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Tagetes patula L. flower methanolic extract exhibited beneficial actions against hepatocellular carcinoma via induction of apoptosis

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Abstract: **Background**: Hepatocellular carcinoma (HCC) is considered as a primary malignant tumor with limited treatment options. *Tagetes patula* flower methanolic extract, containing patuletin as major flavonoid, was reported to exhibit beneficial effects against various cancer cell lines. **Objective**: Keeping this in view, the present study was designed to assess their beneficial action against HCC in both *in vitro* and *in vivo* models. **Methods**: The anti-HCC (Hep G2 cancer cell line) action was determined (Sulforhodamine B assay) followed by apoptosis (Annexin-V) assay. For *in vivo* assessment, diethylnitrosamine (DEN) induced HCC rat model was used. The parameters evaluated involve body and liver weights, CBC, LFT, histopathology and expression of inflammatory cytokines (TNF-α, IL-6 and AFP). **Results**: The extract caused dose-dependent inhibition of growth (IC_{50}=176±23 µg/ml) against Hep G2 cells, while patuletin failed to exhibit any such action. Furthermore, the Tagetes extract treatment resulted in a significant decrease in cell viability and a corresponding increase in apoptosis (both early and late phase), comparable to the effects observed with vinblastine. In rats, DEN treatment also decreased the body weight, altered liver architecture and raised cytokines levels. All of these effects were neutralized by extract in both prevention and treatment protocols. **Conclusion**: Overall, *T. patula* flower methanolic extract has demonstrated beneficial effects in *in vitro* and *in vivo* models of HCC. Hence, it presents itself as a potential source for anti-HCC drug development.

Keywords: Diethylnitrosamine; Hep G2 cell line; Hepatocellular carcinoma; Patuletin; Sulforhodamine B; *Tagetes patula*

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INTRODUCTION

According to Global Cancer statistics 2020, 19.3 million new cases emerged, with nearly 10.0 million cancer deaths worldwide in 2020 (Danpanichkul *et al.*, 2025). Among them, hepatic or liver cancer is the 3rd main cause of cancer deaths across the globe. It also ranks as the second leading premature death from cancers (Huang *et al.*, 2022, Rumgay *et al.*, 2022). Hepatocellular carcinoma (HCC) is the major histologic subtype of liver cancer, representing 80-85 percent of hepatic cancer (Tan *et al.*, 2025). It is the leading cancer cause globally with one third cases residing in the Asia-Pacific region. In this regard, the shift has been noted from viral to non-viral causes such as consumption of alcohol over time (Li *et al.*, 2024, Toh *et al.*, 2023). Unfortunately, there are not many therapeutic options available for its treatment. The chemotherapy has nominal impact on the survival of HCC due to low sensitivity and resistance towards drugs (Liao *et al.*, 2015). Sorafenib is the only (FDA) approved chemotherapeutic drug available with a response rate (2–3%) and overall survival of approximately 3 months. At the moment, surgical resection is the only curative option with a 5-year survival rate of around 30–40% (Aravalli *et al.*, 2008). However, it is reported to be feasible for less than 15% subject with a relapse rate of up to 50% (Roayaie *et al.*, 2009). The aforementioned figures are alarming and require immediate attention from the scientific community to increase the pace of anti-HCC drug development programs. Inflammatory cytokines are reported to play a critical role in the pathogenesis of diseases such as cancers. Among them, tumor necrosis-alpha (TNF-α) and interleukin-6 (IL-6) are key mediators of the inflammatory microenvironment of the liver. High expression of such cytokines in HCC is associated with the activation of oncogenic pathways, such as NF-B and STAT3, that facilitate the proliferation of hepatocytes, angiogenesis and immune evasion (Haque *et al.*, 2015, Jeng *et al.*, 2025). The TNF-α has been reported to be instrumental in the development of HCC (Vachliotis and Polyzos, 2023). The IL-6 is yet another pro-inflammatory cytokine with a reported role in pathogenesis of HCC (Shakiba *et al.*, 2018). Similarly, in response to HCC, alpha-fetoprotein (AFP) is considered as the marker of liver cancer (Arrieta *et al.*, 2007). Taken together, these cytokines, owing to their substantial role in HCC disease development, provide valuable pharmacological targets for managing HCC.

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Humans are granted the wealth of natural flora and fauna. Along with various other attributes, this natural wealth is enriched with diverse chemicals with medicinal values. Researchers have been exploring it for the identification of candidate lead molecules in the drug development process against various disorders. It is worth mentioning that more than 60% of anti-cancer clinically used drugs are obtained for this natural resource (Cragg *et al.*, 2009, Mandlik and Mandlik, 2021). Tagetes (marigold) is a genus of more than 50 species in the Asteraceae family, the majority of which are herbaceous annuals or perennials. *Tagetes patula* L. (*T. patula*) is globally known as French marigold and locally named as Jafri. It is an edible plant and holds significant medicinal and phytochemical values such as, antibacterial, antifungal, nematicidal, antioxidant, anti-inflammatory and antiarthritic properties, highlighting their potential in natural remedies (Faizi *et al.*, 2011, Faizi *et al.*, 2008, Jabeen *et al.*, 2016, Kashif *et al.*, 2015). Its petals were investigated for their potential anticancer activity (Sultana *et al.*, 2025). Numerous phytochemicals, including carotenes, terpenes, steroids, flavonoids, ceramides and thiophenes, have been extensively studied in different parts of the *T. patula* plant (Aati *et al.*, 2022, Bano *et al.*, 2019, Liu *et al.*, 2020, Riaz *et al.*, 2020). In our previous investigation *T. patula* methanol extract and its isolated phenolic compound patuletin (Fig. S1) have been evaluated against human cancer cell lines including HeLa, HT-144, NCI-H460, MCF-7, PC-3 and SF-268 (Faizi *et al.*, 2011, Faizi *et al.*, 2008, Jabeen *et al.*, 2016, Kashif *et al.*, 2015), but these encouraging outcomes have not been validated using an *in vivo* model of HCC. Keeping the aforesaid into account, the present study was designed to assess the effectiveness of *T. patula* flower extract and its active constituent (patuletin) against *in vitro* and *in vivo* model of HCC.

MATERIALS AND METHODS

Chemicals

Doxorubicin, Diethylnitrosamine, Vinblastine, Sulforhodamine B, Trichloroacetic acid, Phosphate Buffer Saline, Trypsin, Hematoxylin, Eosin, Trizol and DMSO were obtained from Sigma Aldrich (USA). The DMEM culture media, Fetal Bovine Serum and Antibiotics for cell culture were provided by Thermo Fisher Scientific (USA).

Collection and identification of T. patula

The fresh, dried and uncrushed mix-color flowers (~5 kg) were gathered from the premises of Karachi University campus. Flowers were identified by the botanical expert from the Department of Botany, University of Karachi, Karachi, Pakistan, and a voucher specimen number (KUH-GH-No. 67280) was placed in the herbarium of Botany Department.

Extraction and isolation of T. patula

Methanol extract and pure component patuletin were obtained by extracting and fractionating the flowers of *T. patula* as mentioned in our previous investigation (Kashif *et al.*, 2015).

Animals

Healthy adult Wistar rats of similar age, weighing 160-180 g (n=5), were obtained by the animal house facility of Dow University of Health Sciences (DUHS), Karachi, Pakistan. The animals were kept under standard conditions [light and dark cycle (12 h each), temperature (25±1°C) and humidity (60–70%)], recommended for the care and use of laboratory animals. Standard food pellets and water were available ad libitum. An acclimatization period of one week was provided prior to experiment. Ethical approval for animal study was obtained from the Institutional Review Board having # 1121/DUHS/Approval/2018/208. All experimental procedures were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines. All animals were monitored regularly for general health, behavior and food and water intake throughout the study.

Cytotoxicity assay

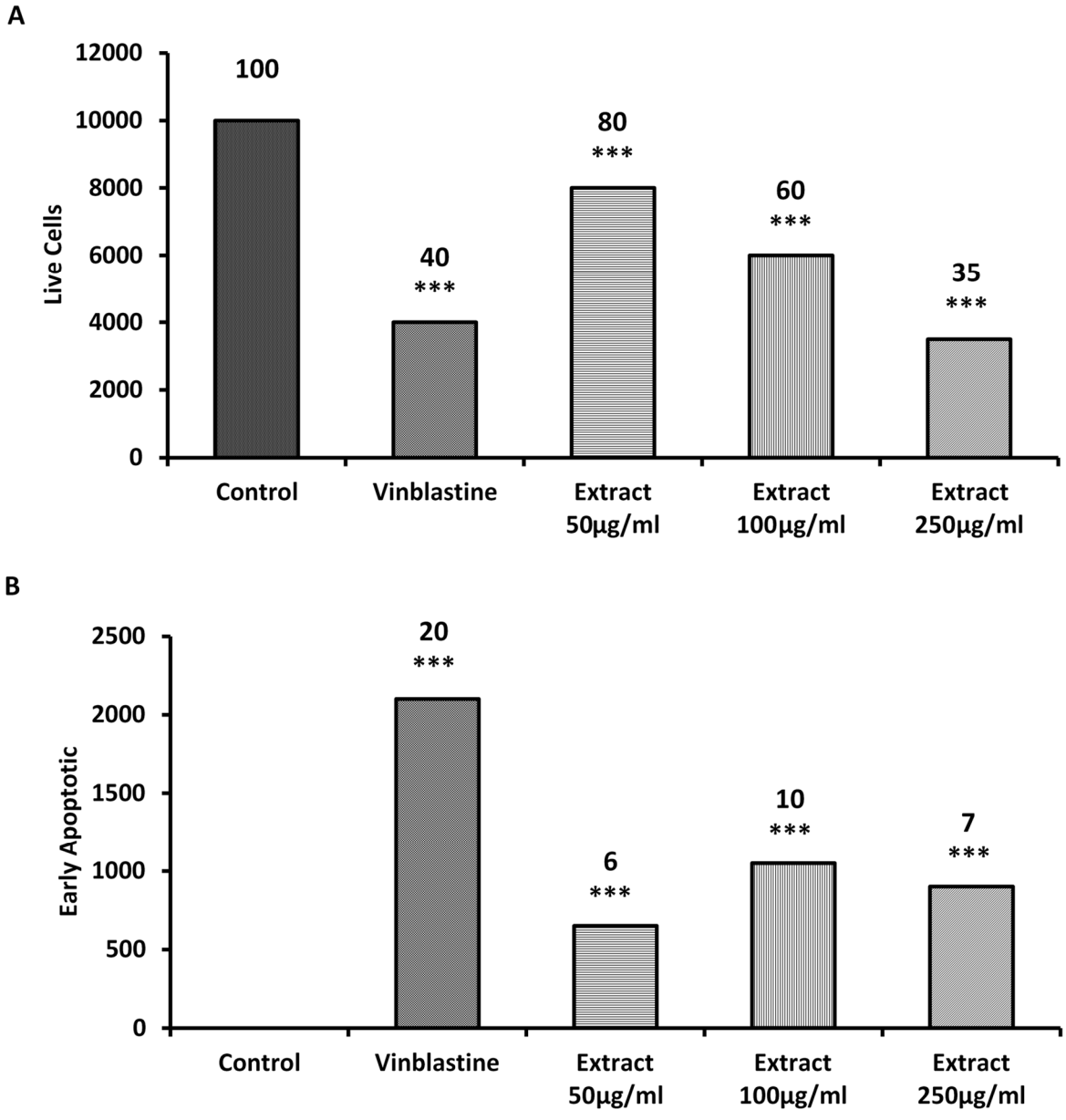
The hepatocellular cancer cells (Hep-G2, ATCC culture) were grown (75 ml cell culture flasks) in DMEM culture medium supplemented with serum (FBS, 10%) having 200 mM L-glutamine, 104 U/mL penicillin, 10 µg/mL amphotericin-B and 100 µg/mL streptomycin. The cell was grown in the incubator (5% CO_2) until a monolayer developed. The growth inhibitory action was assessed as per protocol (Skehan *et al.*, 1990). Briefly, the cells of Hep-G2 were trypsinized and cultured (7500 cells/ well/ 100 µL) in a 96 well plate of cell culture followed by the incubation for 24 hours. After monolayer formation, the test substances i.e., doxorubicin (10 µM), methanolic extract (50, 100 and 250 µg/ml) and patuletin (100 µM) were added (100 µL/well) followed by incubation for 48 hours. After fixation with trichloroacetic acid (50% ice cold), the cells were stained with sulforhodamine B dye (0.4%). The cytotoxic and growth inhibition actions were calculated as described earlier (Arshad Qamar *et al.*, 2010).

Apoptosis assay

The apoptosis induction ability by test substances were evaluated using flow cytometry based Annexin-V assay (Van Engeland *et al.*, 1998). Briefly, the Hep G2 cells (1×10^6 per ml) were treated with *T. patula* methanolic extract (50, 100 and 250µg/ml) and standard drug vinblastine (5 nM) for 48 hours. After trypsinization and washing with PBS, the supernatant was resuspended in 125µl PBS binding buffer (125µl, 25mM $CaCl_2$, 140mM NaCl; 100 mM HEPES). Afterwards, FITC (Annexin-V, 5µl of 1µg/8µl) and propidium iodide (5µl of 1µg/8µl) were added. Cells kept in dark for 15 minutes at ambient temperature followed by OD measurement at 530-585nm (for FITC and propidium iodide) with the flow cytometer (FACS Calibur; USA, Becton Dickinson). A total of 10,000 events were noted and analyzed using software (Cell Quest).

Table 1: The primer sequences

Gene	Primer sequence
TNF-α	F – 5'-ACTACTTACAAAATCTGTTCCTCATTGG-3'
	R – 5'-ATGTAAATGTCGGCCAGTCC-3'
IL-6	F – 5-AAGGACCAAGACCATCCAAC-3
	R – 5-ACCACAGTGAGGAATGTCCA-3
AFP	F – 5-AAACACACGAGACGCTGAAG-3
	R – 5-ATCCAGTGAGTTCCGAAAGC-3
GAPDH	F – GGAAAGCTGTGGCGTGATGG
	R – GTAGGCCATGAGGTCCACCA



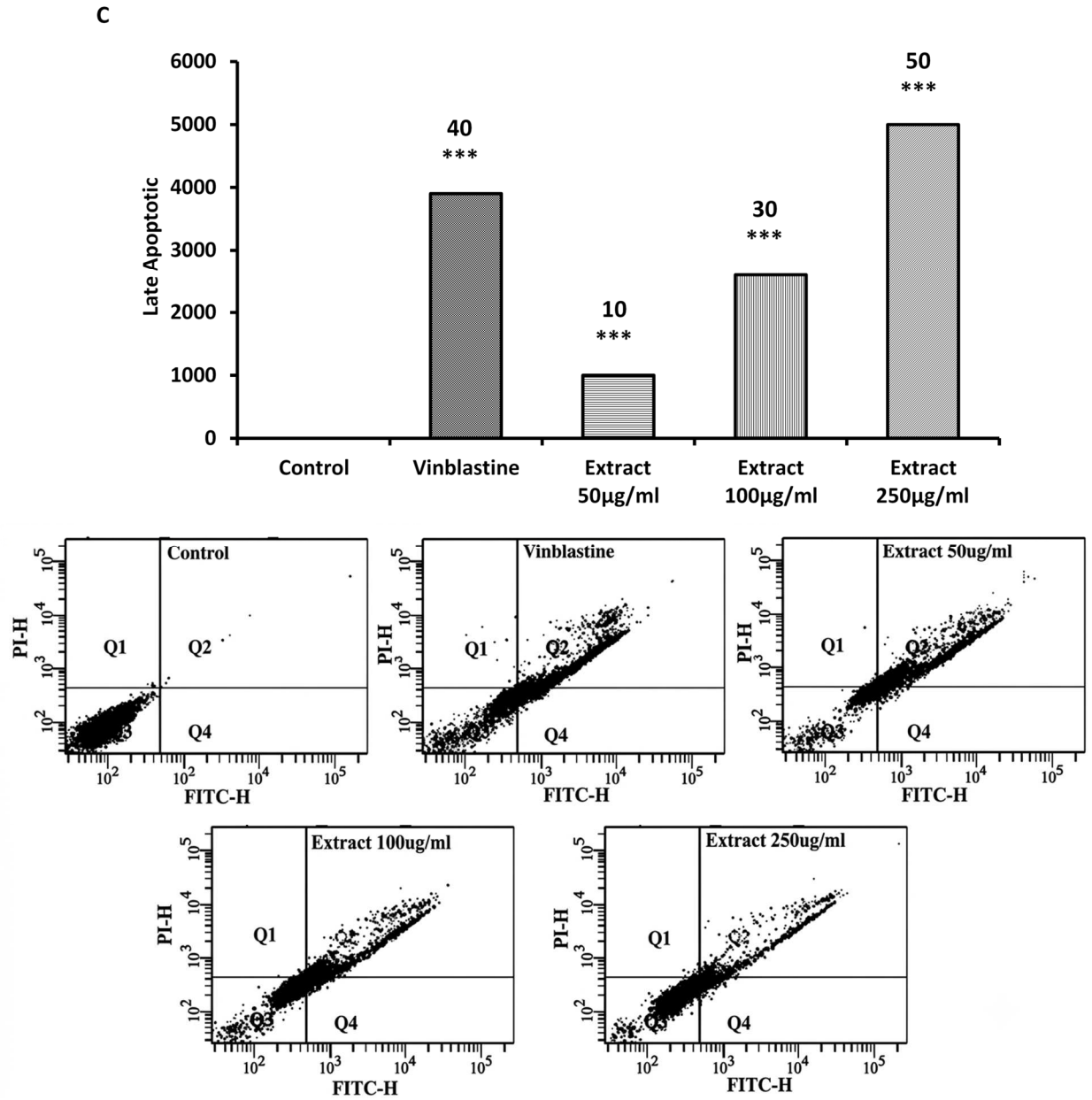


Fig. 1: Effect of *T. patula* methanolic extract on the apoptosis of Hep G2 cells. The figure exhibits the flow cytometry (FITC/PI) based number of cells in various stages, including live cells (A), early apoptotic cells (B) and late apoptotic cells (C), following treatment with *T. patula* flower methanolic extract and vinblastine. A significant reduction in the viability while increase in the apoptotic cells were noted in both standard and test drugs. The representative flow cytometry images (on the bottom) shows detection of cells (total events=10000) in different stages (Q1=Dead / Necrotic cells; Q2=Late apoptotic cells, Q3=Live cells, and Q4=Early apoptotic cells). Data analysis was conducted using one-way ANOVA followed by post-hoc analysis (Least significant difference). Results are presented as the mean ± SEM (n = 5). Asterisks (***) represent significant difference ($p<0.001$) as compared to control.

DEN induced animal model of hepatic cancer

The animal model of hepatocellular carcinoma was developed by administering DEN (Kurma *et al.*, 2021).

Additionally, the test substances and standard drugs were administered as preventive and treatment protocols. The earlier involved the co-administration of test and standard drugs with an objective to evaluate their effectiveness in preventing the development of disease. The later involves development of the disease first, which is followed by treatment with test or standard drugs with the objective of healing the existing disease. The protocols are as follows:

Group 1: Control rats

Group 2: DEN treated rats (60 mg/kg twice daily for 12 weeks, intraperitoneal route)

Preventive (P) protocol
Group 3 and 4: DEN plus Tagetes extract (100 and 250 mg/kg orally from 3rd–18th weeks)
Group 5: DEN plus Silymarin (50 mg/kg orally from 3rd–18th weeks)
Treatment (T) protocol
Group 6, 7 and 8: DEN plus Tagetes extract (100, 250 and 500 mg/kg orally from 12th – 18th weeks)
Group 9: DEN plus Doxorubicin (1 mg/kg orally from 12th – 18th weeks)
After 20th week, the rats were euthanized for the following estimations:

Weight variation
The weight of the animals was noted, followed by euthanasia. Afterwards, the livers were also dissected out, washed and cleaned for unwanted tissue and weighed.

Histopathological evaluation
To conduct histopathological analysis, the liver tissues were fixed with formalin (10% formalin) before being subjected to the Histology Department. Following formalin fixation, tissues were preserved, processed through dehydration and clearing, embedded in paraffin, thinly sectioned and stained using hematoxylin–eosin (H&E) for microscopic study. Subsequently, the slides were examined at 10X and 20X magnification under a microscope and images were recorded for analysis.

Hematology & liver function test (LFT)
The blood was used for the complete blood count (CBC) and liver function test (LFT) including parameters total bilirubin levels (both indirect and direct), SGOT, SGPT and Alkaline Phosphatase. The diagnostics facility, equipped with hematology and chemical analyzers, was used for biochemical estimations.

Expression study
The cells RNA was isolated from rat livers, using the trizol reagent and assessed for purity using nano-drop method. Afterwards, Complementary DNA (cDNA) was synthesized using kit as described by the manufacturer (Thermo Fisher, USA). Using the gene specific primers, the expression of TNF-α, IL-6 and AFP was measured using real time PCR (Qiagen system) with SYBR Green Master Mix PCR (Applied Biosystems, USA). PCR cycles constituted 10 mins at 95°C, 40 cycles for 15 secs at 95°C and one min at 60°C. The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a house keeping gene. The sequences of primer are mentioned in Table 1. The results were computed via comparative CT ($2^{-\Delta\Delta CT}$) method, which demonstrates the fold change or relative expression of the target gene in regard to the internal control in each sample.

The enzyme linked immunosorbent assay (ELISA)
Quantification of TNF-α, IL-6 and AFP was performed using the respective ELISA kits as described by the manufacturer (Invitrogen, USA). Briefly, after the lysis of liver, centrifugation was performed and supernatant was added to pre-coated plates (Biotinylated anti-AFP/IL-6/TNF-alpha antibody). The un-bound antibody was washed followed by the addition of HRP conjugated streptavidin and Tetramethylbenzidine (TMB, chromogenic substrate). Finally, the OD was measured at 450nm.

Statistical analysis
The data is presented as mean ± SEM (triplicate for *in vitro* experiments, while n=5 per group for *in vivo* ones). Statistical differences among various means were found through one-way ANOVA followed by post-hoc analysis (Least significant difference) using IBM, SPSS Statistics, version 19.0 (Chicago, IL, USA). The minimum level of significance was set at $p<0.05$ i.e. 95% confidence interval.

RESULTS

Cytotoxicity assay
The methanolic extract of *T. patula* flower halted the cell growth of Hep-G2 cell line having IC_{50}=76±23 µg/mL. Though, patuletin did not cause any cytotoxic or growth inhibitory actions.

Apoptosis assay
Fig. 1 depicts the flow cytometry analysis, showing the effect of the extract on cell viability and apoptosis. The standard drug-treated group exhibited a significant reduction in viable cells as compared to control group, indicating its cytotoxic effect. The Tagetes extract, in conformity with vinblastine, caused the dose dependent reduction in the viability (significant, $p<0.001$ of Hep-G2 cells) as depicted in Fig. 1 A. This is accompanied by a significant rise in cells at the early (Fig. 1 B) and late apoptotic stage (Fig. 1 C) in a dose dependent manner as compared to the control cells ($p<0.001$). At 50 µg/mL, only a small proportion of cells underwent apoptosis, while 100 µg/ml showed a moderate increase (Figs. 1 B and C). At the highest concentration (250 µg/ml), the extract caused a substantial increase in apoptotic cells, comparable to the effect of Vinblastine (Fig. 1 C). The microscopic diagrams of flow cytometry of different treatments are also described in the Fig. 1.

Weight variation
In the model of HCC, the body weight analysis revealed a significant impact of DEN and the varying efficacy of the treatments (Fig. 2 A). The DEN treatment significantly ($p<0.001$) declined the rodent weight compared to their control, confirming the successful establishment of the HCC model. This decrease was blocked by standard drugs (silymarin and doxorubicin) and extracts (both treatment and preventive protocols). In brief, among the experimental treatments, DEN+P 100 mg and DEN+T 250 mg exhibited partial recovery, though these improvements were not statistically significant. However, Groups including DEN+P 250 mg, DEN+P Syl and DEN+T 100

mg, showed significant recovery of body weight relative to the DEN treated rodents ($p<0.01$). Notably, DEN+T 500 mg demonstrated strong therapeutic efficacy, restoring weight ($p<0.001$), while DEN+T Doxo achieved full recovery, returning to the control level with the highest significance ($p<0.001$). In the case of liver weights, none of the aforementioned treatments have produced any significant effect as compared to control (Fig. 2 B).

Biochemical estimations

The liver function test, bilirubin levels (both indirect and direct), SGOT, SGPT and Alkaline phosphatase) and complete profile of blood (Hemoglobin, hematocrit, MCH, MCHC RBC, WBC, platelets and lymphocytes) data did not reveal any significant alterations in any treatment groups as compared to their controls.

Histopathological evaluation

The H&E staining of liver revealed hepatocytes in a single plane architecture with a different number of portal tracks (Figs. 3 A and B). No evidence of periportal inflammation, dysplasia, fibrosis, or malignancy was seen. However, DEN treated rat liver parenchyma was observed to be disorganized by nodules composed of enlarged, atypical cells having enlarged pleomorphic nucleus with protruding nucleoli and the copious eosinophilic to transparent cytoplasm (Figs. 3 C and D). In the case of treatment, the architecture was noted to be predominantly intact. However, few clusters (focal) of enlarged cells were seen having oval nuclei with profuse cytoplasm. However, overall, the parenchyma was intact of the rat liver tissue (Figs. 3 E-L). In case doxorubicin and silymarin which were used as standard drugs, the architecture of liver was intact initially but enlarged clusters or groups of focal origin were seen with oval nuclei and an abundant eosinophilic cytoplasm was also seen (Figs. 3 M-P).

Expression study

The relative mRNA expression levels of TNF-α, IL-6 and AFP were assessed in all experimental groups (Fig. 4). The DEN caused a rise in hepatic expression of IL-6, TNF-α and AFP as compared to control. In similarity with standard drugs (silymarin and doxorubicin), this rise was found to be inhibited by extract in both prevention and treatment protocols (Figs. 4 A-C).

Briefly, TNF-α expression was significantly increased in DEN treated group compared to the Control group ($p<0.001$). Groups DEN+P Syl and DEN+T Doxo, which received standard drug treatment along with DEN, showed a marked reduction in TNF-α levels compared to the DEN group ($p<0.01$). Similarly, the extract-treated groups DEN+P 100 mg, DEN+P 250 mg, DEN+T 250 mg and DEN+T 500 mg also demonstrated statistically significant improvement as evidenced by decreased TNF-α expression ($p<0.001$, $p<0.01$) (Fig. 4 A). Similarly, IL-6 expression was markedly elevated in the DEN-induced group relative to control, confirming the induction of a pro-inflammatory state ($p<0.001$). Both prevention and treatment protocols resulted in a significant decline in IL-6 levels in a dose dependent manner, with some groups approaching near-normal expression levels ($p<0.001$, $p<0.01$) as shown in Fig. 4 B. AFP expression, a biomarker of hepatocellular carcinoma, was also significantly increased in the DEN treated group compared to control ($p<0.001$), confirming successful establishment of the HCC model. Standard drug-treated groups showed a marked decrease in AFP levels ($p<0.01$). Likewise, extract-treated groups exhibited a significant reduction in AFP expression ($p<0.001$, $p<0.01$), indicating suppression of tumor progression and improvement in hepatic status (Fig. 4 C).

Enzyme linked immunosorbent assay

ELISA analysis revealed the markedly elevated levels of pro-inflammatory cytokines TNF-α and IL-6─in DEN treated group, compared to the control group ($p<0.001$). In conformity with standard drugs (silymarin and doxorubicin), treatment with the plant extracts led to a notable reduction in both TNF-α and IL-6, with several extract-treated groups showing levels approaching those of the control, compared with DEN group ($p<0.001$, $p<0.01$) (Figs. 5 A and B). Similarly, AFP, a marker of tumor progression, was slightly increased in the DEN induced group, although the increase was not statistically significant. But, AFP levels were effectively lowered following treatment with extracts and standard drugs ($p<0.001$, $p<0.01$) (Fig. 5 C).

DISCUSSION

Hepatocellular carcinoma (HCC) is a predominant malignant hepatic cancer, which constitutes 80% of global load of liver cancers (Yeo *et al.*, 2025). Chemotherapy is not effective while surgery is the only option with a low survival rate. Keeping this into account, there is an intense need to look for more therapeutic options to combat this illness. In this regard, *T. patula* with reported anticancer actions against six cancer cell lines of human origin (lung, cervical, skin, prostate, astrocytoma (CNS) and breast cancers) has been tested for its effectiveness against HCC (Sukhikh *et al.*, 2025, Zardeto *et al.*, 2024). For a considerable time, our research groups have had significant experience with *T. patula* extract and its flavonoid contents against various cancer cell lines. These *T. patula* extract-mediated effects demonstrated cytotoxicity in a concentration-dependent manner, exhibiting IC_{50} of the crude extract ranges between 125-250 µg/mL (Kashif *et al.*, 2015). Based on *in vitro* cytotoxic potential, the present study proceeded to *in vivo* testing and determined dosages based on published cytotoxicity and safety data. According to previous reports, Tagetes species at the dose of 100–500 mg/kg were found to be safe when administered orally and no mortality or organ toxicity, or any other adverse effects in animal rat model (Abdel-Haleem *et al.*, 2017, Harikumar *et al.*, 2008, Meurer *et al.*, 2022, Meurer *et al.*, 2019).

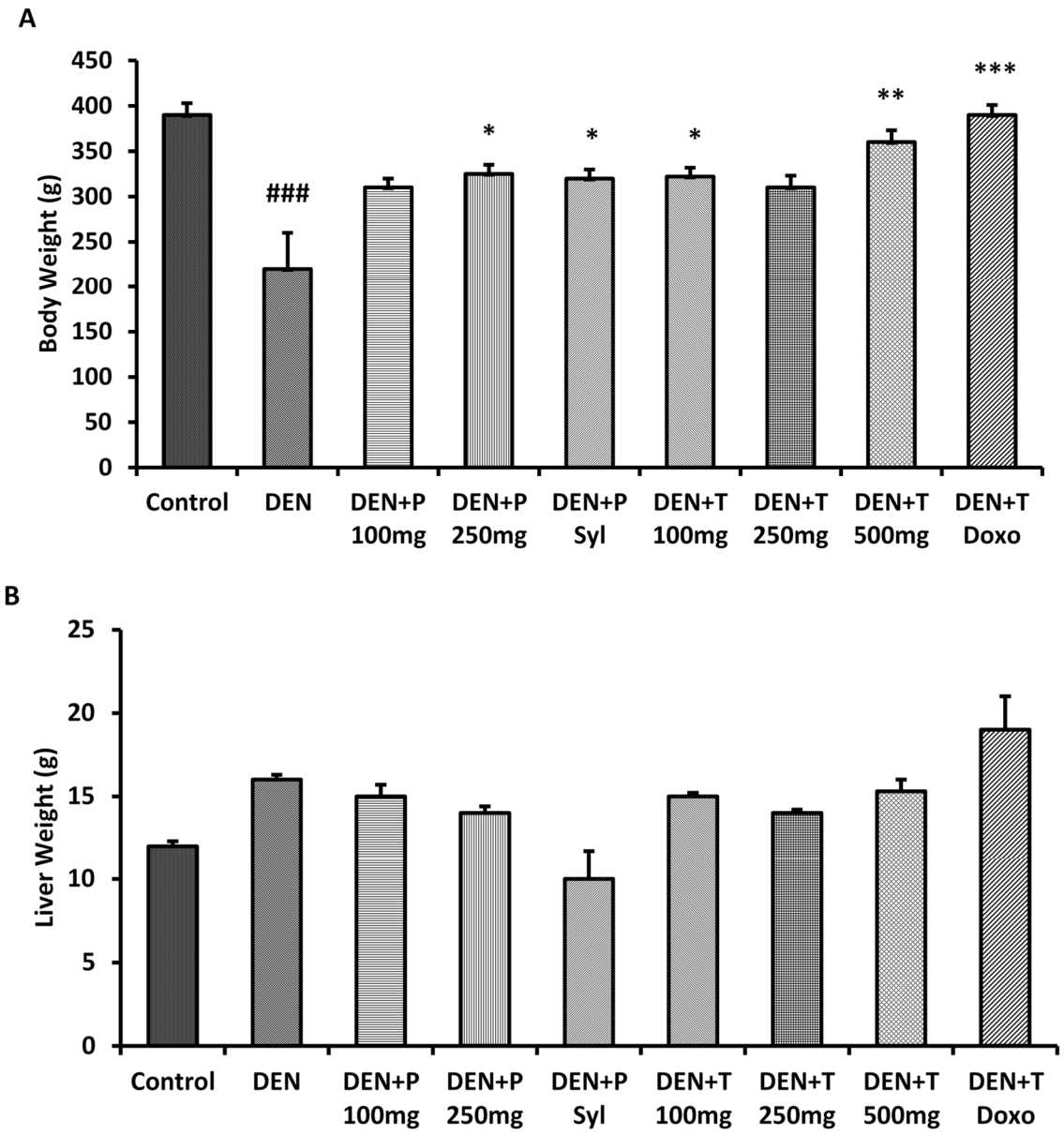


Fig. 2: Effect of *T. patula* extract on the body and liver weight of rats treated with DEN. (A) The DEN exposure significantly decreased rat body weight as compared to control; the effect prevented as well as treated by extract and respective standard drugs. (B) In case of liver weight, no significant change was noted in any of the treatment groups as compared to control. Data analysis was conducted using one-way ANOVA followed by post-hoc analysis (Least significant difference). Results are presented as the mean ± SEM (n = 5). Hash sign (#) shows significance compared to control ($p<0.001$), while asterisk (*) represent significance compared to DEN (P *< 0.05, ** < 0.01 and ***< 0.001).

Considering these outcomes, the current study was considered to assess the *T. patula* medicinal value against the *in vitro* and *in vivo* model of HCC.

Our cytotoxicity data showed growth inhibitory action of *T. patula* flower methanolic extract against human liver cancer cell line (Hep G2) with an IC_{50} value of IC_{50}=176±23 µg/ml. This is in agreement with earlier reports deducing the anticancer potential of Tagetes (Kashif *et al.*, 2015). Patuletin, the famous flavonoid of Tagetes has been considered to be the primary anticancer constituent residing in it (Zardeto *et al.*, 2024). However, in disagreement with earlier reports, patuletin did not cause any significant effect on growth of Hep G2 cell line in present study. This effect could be attributed to the potential anti-HCC activity of extract and combined action of its bioactive constituents and also the potential synergistic interaction between them (Kojima-Yuasa *et al.*, 2015). Additionally, the lack of cancer specific cellular uptake may also underlie this outcome. However, the

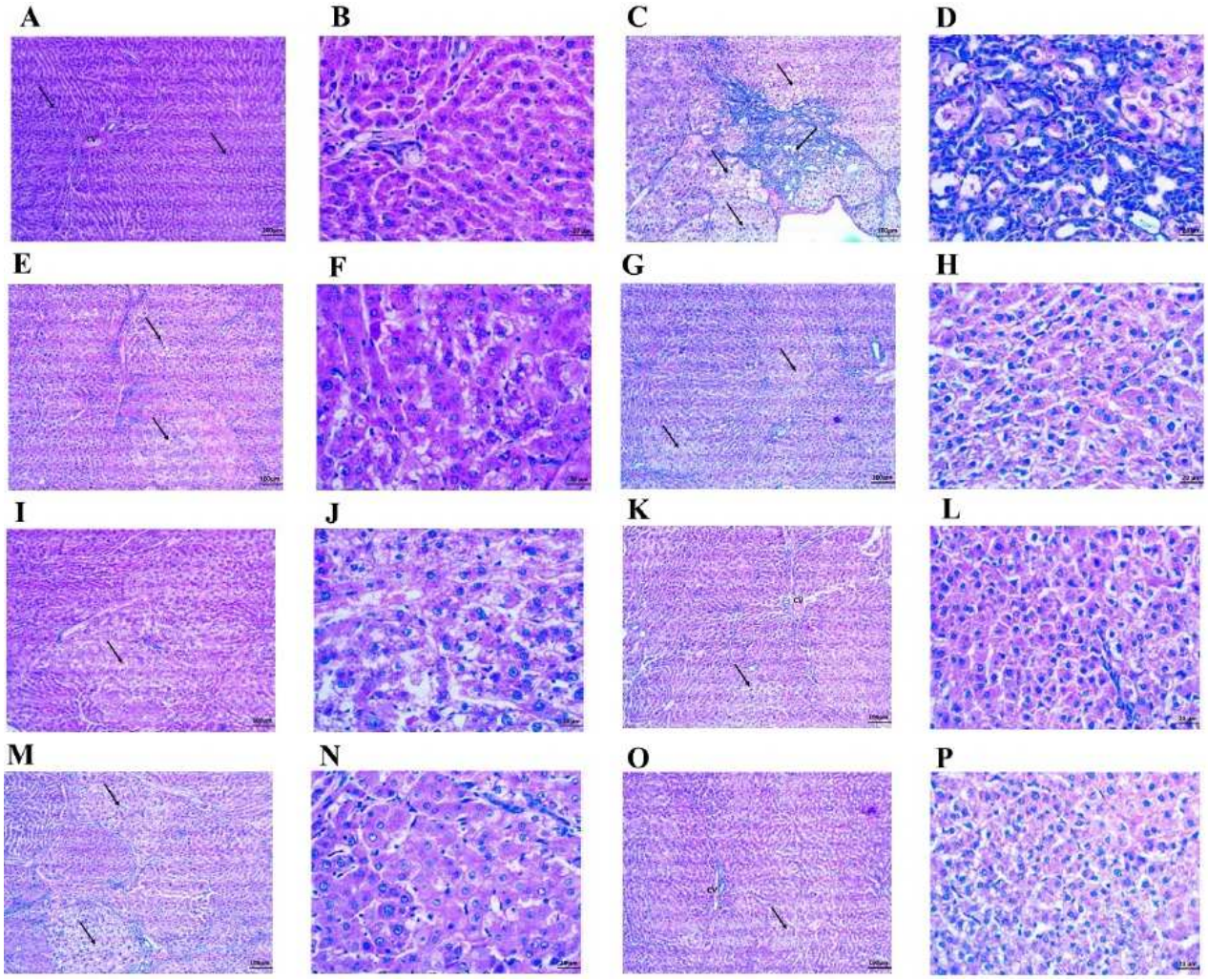


Fig. 3: Representative pictures of liver of rats treated with *T. patula* extract. The control group showed normal hepatocytes morphology with intact cells (A) and (B). The diseased model showed atypical enlarge cells with pleomorphic nuclei (C) and (D). The extract prevention protocol showed partly ephase structure and presence of abundant eosinophilic cytoplasm (E), (F), (G) and (H). The extract treatment protocol groups at different doses showed predominantly intact structure with single focal groups of enlarge cells (I), (J), (K) and (L). The standard drug (Silymarin) showed clusters of atypical enlarge cells, abundant eosinophilic cytoplasm, and vacuolation (M) and (N). Treatment with Doxorubicin showed clusters of enlarge cells with rounded nuclei and abundant eosinophilic cytoplasm (O) and (P). Arrows indicate histological features observed in the microscopic sections of control and treated groups.

situation warrants further experiments to delineate this outcome. Hence, the further testing of patuletin was discontinued from here onwards in present study, while focusing on extract alone.

Our flow cytometry data showed that Tagetes extract resulted in a significant decrease in cell viability through induction of apoptosis in Hep G2 cells, comparable to the effects observed with vinblastine (Fig. 1).

This indicates that the Tagetes extract exhibits dose-dependent cytotoxic and pro-apoptotic effects on these cells, an important pharmacological objective in chemotherapy. Hence, the reported data brought us to evaluate the effectiveness of extract of *T. patula* flower in rat model of HCC.

In this regard, the rodent model of HCC has been effectively developed using a chemical, namely DEN i.e. diethylnitrosamine, which mimics the process of liver injury, fibrosis and malignancy (Kurma *et al.*, 2021). Owing to its significant similarity in histopathological progression with humans, it has gained remarkable popularity among the scientific community for anti-HCC efficacy studies (Ding *et al.*, 2017). Our data showed that the weight of animals was significantly reduced following DEN treatment, the effect nullified by Tagetes extract and standard drugs in both preventive and treatment protocols (Fig. 2).

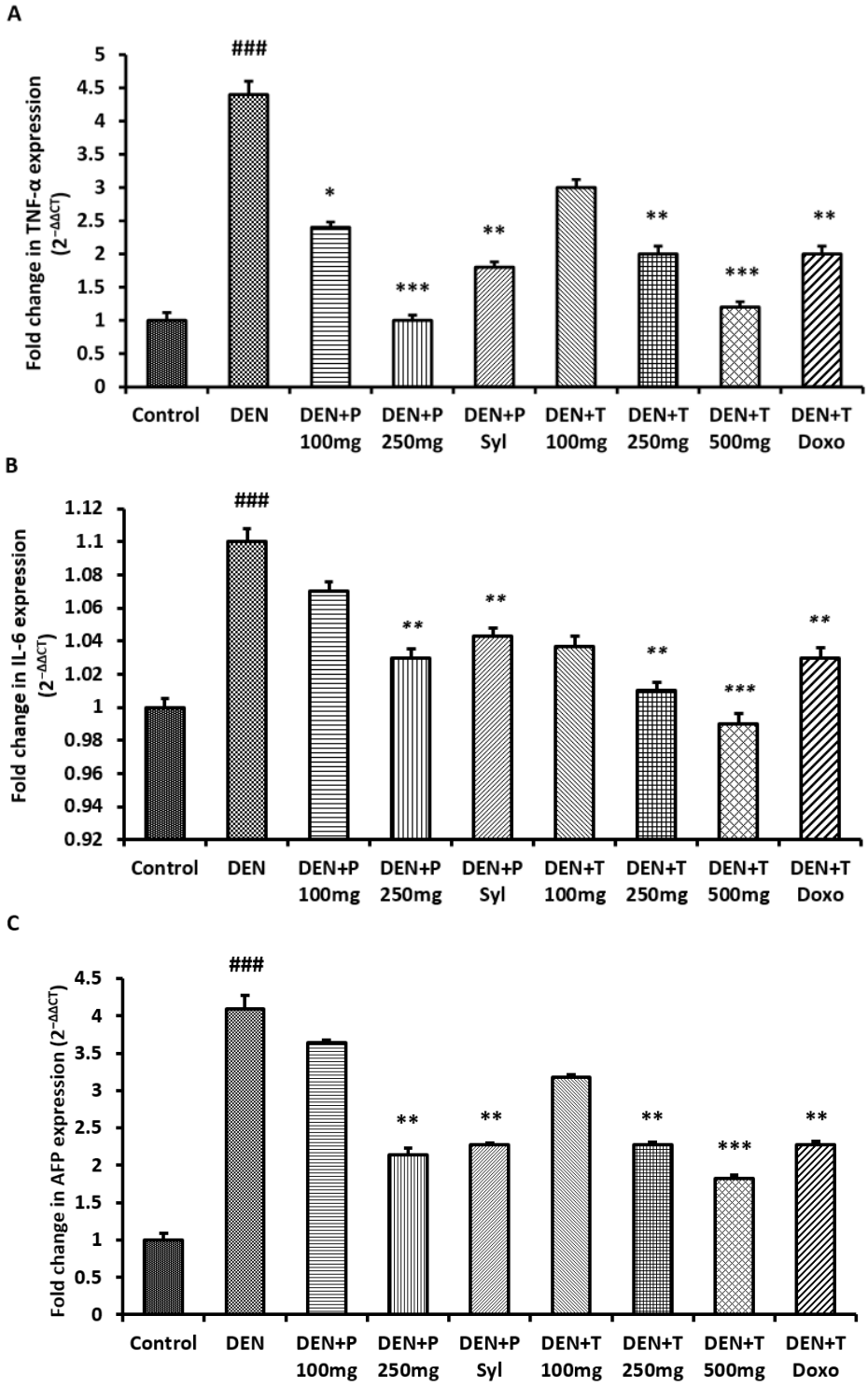


Fig. 4: Effect of *T. patula* extract on expression of TNF-α (A), IL-6 (B) and AFP (C). The figure depicts mean ± S.E.M of gene expression of AFP, IL-6, and TNF-α relative to internal control i.e. GAPDH. The Tagetes extract, in similarity with standard drugs has blocked the DEN induced expression of TNF-α, IL-6 and AFP in both prevention (P) and treatment (T) protocols. Data analysis was conducted using one-way ANOVA followed by post-hoc analysis (Least significant difference). Results are presented as the mean ± SEM (n = 5). Hash sign (#) shows significant difference as compared to control ($p<0.001$), while asterisk (*) represent significant change as compared to DEN (P-value *< 0.05, ** < 0.01 and ***< 0.001).

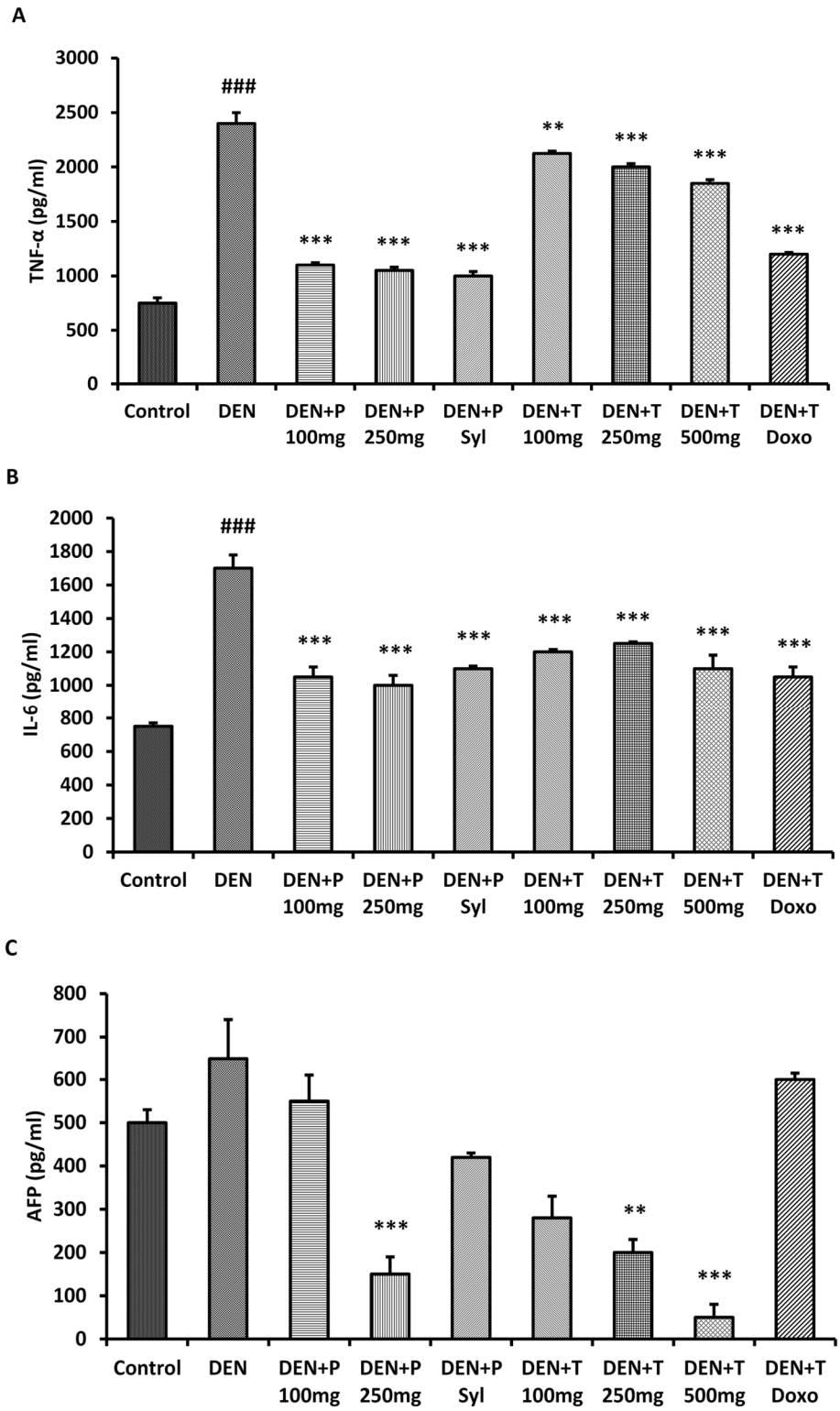


Fig. 5: Effect of *T. patula* extract on levels of TNF-α (A), IL-6 (B) and AFP (C). The figure shows that the DEN treatment has significantly enhanced the levels of TNF-α and IL-6, which was resisted by Tagetes extract in both prevention and treatment protocols. Data analysis was conducted using one-way ANOVA followed by post-hoc analysis (Least significant difference). Results are presented as the mean ± SEM (n = 5). Hash sign (#) shows significant difference as compared to control ($p<0.001$), while asterisk (*) represent significant change as compared to DEN (P-value *< 0.05, ** < 0.01 and ***< 0.001).

This suggests a beneficial effect of the Tagetes extract towards HCC. Furthermore, the DEN treatment did not remarkably alter the liver weights. This is in line with the existing literature using mild DEN treatment (Imamoto *et al.*, 2014). In DEN-induced hepatocarcinogenesis, serum liver function enzymes (LFTs) are prominently increased and are generally indicative of acute hepatic injury or necrosis. However, with chronic or low doses of DEN treatment, biochemical changes can be subtle (El-Serag and Rudolph, 2007). DEN model in the present study was mainly focused to cause progressive carcinogenesis, not on a massive hepatocellular injury. Presumably, the LFT parameters remained unchanged (El–Serag and Rudolph, 2007). Having said that, the lack of significant alterations in both LFT and CBC following treatment with Tagetes extract is also indicative of its safe and non-toxic nature, thereby supporting its use in clinical settings. The histopathological images depict the structural abnormalities in DEN exposed livers, such as disorganized parenchyma along with multiple large nodules constituted of large, typical cells and abundant eosinophilic granular cytoplasm (Fig. 3). These changes are reported earlier too thereby supporting our methodology (Imamoto *et al.*, 2014). In the case of chemo-prevention protocol, the aforementioned changes were observed in the liver but with reduced intensity. However, the hepatic architecture was found to be intact following treatment protocol. In case of standard drugs (silymarin and doxorubicin), small number of focal enlarge clusters were seen with rounded nuclei and abundant eosinophilic cytoplasm. Hence, the histopathological data exhibit that the Tagetes extract slowed the progression of HCC in treatment protocol as compared to the prevention. This is a favorable outcome because the acceptability to take drugs is higher following the diagnosis of disease.

Cytokines are believed to play a pivotal role in the inflammatory processes, thereby affecting the progression of disease. It is of note that the DEN treatment has been reported to increase the pro-inflammatory cytokines expression TNF-α and IL-6 in animal model of HCC (Imamoto *et al.*, 2014, Qiu *et al.*, 2016, Shakiba *et al.*, 2018). Our data showed an increase in its hepatic transcription (Fig. 4) and translation (Fig. 5) following DEN treatment. It is worth noting that the Tagetes extract has significantly decreased gene expression and cytokine levels in both prevention and treatment protocols. Similar findings of potential protective effects are also reported in earlier investigations against chronic nonbacterial prostatitis by modulating key metabolic pathways (Liu *et al.*, 2019, Shakiba *et al.*, 2018). Hence, these results regarding important cytokines validate aforesaid outcomes suggesting anti-HCC ability of Tagetes. Alpha-fetoprotein (AFP) is mainly produced by the liver and while increased levels are commonly associated with liver cancer, serving as an indicator of pathological changes in inflammatory conditions (Arrieta *et al.*, 2007). The DEN treatment group showed increased expression of AFP, indicating successful induction of hepatocellular carcinoma. After Tagetes extract treatment, AFP levels decreased significantly in both protocols (Figs. 4 and 5), suggesting improvement in liver condition and suppression of tumor progression. However, AFP levels, serving as a protein marker, were not significantly affected, following DEN treatment (Fig. 5). The mRNA levels do not necessarily correlate with their respective proteins. It can be attributed to earlier stages of disease, enhanced protein degradation, or involvement of some post-translational phenomenon (Marubashi *et al.*, 2011). Overall, DEN induction elevated pro-inflammatory cytokines and tumor marker expression, while Tagetes extract treatment effectively reduced these alterations, demonstrating anti-inflammatory and hepatoprotective effects.

CONCLUSION

In conclusion, *T. patula* flower methanolic extract demonstrated the potential therapeutic activity against the DEN induced hepatocellular carcinoma. DEN induction severely compromised body weight and liver weight and markedly elevated pro-inflammatory cytokines (TNF-α, IL-6) and tumor marker expression (AFP), while extract treatment effectively reduced these alterations, demonstrating anti-inflammatory and hepatoprotective effects. Therefore, *T. patula* flower presents as a potential candidate for the anti-HCC drug discovery screening program.

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Authors' contributions
Asfandyar Ajab Khan: Investigation, writing-original draft, writing-review & editing, formal analysis. Muhammad Kashif: Conceptualization, Data curation, writing-original draft, visualization. Hazar Khan: Data curation, validation. Anam Razzak: Investigation, writing-original draft, writing-review & editing. Shazmeen Aslam: Writing-review & editing. Samina Bano: Methodology, writing-review & editing. Shaheen Faizi: Supervision, Data curation, visualization, Talat Roome: Conceptualization, supervision, project administration, validation, visualization.

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All data generated or analyzed during this study are included in this published article.

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The study was conducted by the approval of Institutional Review Board of Dow University of Health Sciences (IRB-

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Conflict of interest

The authors declare that they have no conflict of interests.

Supplementary data

https://www.pjps.pk/uploads/2026/06/SUP1782202680.pdf

REFERENCES

Aati HY, Emam M, Al-Qahtani J, Aati S, Aati A, Wanner J and Seif MM (2022). Chemical composition of tagetes patula flowers essential oil and hepato-therapeutic effect against carbon tetrachloride-induced toxicity (*in-vivo*). *Molecules,* **27**(21): 7242.

Abdel-Haleem SA, Ibrahim AY, Ismail RF, Shaffie NM, Hendawy S and Omer E (2017). *In-vivo* hypoglycemic and hypolipidemic properties of Tagetes lucida alcoholic extract in streptozotocin-induced hyperglycemic Wistar albino rats. *Ann. Agric. Sci.,* **62**(2): 169-181.

Aravalli RN, Steer CJ and Cressman EN (2008). Molecular mechanisms of hepatocellular carcinoma. *Hepatology,* **48**(6): 2047-2063.

Arrieta O, Cacho B, Morales-Espinosa D, Ruelas-Villavicencio A, Flores-Estrada D and Hernandez-Pedro N (2007). The progressive elevation of alpha fetoprotein for the diagnosis of hepatocellular carcinoma in patients with liver cirrhosis. *BMC Cancer,* **7**(1): 28.

Arshad Qamar K, Dar A, S Siddiqui B, Kabir N, Aslam H, Ahmed S, Erum S, Habib S and Begum S (2010). Anticancer activity of ocimum basilicum and the effect of ursolic acid on the cytoskeleton of MCF-7 human breast cancer cells. *Lett. Drug Des. Discov.,* **7**(10): 726-736.

Bano S, Faizi S, Lubna, Fayyaz S and Iqbal EY (2019). Isolation of ceramides from Tagetes patula L. yellow flowers and nematicidal activity of the fractions and pure compounds against cyst nematode, Heterodera zeae. *Chem. Biodivers.,* **16**(6): e1900092.

Cragg GM, Grothaus PG and Newman DJ (2009). Impact of natural products on developing new anti-cancer agents. *Chem. Rev.,* **109**(7): 3012-3043.

Danpanichkul P, Pang Y, Diaz LA, Arab JP, Hwang SY, Dejvajara D, Suresh V, Suenghataiphorn T, Wannaphut C, Suparan K, Wijarnpreecha K, Kim H, Kim D, Singal AG and Yang JD (2025). Young adults and alcohol-associated liver cancer: Incidence and death from 2000 to 2021. *Cancers (Basel),* **17**(4): 609.

Ding YF, Wu ZH, Wei YJ, Shu L and Peng YR (2017). Hepatic inflammation-fibrosis-cancer axis in the rat hepatocellular carcinoma induced by diethylnitrosamine. *J. Cancer Res. Clin. Oncol.,* **143**(5): 821-834.

El–Serag HB and Rudolph KL (2007). Hepatocellular carcinoma: Epidemiology and molecular carcinogenesis. *Gastroenterology,* **132**(7): 2557-2576.

Faizi S, Fayyaz S, Bano S, Yawar Iqbal E, Siddiqi H and Naz A (2011). Isolation of nematicidal compounds from *Tagetes patula* L. yellow flowers: Structure–activity relationship studies against cyst nematode Heterodera zeae infective stage larvae. *J. Agric. Food Chem.,* **59**(17): 9080-9093.

Faizi S, Siddiqi H, Bano S, Naz A, Lubna, Mazhar K, Nasim S, Riaz T, Kamal S and Ahmad A (2008). Antibacterial and antifungal activities of different parts of *Tagetes patula*.: Preparation of patuletin derivatives. *Pharm. Biol.,* **46**(5): 309-320.

Haque S, Muzaffar M, Singh B, Tanweeruddin M, Kumar A, Mahmood K and Kumar A (2015). Estimation of serum Alpha Feto-Protein (AFP), Interlukin-6 and Des-γ-Carboxyprothrombin (DCP) in case of hepatocellular carcinoma. *Glob. J. Pathol. Microbiol.,* **3**: 16-19.

Harikumar KB, Nimita CV, Preethi KC, Kuttan R, Shankaranarayana ML and Deshpande J (2008). Toxicity profile of lutein and lutein ester isolated from marigold flowers (Tagetes erecta). *Int. J. Toxicol.,* **27**(1): 1-9.

Huang DQ, Singal AG, Kono Y, Tan DJ, El-Serag HB and Loomba R (2022). Changing global epidemiology of liver cancer from 2010 to 2019: NASH is the fastest growing cause of liver cancer. *Cell Metab.,* **34**(7): 969-977. e962.

Imamoto R, Okano J-I, Sawada S, Fujise Y, Abe R and Murawaki Y (2014). Null anticarcinogenic effect of silymarin on diethylnitrosamine-induced hepatocarcinogenesis in rats. *Exp. Ther. Med.,* **7**(1): 31-38.

Jabeen A, Mesaik MA, Simjee SU, Bano S and Faizi S (2016). Anti-TNF-α and anti-arthritic effect of patuletin: A rare flavonoid from *Tagetes patula. Int. Immunopharmacol.,* **36**: 232-240.

Jeng LB, Shih FY, Chan WL and Teng CF (2025). Cytokine biomarkers for independent prediction of hepatocellular carcinoma prognosis. *Discov. Oncol.,* **16**(1): 1-11.

Kashif M, Bano S, Naqvi S, Faizi S, Lubna, Ahmed Mesaik M, Azeemi KS and Farooq AD (2015). Cytotoxic and antioxidant properties of phenolic compounds from *Tagetes patula* flower. *Pharm. Biol.,* **53**(5): 672-681.

Kojima-Yuasa A, Huang X and Matsui-Yuasa I (2015). Synergistic anticancer activities of natural substances in human hepatocellular carcinoma. *Diseases,* **3**(4): 260-281.

Kurma K, Manches O, Chuffart F, Sturm N, Gharzeddine K, Zhang J, Mercey-Ressejac M, Rousseaux S, Millet A and Lerat H (2021). DEN-induced rat model reproduces

key features of human hepatocellular carcinoma. *Cancers,* **13**(19): 4981.

Li S, Lin Y, Gao X, Zeng D, Cen W, Su Y, Su J, Zeng C, Huang Z and Zeng H (2024). Integrative multi-omics analysis reveals a novel subtype of hepatocellular carcinoma with biological and clinical relevance. *Front. Immunol.,* **15**: 1517312.

Liao CY, Lee CC, Tsai CC, Hsueh CW, Wang CC, Chen IH, Tsai MK, Liu MY, Hsieh AT and Su KJ (2015). Novel investigations of flavonoids as chemopreventive agents for hepatocellular carcinoma. *Biomed Res. Int.,* **2015**(1): 840542.

Liu L, Luo S, Yu M, Metwaly AM, Ran X, Ma C, Dou D and Cai D (2020). Chemical constituents of tagetes patula and their neuroprotecting action. *Nat. Prod. Commun.,* **15**(11): 1934578X20974507.

Liu X, Ran X, Riaz M, Kuang H, Dou D and Cai D (2019). Mechanism investigation of Tagetes patula L. against chronic nonbacterial prostatitis by metabolomics and network pharmacology. *Molecules,* **24**(12): 2266.

Mandlik DS and Mandlik SK (2021). Herbal and natural dietary products: upcoming therapeutic approach for prevention and treatment of hepatocellular carcinoma. *Nutr. Cancer,* **73**(11-12): 2130-2154.

Marubashi S, Nagano H, Wada H, Kobayashi S, Eguchi H, Takeda Y, Tanemura M, Umeshita K, Doki Y and Mori M (2011). Clinical significance of alpha-fetoprotein mRNA in peripheral blood in liver resection for hepatocellular carcinoma. *Ann. Surg. Oncol.,* **18**(8): 2200-2209.

Meurer M, de Oliveira BM, Cury BJ, Jerônimo DT, Venzon L, Franca TC, Mariott M, Silva-Nunes R, Santos AC and Roman-Junior WA (2022). Extract of Tagetes erecta L., a medicinal plant rich in lutein, promotes gastric healing and reduces ulcer recurrence in rodents. *J. Ethnopharmacol.,* **293**: 115258.

Meurer MC, Mees M, Mariano LNB, Boeing T, Somensi LB, Mariott M, Dos Santos AC, Longo B, França TCS and Klein-Junior LC (2019). Hydroalcoholic extract of Tagetes erecta L. flowers, rich in the carotenoid lutein, attenuates inflammatory cytokine secretion and improves the oxidative stress in an animal model of ulcerative colitis. *Nutr. Res.,* **66**: 95-106.

Qiu B, Wang J, Yu Y, Zhen C, Gu J, Liu W, Wen Y, Chen L, Gao Y and Xia Q (2016). DJ-1 promotes development of DEN-induced hepatocellular carcinoma and proliferation of liver cancer cells. *Oncotarget,* **8**(5): 8499.

Riaz M, Ahmad R, Rahman NU, Khan Z, Dou D, Sechel G and Manea R (2020). Traditional uses, Phyto-chemistry and pharmacological activities of *Tagetes patula* L. *J. Ethnopharmacol.,* **255**: 112718.

Roayaie S, Blume IN, Thung SN, Guido M, Fiel MI, Hiotis S, Labow DM, Llovet JM and Schwartz ME (2009). A system of classifying microvascular invasion to predict outcome after resection in patients with hepatocellular carcinoma. *Gastroenterology,* **137**(3): 850-855.

Rumgay H, Arnold M, Ferlay J, Lesi O, Cabasag CJ, Vignat J, Laversanne M, McGlynn KA and Soerjomataram I (2022). Global burden of primary liver cancer in 2020 and predictions to 2040. *J. Hepatol.,* **77**(6): 1598-1606.

Shakiba E, Ramezani M and Sadeghi M (2018). Evaluation of serum interleukin-6 levels in hepatocellular carcinoma patients: A systematic review and meta-analysis. *Clin. Exp. Hepatol.,* **4**(3): 182-190.

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S and Boyd MR (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. *JNCI: J Natl Cancer Inst.*, **82**(13): 1107-1112.

Sukhikh S, Ivanova S, Babich O, Shepel E and Kriger O (2025). Study of biological activity of aqueous extracts of *Tagetes Patula* L. flowers. *Fitoterapia,* 106712.

Sultana H, Alakeel KA, Hassan J, Mallick SR, Zakaria M, Kayesh E, Gomasta J, Zubayer M, Billah MM and Ozaki Y (2025). Nutrients, bioactive compounds and antinutritional properties of marigold genotypes as promising functional food. *Sci. Rep.,* **15**(1): 4867.

Tan EY, Danpanichkul P, Yong JN, Yu Z, Tan DJH, Lim WH, Koh B, Lim RYZ, Tham EKJ and Mitra K (2025). Liver cancer in 2021: Global burden of disease study. *J. Hepatol.,* **82**(5): 851-860.

Toh MR, Wong EYT, Wong SH, Ng AWT, Loo L-H, Chow PK-H and Ngeow J (2023). Global epidemiology and genetics of hepatocellular carcinoma. *Gastroenterology,* **164**(5): 766-782.

Vachliotis ID and Polyzos SA (2023). The role of tumor necrosis factor-alpha in the pathogenesis and treatment of nonalcoholic fatty liver disease. *Curr. Obes. Rep.,* **12**(3): 191-206.

Van Engeland M, Nieland LJ, Ramaekers FC, Schutte B and Reutelingsperger CP (1998). Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry,* **31**(1): 1-9.

Yeo YH, Abdelmalek M, Khan S, Moylan CA, Rodriquez L, Villanueva A and Yang JD (2025). Current and emerging strategies for the prevention of hepatocellular carcinoma. *Nat. Rev. Gastroenterol. Hepatol.,* **22**(3): 173-190.

Zardeto G, Krzyzaniak LM, de Mello JCP, Ueda-Nakamura T, Dias-Filho BP, de Oliveira Silva S and Nakamura CV (2024). Isolation of flavonoids from Tagetes patula (French Marigold) flowers: Cytotoxic and oxidant activity in human cervical carcinoma cells. *Bol. Latinoam. Caribe Plantas Med. Aromat.,* **23**(3): 448-459.