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IN-VITRO ANTICANCER ACTIVITY OF *CASSIA FISTULA* FRUIT AND LEAF EXTRACT ON LIVER CANCER CELL LINE

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Keywords:

Hepatocellular carcinoma, *Cassia fistula*, Cytotoxicity, Interleukins

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ABSTRACT: Among different neoplastic lesions, hepatocellular carcinoma plays a pivotal role due to the extreme difficulty of treating it in advanced conditions. Research shows that there is a high intractability towards chemotherapy for this disease. Therefore, using medicinal plants can be another approach to fend off this grim scenario and pave a new way to prolong survival time. For this purpose, the ethanolic extracts of leaves and fruits of *Cassia fistula* (Golden shower) as a probable anticancer drug have been administered in the liver cancer cell line, HEPG2 and the gene expression of cytokines (interleukin-6, interleukin-8, interleukin IL-1β, transforming growth factor-TGF-β1, tumour necrosis factor-TNF-α) against this treatment has been analyzed in the present work. The current study revealed a significant reduction in the expression of pro-inflammatory cytokines by the application of these extracts demonstrating their vital role in suppressing tumour growth and proliferation in liver cancer. It appears that important phytochemicals played a key role in decreasing the expression of these cytokines. The combination of leaves and fruit extracts of *Cassia fistula* can be a promising agent to treat these patients.

INTRODUCTION: The uncontrolled proliferation of abnormal cells in the body leads to the development of cancer. One of the important factors in carcinogenesis is multiple changes in the genes. It can develop anywhere in the body. Among the various types of cancer, liver cancer is one of India's leading causes of mortality. Non-alcoholic fatty liver disease is considered to be one of the major risk factors of Hepatocellular Carcinoma (HCC) [1]. The major risk factors for HCC includes associated infection with Hepatitis B or C viruses, Cirrhosis, Chronic liver disease, exposure to aflatoxins, nonalcoholic fatty liver disease, genetic susceptibility and certain dietary factors.

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Most conventional human liver cancer cell lines, such as HEPG2, and Hep3B, are recurrently employed to carry out the *in-vitro* experiments originally developed from HCC. Surgical resection, transplantation, and ablation are the most common treatment for this deadly disease. Monitoring HCC with alpha-fetoprotein and liver ultrasound are extensively used in patients suffering from HCC. These treatments can have side effects on normal cells. Utilization of medicinal plants in the new medical and pharmacological treatment strategy of cancer, which is considered to be the alternative approach to replace these often adverse effect-producing methods [2].

Numerous medicinal plants bearing phytopharmaceuticals have been extensively used in agricultural, veterinary, and medical fields. *Cassia fistula*, herbarium number 007670, is one of the most beneficial plants applied in herbal therapies [3]. It is a deciduous plant bearing yellow flowers, alternate leaves, pendulous fruit, grey

bark, with light brown seeds [4]. This plant belongs to the family Fabaceae and has been efficacious in anti-tumor, hepatoprotective, anti-diabetic activities [4]. Most of these species are rich in oxy anthraquinones-and anthraquinone derivatives, flavonoids, and tannins [5]. This plant's leaves, fruits, flower, stem, and roots comprise phytochemical active agents [6].

Notable phenolic compounds with novel structures such as epiafzelechin-3-O-glucoside, bioflavonoids are generated by different organs of this species. Therefore, this study aimed to analyze the anticancer efficacy of crude ethanolic extract of leaves and fruits of *Cassia fistula* against hepatocellular carcinoma cell line, HEPG2 with respect to normal cell line HEK 293.

MATERIALS AND METHODS:

Plant Collection: The leaves and fruits of *C. fistula*, family Fabaceae, (Floral formula % ▪ K(5) C1+2+(2) A(9)+1 G1) were collected from Heritage Institute of Technology Campus, West Bengal, India in June 2022. A Botanist identified the plant. It is a common plant of Bengal. Then the collected samples were cleansed, air-dried and ground into semi-granulated powder using a mortar and pestle for further processing.

Preparation of Extract and Isolation: The fresh leaves, about 10 gw as subjected to extraction at room temperature using 100 mL of 70% ethyl alcohol solution prepared with deionized water. Similarly, for dried samples 5g of *Cassia* leaves in 50 ml and 2g of *Cassia* fruit in 20 ml, were subsequently extracted using 70% ethyl alcohol solution prepared with deionized water.

The extracts were kept in dark for 48 hours. After two days, the extract was filtered through Whatman No.1 paper to remove the debris. Following filtration, the extract was further passed through 0.55µm Merck Millipore filter for sterilization purpose. All the extracts were stored in amber glass bottles at 4^{o}C throughout the study period.

We procured the two cell lines namely normal kidney cell line (HEK 293) and liver cancer cell line (Hep G2) from the Cell Culture: National Center for Cell Science, Pune. All the cells were cultured in Dulbecco's modified eagle medium (DMEM) media containing 10% fetal bovine serum (FBS), 10% Ham's F-12 Nutrient Mix, 100 units/mL of Penicillin and 100 units/mL of Streptomycin and L-glutamine (2mM). Cell culture medium was maintained at 4^{o} C for cell culture assay.

Cytotoxicity by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide:

(MTT) Assay: HEPG2 cells were seeded on to 96-well microtiter plate in a total volume of 100µL and left overnight to maintain confluency. Six vehicle controls with media were run as controls. After 24 hours, the media was removed from each well, including the control and 100µL of fresh media was added and kept for incubation for 24 hours. At the end of 24 hours, the extracts used as a drug were inoculated in triplicates (dosage- 0, 2, 4, 6, 8, 10, 12, 15µl).

The plate was kept for incubation at 37^{o}C in 5% CO_2 for 24 hours. At the end of the incubation period, 10µL of MTT Reagent, including controls, was added to each well. Then the plates were wrapped with aluminium foil to avoid exposure to light. The plates were then incubated for 2 hours at 37^{o}C. After incubation, the cells were observed under an inverted microscope to ensure the formation of purple formazan crystals.

These crystals were formed due to the ability of the living cells (HEPG2) to reduce the yellow dye MTT to purple crystals. After observation, 100µL of solubilization solution was added to each well. Then the plates were incubated overnight. The next day, the absorbance was read at 570nm in an ELISA plate reader. Calculation of cell death percentage was done using the formula-

$$\% \text{ cell death} = 100 - [At / Ac \times 100]$$

Whereas, At: absorbance of treated cells, Ac: absorbance of control.

The concentration of the extract that causes half-maximal inhibition of cell proliferation (IC_{50}) was determined by plotting the normalized absorbance values against cell number.

Drug Administration: After seeding the cells in a 12-well plate for 24 hours incubation in 5% CO_2 at 37^{o}C, the plates were removed from the incubator, and 50µL of the leaf and fruit extract of *C. fistula*

as a drug was administrated in the respective sets – *Cassia* leaves, dry *Cassia* leaves, and *Cassia* fruit along with their vehicle controls. All the sets were performed in triplicates to get statistically validated results. The plates were then kept for 24 hours of incubation.

Cytopathic Effect Study: After the incubation period, the plates were observed under an inverted microscope for analysis of the cell morphology after drug administration.

Cell Viability Assay:

Methylene Blue Staining (MBS): To illustrate the viability of the cell population, methylene blue staining was performed. The methylene blue solution was prepared using Phosphate buffer saline solution (1X, PBS), 0.06% methylene blue 1.25% glutaraldehyde.HEPG2 cells were plated in 12-well plates after incubation was taken out, and the medium was discarded.

Cells were then stained by adding methylene blue solution (1 mL) and kept for 1-hour incubation. After an incubation period at 37^{o}C in 5% CO_2, the plates were examined under an inverted microscope for the viable and dead cells. Following incubation, the cells were rinsed with PBS solution and 1 mL of RNA isoplus was added to each well to harvest cells. Then using a cell scraper, the cells were scraped off the bottom of the wells and stored in Eppendorf tubes for further molecular biological assays [7].

Gene Expression Analysis of Cytokines: Total RNA extraction was performed following the guidelines of TRIZOL RNA Isolation. Then RNA purity was analyzed by measuring A260/280 ratio using UV-Vis spectrophotometer (Agilent, USA). Complementary DNA (cDNA) synthesis was carried out using a cDNA synthesis kit (iscript Reverse Transcriptase, Bio-Rad, USA) with Thermal Cycler PCR Machine (T 100, Bio-Rad, USA).

Further, Real-Time Quantitative PCR Method was carried out in CFX-96 model of RT PCR (Bio-Rad, USA) for gene expression analysis of six cytokines namely- Tumour Necrosis Factor (TNFα), Transforming growth factor (TGF)–β1, (Interleukins- IL-6, IL-10, IL-8,IL-1β) against the housekeeping gene, βactin.

Statistical Analysis: The statistical analysis of data was performed using one-way ANOVA using statistical software EZ SPSS.

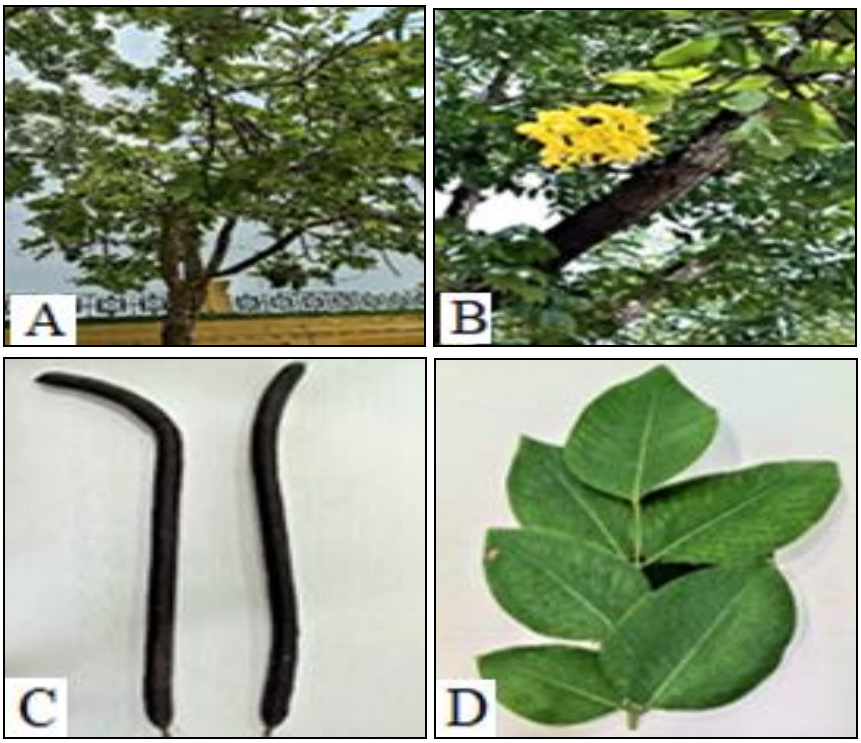

FIG. 1: (A) REPRESENTS *CASSIA FISTULA* TREE B) SHOWS *CASSIA FISTULA* BEARING YELLOW FLOWERS C) ILLUSTRATES *CASSIA FISTULA* HAVING PENDULOUS FRUITS) SHOWS ITS LEAVES

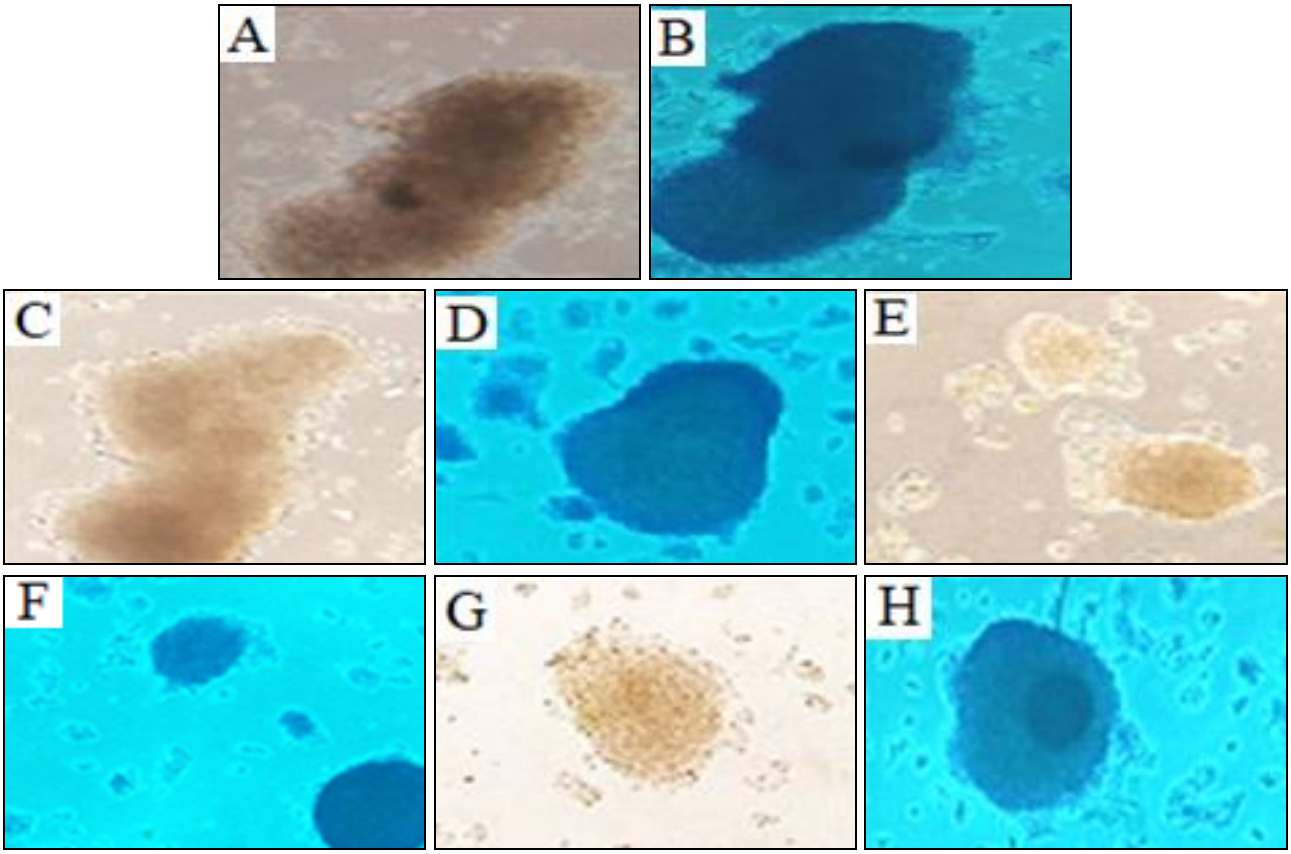

FIG. 2: CELL CYTOPATHIC EFFECT OF HEPG2 CELLS BY THE EXTRACT ALONG WITH VIABILITY ASSAY AFTER MBS: The HEPG2 cell morphology was determined under an inverted microscope after exposure to the ethanolic plant extract used as a drug treatment. The inoculum was added when cells were 70 to 80% confluent. A) represents HEPG2 cell line after 24 hours of fruit extract of *Cassia* plant as a drug and B) with cells after 24 hours of drug treatment, fruit extract after mbs C) HEPG2 cells after 24 hours of drug inoculation with vehicle control set (alcohol) and D) with vehicle control set after MBS E) represents HEPG2 cells after 24 hours of leaf extract of *Cassia* plant and F) with leaf extract after MBS G) HEPG2 cells after 24 hours of drug inoculation with vehicle control set (alcohol) and H) with vehicle control set after MBS.

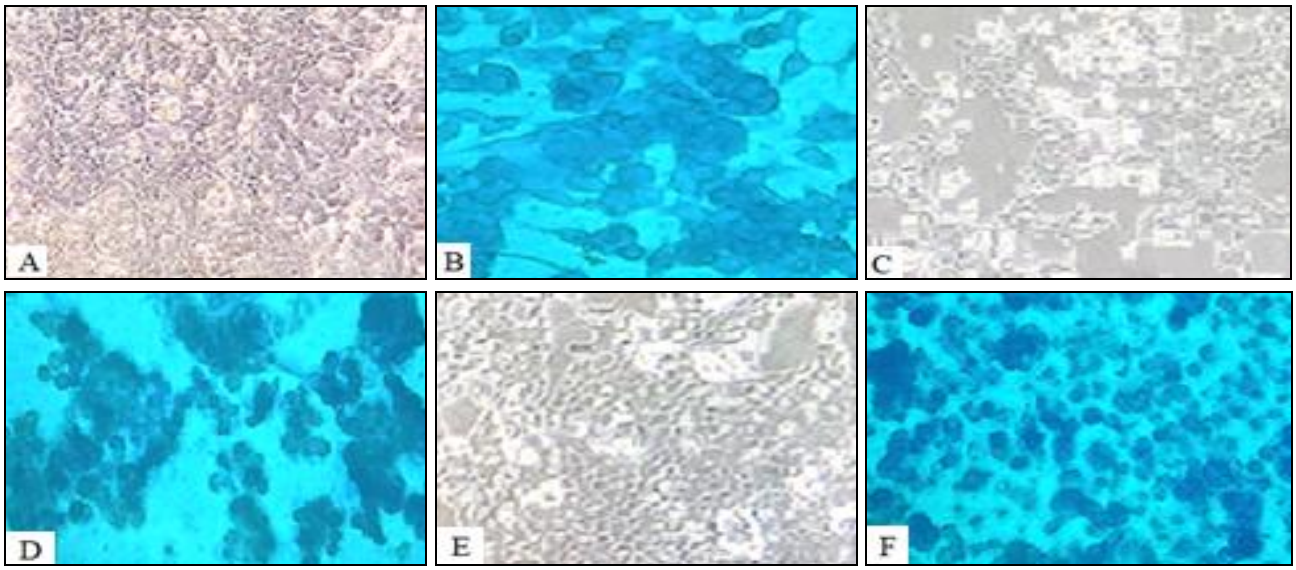

FIG. 3: CELL CYTOPATHIC EFFECT OF HEK293 CELLS BY THE EXTRACT ALONG WITH VIABILITY ASSAY BY METHYLENE BLUE STAINING: (A) represents control HEK cell line after 24 hours (B) control HEK cells after MBS (C) HEK cells after 24 hours of drug inoculation of ethanolic fruit extract of *cassia* plant (D) HEK cells after 24 hours of drug inoculation of ethanolic fruit extract of *cassia* plant after MBS (E) HEK cells after 24 hours of drug inoculation of ethanolic leaf extract of *cassia* plant (F) HEK cells after 24 hours of drug inoculation of ethanolic leaf extract of *Cassia* plant after MBS.

RESULTS:

Cell Morphology and Cytopathic Effect: Fig. 2 (A-H) represents morphological observation in the HEPG2 cell line. The cells usually grow with big vacuoles and tend to form clumps. The inoculum was added when the cells were around 70-80% confluent.

Treating the HEPG2 cells with the ethanolic fruit and leaf extract of *Cassia* plant, the cells started to change their integrity, showed blebs, and became round, indicating apoptosis and cell death.

The cells also got detached from the surface after 24 hours of drug inoculation. In vehicle control set (alcohol) apoptotic bodies and non-apoptotic bodies were seen. The cell size was reduced in the vehicle control set, and membrane blebs were seen. After methylene blue staining, some cells retained the stain indicating its viability, and most of them were dead, suggesting apoptosis or necrosis had occurred.

Fig. 3(A-G) illustrates images to show the morphological changes in the HEK293 cell line. In the control set, the cells were intact, usually in shape showing dendrite-like extensions.

After methylene blue staining, the cells in the control set exhibit epithelial morphology. In the treatment group with Cassia plant's ethanolic fruit and leaf extract, the cells detached from the surface and became round in shape, indicating apoptosis.

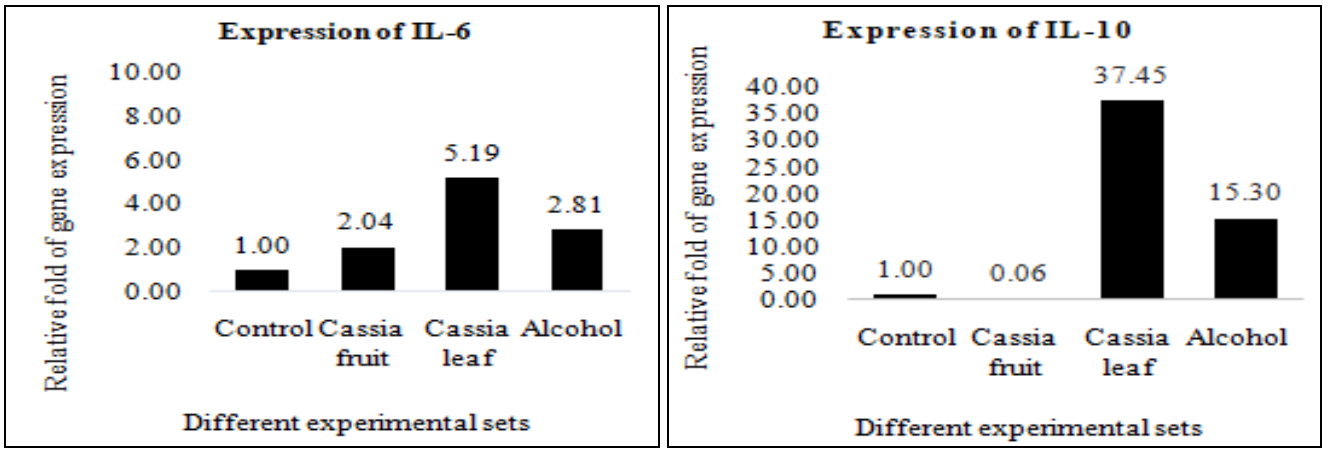

FIG. 4: DIFFERENTIAL EXPRESSION OF IL-6 AND IL-10 IN DIFFERENT EXPERIMENTAL SETS. The column graph represents mean value ± SEM of four independent experimental findings A) IL-6, which is considered to be pro-inflammatory cytokine was increased by the drug treatment compared to vehicle control and normal control in liver cancer cell line B) IL-10 also got reduced by the ethanolic leaves and fruit extract of *Cassia* plant compare to vehicle control and normal control in the liver cancer cell line.

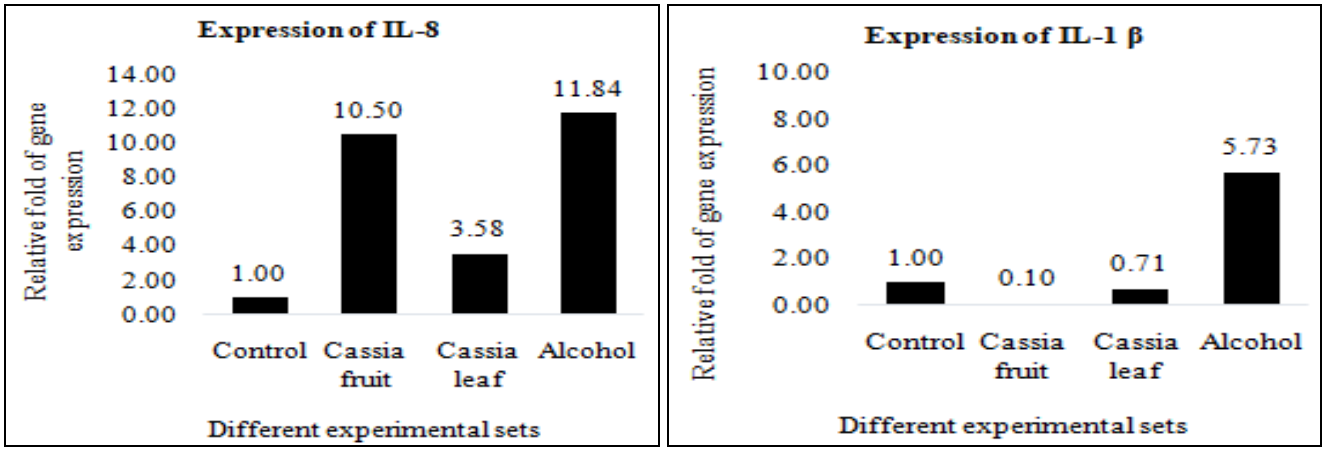

FIG. 5: DIFFERENTIAL EXPRESSION OF IL-8 AND IL-1B IN DIFFERENT EXPERIMENTAL SETS. The column graph represents mean value ± SEM of four independent experimental findingsA) IL-8 which helps in tumor progression got significantly reduced by ethanolic leaf extract of *Cassia* plant as the drug treatment compared to vehicle control in liver cancer cell line B) IL-1β showed down-regulation by the ethanolic fruit and leaves extract of *Cassia* plant as the drug treatment compare to vehicle control and normal control in the liver cancer cell line.

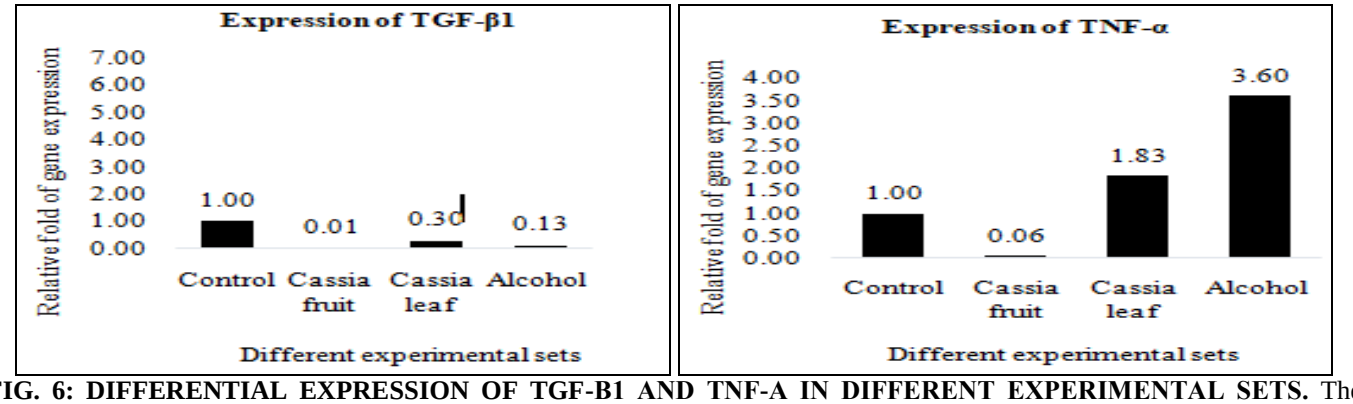

FIG. 6: DIFFERENTIAL EXPRESSION OF TGF-B1 AND TNF-A IN DIFFERENT EXPERIMENTAL SETS. The column graph represents the mean value ± SEM of four independent experimental findingsA) The pro-fibrogenic cytokine TGF-β1 got decreased in experimental sets compared to vehicle control and normal control following the drug treatment. B) TNF-α, another pleiotropic cytokine got merely reduced by ethanolic fruit extract of *Cassia* plant compared to vehicle control and normal control in the liver cancer cell line.

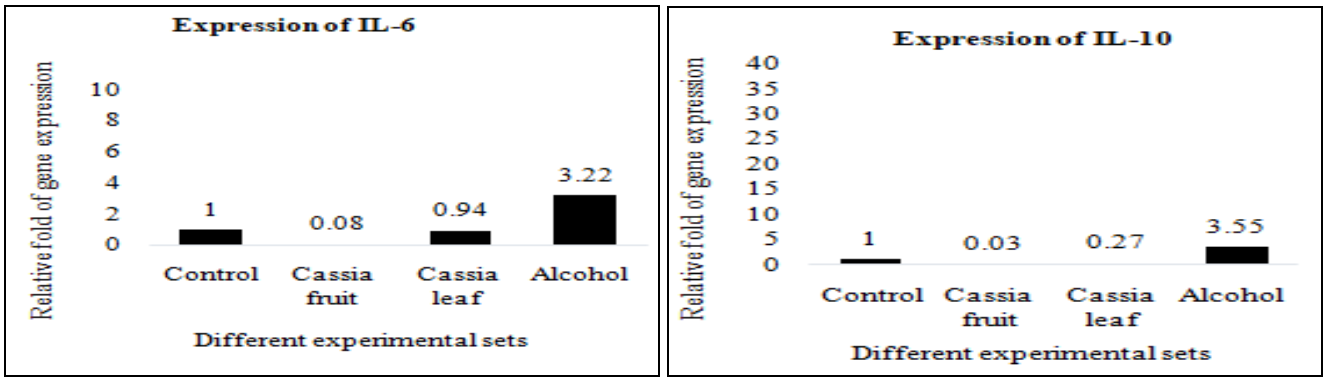

FIG. 7: (A) REPRESENTS DIFFERENTIAL EXPRESSION OF IL-6 AND (B) IL-10 IN NORMAL CELL LINE IN HEK293 WITH OR WITHOUT CASSIA LEAVES AND FRUIT EXTRACT

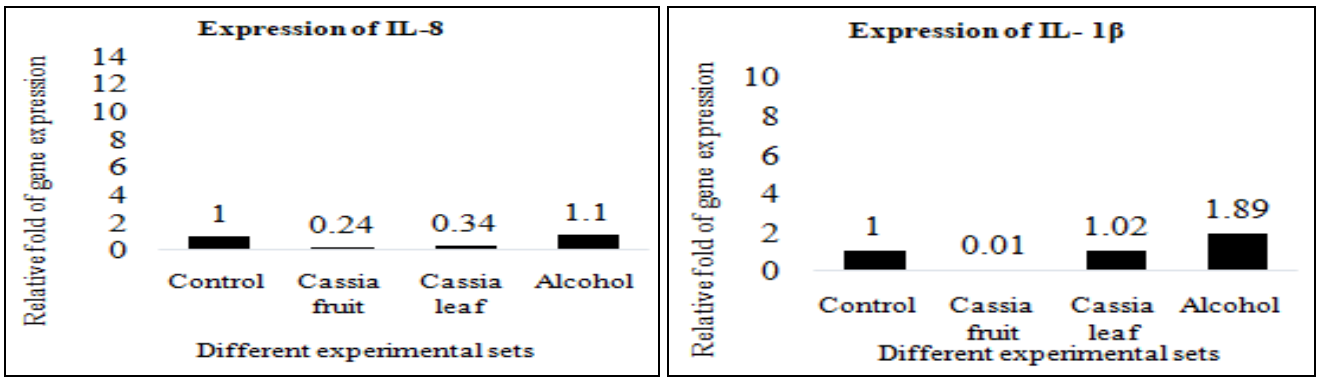

FIG. 8: (A) DEMONSTRATES DIFFERENTIAL EXPRESSION OF IL-8 AND (B) IL-1BIN NORMAL CELL LINE IN HEK293 WITH OR WITHOUT *CASSIA* LEAVES AND FRUIT EXTRACT

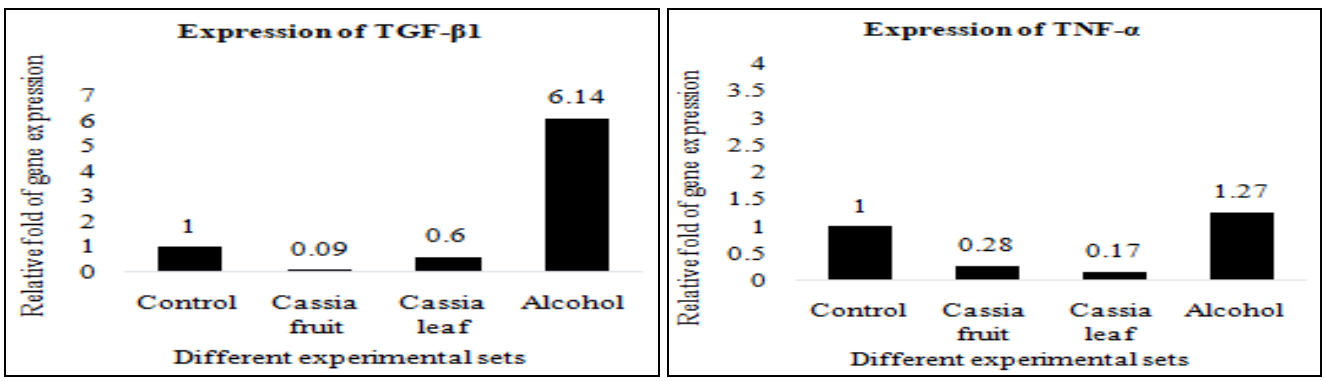

FIG. 9: (A) REPRESENTS DIFFERENTIAL EXPRESSION OF TGF-B1 AND (B) TNF-A IN NORMAL CELL LINE IN HEK293 WITH OR WITHOUT *CASSIA* LEAVES AND FRUIT EXTRACT

Differential Expression of Cytokine Genes using RT-PCR Technique: The difference in the expression of cytokine genes has been demonstrated with the help of column diagrams (Figure 5 -10). IL-6 gene expression got mildly increased in vehicle control (alcohol) compared to normal control. Concerning normal control, IL-6 gene expression was found to be increased with the treatment of *Cassia* fruit and leaf extract in cancer cell line HEPG2. On the contrary, IL-6 gene expression was marginally reduced in HEPG2 cell line by the treatment with Cassia fruit extract compared to the vehicle control set. IL-10 gene expression was increased with the drug treatment compared to vehicle control in cancer cell line, HEPG2.

But, IL-10 gene expression was significantly reduced in the HEPG2 cell line with the treatment of Cassia leaf extract compared to the vehicle control group. About vehicle control, only fruit extract was seen to be effective. Regarding vehicle and normal control, IL-8 gene expression increased in the experimental sets. IL-1β gene expression increased in vehicle control compared to control in the cancer cell line. But IL-1β gene expression significantly reduced in the treatment sets compared to the control set in a cancer cell line, with HEPG2 indicating its role in hepatoprotective activities. Regarding normal control, TGF- β1 gene expression decreased in vehicle control in cancer cell lines. TGF- β1 gene expression was also found to decrease with the drug treatment in the treatment sets. Regarding control and vehicle control, both leaf and fruit extract was seen to be effective in preventing liver cancer. TNF-α gene expression got increased in vehicle control in cancer cell lines. TNF-α gene expression decreased in the HEPG2 cell line with the drug treatment compared to vehicle control in the cancer cell line. On the contrary, TNF-α gene expression was markedly reduced with the treatment of *Cassia* fruit extract compared to normal control in a cancer cell line.

Statistical Analysis: The data demonstrate statistical significance for the cytokine parameters-IL-10, IL-1β, TGF-β1, with P value less than equal to 0.05.The targeted cytokine changes for anticancer activity, when explored upon different experimental sets in a normal cell line, HEK293 revealed that the fold increase concerning the housekeeping gene was within the normal range in all experimental sets. Thus the result showed no such significant differences by One-way ANOVA.

TABLE 1: STATISTICAL ANALYSIS OF GENE EXPRESSION VALUES USING ONE-WAY ANOVA

Sl. no.	Cytokines	F-value	P-value
1	IL-6	1.45	0.25(NS)
2	IL-10	4.73	0.00(S)*
3	IL-8	2.39	0.09(NS)
4	IL-1β	5.69	0.00(S)*
5	TGF-β1	11.15	0.00(S)*
6	TNF-α	1.55	0.22(NS)

S: significant; NS: non-significant *Significant at 0.05 level.

DISCUSSION: Our study focuses on the anticancer activities in the plant parts like leaves, fruits of *Cassia* fistula against liver cancer. Researchers also confirmed that *Cassia fistula* bears hepatoprotective, wound healing activities [8]. Previous studies have reported the anti-oxidant, hepatoprotective potential of ethanolic extract of *Cassia fistula* fruits, leaves and methanolic extract of *Cassia fistula* seeds against hepatotoxin in liver injury mice [9]. IL-6 is a pleiotropic cytokine that plays a multifunctional role in liver cancer [10]. IL-6 plays an important role in liver proliferation by activating various signaling pathways like Janus kinase (JAK), activator and transducer of transcription 3(STAT3) [11]. On the contrary, it is seen that under harmful conditions, IL-6 can block apoptotic activity, thereby protecting cell [12]. The inflammatory cytokine IL-6 stimulates acute-phase reactants and increases its long-term level, leading to metastatic potential in HCC [13]. A significant positive correlation of IL-6 serum concentration with the octamer binding transcription factor (OCT_4) contributed to the poor outcome in HCC [14]. Our experiment showed a study of the gene expression of IL-6 in the liver cancer cell line HEPG2. In our data, the expression of IL-6 was increased by the *Cassia* leaf and fruit extract compared to the control group.

Similarly, another cytokine, IL-10, is considered a pleiotropic cytokine produced in the liver by kupffer cells, macrophages, and lymphocytes. High IL-10 serum level was increased by ELISA method among HCC groups investigated by the researchers. Thus, increased IL-10 had a positive correlation with tumour size, indicating its role in tumour metastasis [15].

In our study, the gene expression of IL-10 was markedly reduced after the administration of ethanolic fruit extract of *Cassia* plant as a drug compared to normal control and vehicle control (alcohol) set in liver cancer cell line. Thus, our data shows that ethanolic fruit extract of Cassia plant could control IL-10 gene expression, thereby contributing towards anti-tumour activity in HCC.

Previous studies show that the chemoattractant cytokine IL-8 is considered a pro-inflammatory cytokine. A group of researchers examined mRNA levels of IL-8 in patients with liver cancer by RT-PCR and found IL-8 levels to be significantly high than the normal control group indicating its role in tumour invasion and metastasis [16]. According to our experimental data, we found the gene expression of IL-8 got significantly reduced by the ethanolic fruit extract of *Cassia* plant as a drug treatment in HEPG2 compared to vehicle control (alcohol) group in liver cancer cell line.

It is seen from previous studies conducted by researchers that IL-1β can be considered as pro or anti-inflammatory cytokine. In the tumour environment, they promote tumour progression metastasis. Hepatic inflammation triggers IL-1β signalling, which contributes to fibrinogenesis and promotes HCC [17]. Our study showed down-regulation of IL-1β by the ethanolic extract of Cassia leaf and fruit in the liver cancer cell line compared to the control and vehicle control set. Evidence shows that the pro-fibrinogenic cytokine TGF-β1 suppresses the tumour metastasis by apoptosis at early stages. Still, its over-expression alternates the gene expression by maintaining cell survival, activating anti-apoptotic signal, liver injury, which leads to cirrhosis and, finally HCC during advance stage [18]. It can also help HCC cells to disperse in the portal venous system and promote tumour progression [19]. Similarly, in our finding, we could observe the down-regulation of the expression of TGF-β1 by the administration of ethanolic leaf and fruit extract of *Cassia* plant as a drug in liver cancer line compared to control and vehicle control group. One of the most important inflammatory cytokine TNF-α gets activated by a death-inducing signalling complex which leads to the activation of caspases and finally cell death [20].

This complex gets activated by the death receptor TRAIL which is found mostly in HCC. Researchers reported that TNF-α serum level were high in chronic hepatitis patients compared to the healthy group, which indicates a positive correlation between TNF-α and liver fibrosis [21]. Similarly, another study was also performed to evaluate the expression of TNF-α protein in HCC patients comparedd to normal control. It revealed high expression of TNF-α in HCC group, indicating TNF-α to be a key mediator in HCC [22]. The results of our experiment showed a reduction in the expression of TNF-α by the ethanolic fruit extract of *Cassia* plant as a drug in liver cancer line compared to control and vehicle control which indicates its role in anticancer activity.

Previous literature examined the hepatoprotective activity of Procyanidin B2 in mouse liver and concluded that this compound decreased the expression of IL-1β, TNF-α in liver cancer [23]. Another study investigated by researchers also supported it that this compound prevents the growth of HEPG2 cells [24]. Thus, this secondary metabolite procyanidin B2 not only induces apopotosis and prevents the growth of hepatic stellate cells but also helps in the down-regulation of the expression of IL-1β, TNF-α, TGFβ1 [25].

The leaf extract of *Cassia*plant possess this compound [26]. Hence, it can be concluded that leaf extract of this plant used as a drug in our experiment played a major role in decreasing the level of IL-1β, TGF-β1 thus inhibiting the growth of HEPG2 cells and preventing HCC to some extent. The fruit of *Cassia* plant bears one of the most important anticancer compounds –1,8-dihydroxy-3-anthraquinone, commonly known as Rhein [27]. A group of researchers considered the activity of rhein to be pro- and anti-inflammatory. They found that rhein inhibits the activity of reactive oxygen species such as Nitric oxide synthase and thus suppresses the down-regulation

of IL-1β, TNF-α by blocking nuclear factor-κβ pathway [28].

CONCLUSION: Thus, from our finding we can conclude that the leaves extract of *Cassia* plant exhibits epiafzelechin, epiafzelechin-3-O-glucoside, procyanidin which possess anti-oxidant activity and can suppress carcinogenesis in cancer, including liver cancer. Similarly, the fruit extract of Cassia plant bears compounds like rhein and other resinous derivatives that can inhibit hepatocarcinogenesis.

Because of our data, we could observe that this drug down-regulates many pro-inflammatory cytokines (IL-10, IL-1β, TGF-β1, TNF-α), which decreases proliferative activity and decreases the viability of liver cancer cells, thereby preventing tumour progression. We can further study the synergistic effect of crude extract of leaves, fruits of *Cassia fistula* against hepatocellular carcinoma (HEPG2) to potentially enhance the efficacy of this drug.

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CONFLICTS OF INTEREST: The authors have declared no competing interests.

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