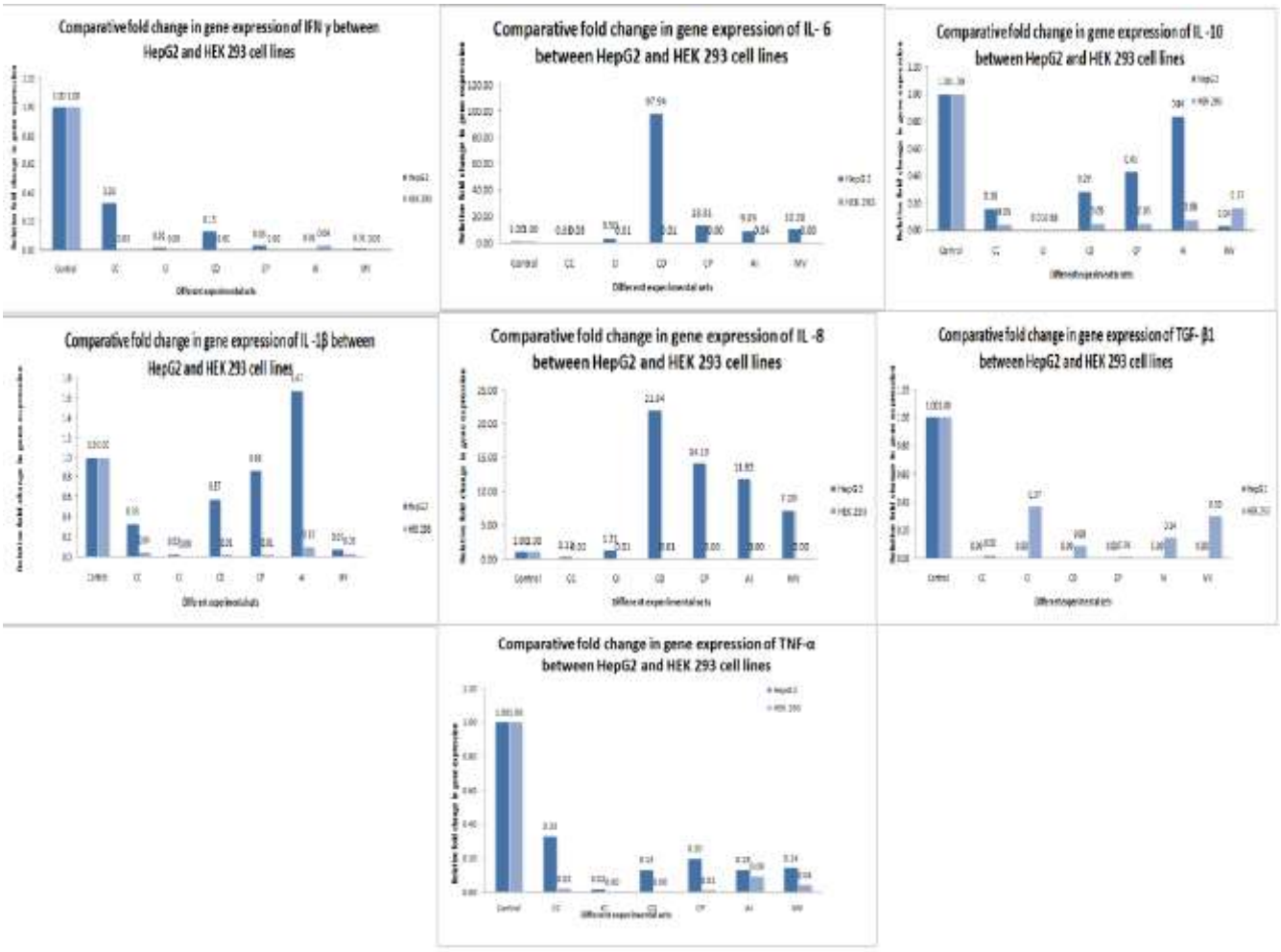

Fig. 3: All the medicines suppressed the production of IFN-γ. The induction of IL-6 by the medicines Carduus marinus, Arsenic iodatum, Calcarea phosphoric and Mercurius vivus may have a worsening effect on the condition of hepatic cancer and the prognosis of patients with HCC. All the medicines showed down-regulation of the gene expression of IL-10 and it was remarkably suppressed by Calcarea iodata, thus indicating towards the anti-cancer activity of the medicines. The anti-tumerogenic action of IL-1β counterbalanced the potential increase in IL-8 (angiogenesis factor) production caused by these four medications. The anti-tumerogenic properties of IL-1β counterbalanced the increased production of IL-8 (angiogenesis factor) caused by the medicines Carduus marinus, Arsenic iodatum, Calcarea phosphorica and Mercurius vivus. Calcarea iodata exhibited a marginal up-regulation of TGF-β1 but it is much less when compared to control HepG2 cells. In this *in vitro* investigation using the HepG2 cell line, Calcareaiodata showed a slight up-regulation of TGF-β1 and a significant suppression of TNF-α, suggesting that it may have anti-cancer properties. [Note: Carduus marinus (CD); Arsenic iodatum (AI); Calcarea phosphorica (CP); Calcarea carbonica (CC); Calcarea iodata (CI);Mercurius vivus (MV)]

In this *in vitro* investigation using the HepG2 cell line, Calcarea iodata showed a slight up-regulation of TGF-β1 and a significant suppression of TNF-α, suggesting that it may have anti-cancer properties. (Note: Carduus marinus (CD); Arsenic iodatum (AI); Calcarea phosphoric (CP); Calcarea carbonica (CC); Calcarea iodata (CI); Mercurius vivus (MV)) (Fig. 3).

Discussion

Hepatocellular carcinoma (HCC) is a global health concern that entails a significant healthcare burden with elevated incidence rate, followed by unfavorable prognosis. Though it usually arises from Hepatitis B infection and liver cirrhosis, its pathogenesis differs mostly based on the underlying etiological causes[10]. The development of HCC is a result of a complex interplay between pro- and anti-inflammatory cytokines (TNF-α, IL-6), pro-angiogenic molecules (VEGF, Angiopoietins, PECAM-1, HIF-1α), transcription factors (STAT-3, NF-kB) and their signaling pathways. Since cytokines are expressed and released at different times during the evolution of HCC, measuring them using various techniques can offer comprehensive information on the diagnosis and treatment of HCC[19].

Pathological angiogenesis is considered to be a precondition for liver disease and also for the development of HCC. Angiogenesis is the process that extends the growth of tumors and is the outcome of multiple physiological events. Under typical circumstances, the ratio of angiogenic inducers to inhibitors maintains control over the angiogenic process and guards against unwarranted tissue vascularization[15]. In the liver, interleukin IL-6 plays a significant role in inducing the acute phase response[9]. To initiate intracellular signaling, IL-6 binds to the signal-transducing subunit gp130 on target cells, either in combination with the soluble or membrane-bound IL-6 receptor[9,15]. IL-6 can trigger monocyte chemotaxis and sustain chronic inflammation towards any wounded tissue using the latter "trans-signaling" mechanism[18,19].

The process of hepatocarcinogenesis is intricately associated with transforming growth factors (TGF)-α and TGF-β[8]. TGF-α expression is lower in normal hepatocytes than in malignant cells. TGF-β is a crucial regulator of the late phase of inflammatory processes. It does this, at least in part, by controlling adhesion molecules on parenchymal cells. TGF-β blocks pro-inflammatory cytokine actions in this way, the phenomena inhibits several cellular functions like survival, differentiation and proliferation[8]. Ironically, these changes in the microenvironment can be advantageous to cancer cells[13]. One powerful cytokine that reduces inflammation, is IL-10. Compared to viral infections, its mechanism of action in HCC is less explored in previous research studies.

According to a recent meta-analysis, IL-10 levels in HCC patients rose in comparison to healthy controls and cirrhosis patients, but not in comparison to patients with viral hepatitis[23]. According to a study[13], serum levels of IL-10 functioned as a negative predictive indicator in HCC that was incurable. The cytokine known as tumor necrosis factor (TNF), is generated through proteolytic cleavage of a trans-membrane protein precursor (mTNF) into soluble TNF (sTNF). TNF triggers a variety of hepatic biological reactions including apoptosis, hepatocyte necrosis, inflammation of hepatic cells and regeneration, in addition to accelerating the development of HCC[18]. When chronic liver injury occurs, the transcription factor signal transducer and activator of transcription 3 - STAT3's downstream transduction pathway is activated by the expression of IL-6 and TNF-α, leading to neoplastic change in the hepatic milieu[13,18]. Regardless of the cause, IL-6's pro-proliferative effects are mediated via activating and directly interacting with the p65 subunit of Nuclear factor kappa B (NF-κB), whose activation is linked to a frequent and early event in liver fibrosis and HCC[5,7,23].

Our study showed the gene expression analysis of these above mentioned pro-inflammatory and anti-inflammatory cytokines within the HCC cells in *in vitro* condition. IFN-γ's anti-tumerogenic effect is founded on its anti-tumerous, pro-apoptotic and cytostatic properties. As a result of these properties, it plays a vital role in adjuvant immune therapy against a variety of cancer types. All the medicines suppressed the production of IFN-γ[6]. According to the study's findings, IL-6 may contribute to the development of HCC by lowering immune surveillance and functioning as an autocrine tumor growth factor[18].

The induction of IL-6 by the medicines Carduus marinus, Arsenic iodatum, Calcarea phosphorica and Mercurius vivus may have a worsening effect on the condition of hepatic cancer and the prognosis of patients with HCC18. IL-10 was relatively high and that, along with AFP and IL-6, could serve as a biomarker for the patient[18]. All the medicines showed down-regulation of the gene expression of IL-10 and it was remarkably suppressed by Calcarea iodata, thus indicating towards the anti-cancer activity of the medicines. The liver cancer tissues had a high level of the cytokine IL-8[1]. Clinical examination revealed metastases with a higher frequency of portal vein, venous and bile duct invasions.

The anti-tumerogenic properties of IL-1β counterbalanced the increased production of IL-8 (angiogenesis factor) caused by the medicines Carduus marinus, Arsenic iodatum, Calcarea phosphorica and Mercurius vivus. It is clear that TGF beta inhibits the growth of tumors in the early stages of liver cancer by causing apoptosis and cytostasis; yet, in the latter stages, TGF beta can worsen malignant situations[6]. Calcarea iodata exhibited a little up-regulation of TGF-β1. Tumor necrosis factor alpha (TNFα) is the final and most significant element. Research studies have shown that TNFα promotes tumor growth and isa poor prognosis for HCC patients[6]. In this *in vitro* experiment, using the HepG2 cell line, Calcarea iodata showed a slight up-regulation of TGF-β1 and a significant suppression of TNF-α, highlighting its role in the anti-cancer treatment among the screened alternative medications.

Conclusion

In this *in vitro* investigation using the HepG2 cell line, *Calcarea iodata* showed an up-regulation of TGF-β1 and a significant suppression of TNF-α, highlighting its role in the anti-cancer treatment among the screened homeopathic medications.

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