Vitamin D potentiates anti-tumor activity of 5-fluorouracil via modulating caspase-3 and TGF-β1 expression in hepatocellular carcinoma-induced in rats

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Canadian Journal of Physiology and Pharmacology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID</td>
<td>cjpp-2018-0445.R1</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>28-Aug-2018</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>R Ebrahim, Amal; Faculty of Pharmacy, Mansoura University, Egypt, Biochemistry Department El-Mesery, Mohamed; Faculty of Pharmacy, Mansoura University, Egypt El-Karef, Amro; Faculty of medicine mansoura university, pathology Department Eissa, Laila; Faculty of Pharmacy, Mansoura University, Egypt, Biochemistry department</td>
</tr>
<tr>
<td>Is the invited manuscript for consideration in a Special Issue:</td>
<td>Not applicable (regular submission)</td>
</tr>
<tr>
<td>Keyword:</td>
<td>Hepatocellular carcinoma, vitamin D, 5-fluorouracil, TGF-β1, caspase-3</td>
</tr>
</tbody>
</table>

https://mc06.manuscriptcentral.com/cjpp-pubs
Vitamin D potentiates anti-tumor activity of 5-fluorouracil via modulating caspase-3 and TGF-β1 expression in hepatocellular carcinoma-induced in rats

Amal R Ebrahim 1, Mohamed El-Mesery 1, Amro EL-Karef 2, Laila A Eissa1*

1 Biochemistry Department, Faculty of Pharmacy, Mansoura University, Mansoura, 35516, Egypt

2 Pathology Department, faculty of medicine, Mansoura University, Mansoura, 35516, Egypt

1*corresponding author:

Laila Ahmed Eissa

Adress: Biochemistry Department, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt.

Email: lailaeissa2002@yahoo.com

Phone: +2/01097400781
Abstract
The role of vitamin D (Vit D) either alone or in combination with 5-fluorouracil (5-FU) in thioacetamide (TAA) induced-hepatocellular carcinoma (HCC) in rats was investigated. Fifty male Sprague-Dawely rats were randomized into: control group and four groups receiving TAA (200 mg/Kg) i.p twice per week for 16 weeks that were further divided into HCC group, 5-FU group (75 mg/kg; ip; once weekly for 3 weeks starting from the 12th week), Vit D group (200 IU/kg/day; daily by oral tube; for 16 weeks) and 5-FU+Vit D group receiving the previously mentioned dosage regimens of 5-FU and Vit D. HCC was detected by histopathological changes in liver sections and the elevation of serum alpha-fetoprotein (AFP) level. 5-FU+Vit D significantly decreased gene expression of nuclear factor Erythroid 2-related factor 2 (NrF2) and TGF-β1 at both gene and protein level and serum AFP concentrations in comparison with their corresponding monotherapy. Moreover, 5-FU+Vit D enhanced apoptosis by increasing caspase-3 gene and protein expression. Conclusively, Vitamin D enhances anti-tumor activity of 5-FU in HCC-induced model and improves liver function of treated animals. Moreover, combination therapy in TAA induced-HCC rat model was more effective than 5-FU or Vit D by modulating TGF-β1, caspase-3 and NrF2 expressions.

Keywords:
Hepatocellular carcinoma; vitamin D; 5-fluorouracil; TGF-β1; caspase-3.
**Introduction**

Hepatocellular carcinoma (HCC) is considered one of the most common and the third fatal malignancy worldwide (Njei et al. 2015). The incidence and mortality of HCC are elevated rapidly every year (Forner and Bruix 2012). Therefore, early HCC detection is urgently needed, due to fact that most HCC patients at the advanced stage are prone to present a poor prognosis (Wang and Ding 2010; Shi et al. 2014). Hepatitis B and C viral infections are the main HCC risk factors (Chuang et al. 2010). In addition, dietary aflatoxin exposure, chronic alcohol consumption and non-alcoholic cirrhosis are considered risk factors of HCC (Bouchard and Navas-Martin 2011). Non-alcoholic fatty liver disease (NAFLD) is closely linked with the metabolic syndrome, particularly type 2 diabetes mellitus and obesity (Bugianesi et al. 2007). Metabolic syndrome may lead to HCC by NAFLD (Scalera and Tarantino 2014). Moreover, Non-alcoholic Steatohepatitis (NASH) in association with multiple components of the metabolic syndrome is thought to increase the risk for developing chronic liver disease, cirrhosis, and HCC (Bugianesi et al. 2007). Treatment of HCC is quite challenging and complicated especially when underlying chronic liver diseases are present (Kalogeridi et al. 2015). A number of treatment options are available for HCC such as surgical resection (Capussotti et al. 2009) and transarterial chemoembolization (Kawamura et al. 2013). However, liver transplantation is the most important option for HCC treatment (Chen et al. 2014). However, newer treatments are needed for the effective management of HCC.

Chemotherapy is the main treatment for advanced or recurrent liver cancer (Hirai et al. 2001) such as 5-flurouracil that is commonly used for solid tumors such as liver,
colorectal and gastrointestinal cancers in clinical practice either alone or in combination with other drug (Diasio and Johnson 2000). It initiates apoptosis and cell cycle arrest by interfering with DNA replication and repair (Ardalan and Glazer 1981). Unfortunately, 5-FU induces gastrointestinal toxicity and hematologic side effects (He et al. 2003). Therefore, recent treatment strategies aim to decrease the required dose and enhance its efficacy by using the new trends of combination therapy.

Vitamin D is fat-soluble vitamin whose primary function is to maintain calcium and phosphorus homeostasis (Stokes et al. 2013), and it has a direct influence on the liver that is considered the main synthesis organ for vitamin D-binding protein and 25-hydroxy vitamin D (Fedirko et al. 2014). Regarding vitamin D functions, Vit D and its derivatives have immune, neuroendocrine activities, anti-carcinogenic properties and protecting DNA against oxidative damage (Welsh et al. 2003; Bikle et al. 2004). Recently, it was reported the ability of Vit D to enhance anti-tumor activity of chemotherapeutic drugs by activating apoptosis (Diaz et al. 2015). The synergistic effects of Vit D have been confirmed in combination chemotherapy in several carcinomas of somatic cells in vitro and in vivo (Fleet 2008; Krishnan et al. 2010). Moreover, Vit D deficiency has been reported in HCC patients (Finkelmeier et al. 2014; Wu et al. 2018) and a causal relationship of low Vit D levels with HCC development has been proposed (Fedirko et al. 2014). Epidemiological researches clearly indicated that a suitable level of serum Vit D correlates with a low cancer incidence (Marcinkowska et al. 2016).

Transforming growth factor β1 (TGF-β1) is one of three members of the TGF-β superfamily of cytokines that is considered as one of the most important factors controlling the progression of carcinogenesis of HCC (Lee et al. 2012). Although the anti-cancer activity of Vit D was proved before, its role in HCC-induced model
in rats has not done yet. Thus, the current study intended to examine Vit D role in thioacetamide (TAA) induced HCC in rats. In addition, the effect of Vit D on 5-FU anti-tumor activity was analyzed. Moreover, this study targeted to explore the molecular mechanism of Vit D action by analyzing expression levels of NrF2, TGF-β1 and caspase-3.

**Materials and methods**

**Drugs and chemicals**

TAA was purchased from Sigma-Aldrich, St. Louis, MO, USA. 5-FU ampoules (250 mg/ml) was purchased from pharco company, Alexandria, Egypt. Vit D3 (vidrop, cholecalciferol 2800 IU/mL) was purchased from medical union pharmaceuticals, Egypt.

**Animals**

Fifty adult male Spargue-Dawely rats were used in this research weighing 180-200 g. All rats were kept for acclimatization under standard conditions of temperature with normal photoperiod (12 h light: dark cycles) and allowed free access to food and water throughout the study period. The animal experiment protocol was approved by "Research Ethics Committee" Faculty of Pharmacy, Mansoura University, Egypt (protocol code-No. 2018-9), in accordance with "Principles of Laboratory Animal Care" (NIH publication No. 85-23, R- 1985).

**Experimental design**

Animals were randomly classified into control group (n=10) that did not receive any treatment and TAA receiving groups that received TAA 200 mg/kg in normal saline was injected i.p. twice per week for 16 weeks to induce HCC (Dasgupta et al. 1981). TAA receiving animals were divided into 4 groups (n=10):
**HCC groups:** received only TAA.

**5-FU group:** received TAA and 5-FU (ip. injection of 75 mg/kg) once per week for 3 consecutive weeks starting from 12th week (Abdel-Hamid and Morsy 2010; Abdel-Hamid et al. 2011).

**Vit D group:** received TAA and cholecalciferol (200 IU/kg) every day by oral gavage tube for 16 week (Gurel et al. 2015).

**5-FU+Vit D group:** received TAA and a combination treatment of 5-FU and Vit D in the same regimen as mentioned in the last two previous groups.

**Blood and liver sampling**

At ending of 16th week, animals were fasted and allowed access to water. Blood samples were collected from heart under diethyl ether anesthesia and then centrifuged at 4000 r.p.m for 5 minutes. Then, sera were immediately kept frozen at -20 °C for biochemical analysis. After the decapitation of rats, livers were isolated and divided into two parts: One part was fixed in 10% buffered formalin for histopathological and immunohistochemical assay. The other part was immersed in liquid nitrogen and stored at -80°C for gene expression analysis of caspase-3, TGF-β1 and NrF2.

**Histopathology study**

Liver specimens were immersed immediately in 10% buffered formalin and they placed in ascending grades of ethanol for dehydration. They were embedded in paraffin and 5 micrometer-thickness sections were cut and stained with hematoxylin and eosin (H&E) to be viewed under microscope. Liver specimens were anonymously coded and examined in masked manner. A digital camera–aided computer system (Nikon Digital Camera, Japan) was used for photographing of histopathological alteration.
**Immunohistochemistry**

Five-micrometer-thick sections were cut, deparaffinized by heating and rehydrated using xylene and descending concentrations of ethanol then immersed in an antigen retrieval (citrate buffer solution, pH 6.0). Endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 15 min. All non-specific binding sites were blocked using 1% bovine serum albumin for 1 h. Sections were incubated over night with monoclonal TGF-β1 and caspase-3 antibodies at 4°C. Afterwards, sections were incubated with horseradish peroxidase conjugated anti-mouse antibody. The chromogen used was 2% 3, 3'-diaminobenzidine (DAB) in 50 mM Tris-buffer (pH 7.6). Then, slides were counterstained with hematoxylin. the slides were examined under light microscope (Nikon Digital Camera, Japan).

**Biochemical analysis of liver function**

Activities of serum [alanine aminotransferase (ALT), aspartate aminotransferase (AST) (ELITech Clinical Systems, Zone Industrielle, France), gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) (Egyptian Company of Biotechnology, Cairo, Egypt)] were assayed kinetically according to the manufacturer’s protocol. Total bilirubin and albumin concentration (Diamond, diagnostic, Giza, Egypt) were measured according to the manufacturer's instructions.

Serum AFP concentration was estimated using ELISA kit from (Diametra S.r.I, Italy).

**Determination of relative values of hepatic gene expression for TGF-β1, caspase-3 and NrF2 mRNA**

Total RNA for TGF-β1, caspase-3 and NrF2 was isolated from liver tissues using GF-l extraction kit (vivantis, Malasyia). According to manufacture instructions, RNA concentration and purity were monitored by measuring the absorbance at 260
nm and the absorbance ratio at 260/280 nm, respectively using NanoPhotometer. QuantiTect reverse transcription kit (Qiagen Valencia, USA) used for reverse-transcription of 1μg of total RNA into single-stranded complementary DNA (cDNA). NrF2, caspase-3 and TGF-β1 mRNA levels in different rats hepatic tissues were determined using SensiFAST SYBR® No-ROX Kit (Bioline, USA). Meanwhile, rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene and an internal reference control. Gene specific PCR primers (Table 1) were designed using Primer Express 3.0 (Applied Biosystems, USA) according to the nucleotide sequence obtained from the Gene Bank.

**Statistical analysis**

SPSS version 18 (Chicago, IL, USA) was used for statistical evaluation. Results were represented as mean ± standard deviation (SD) and analyzed with One-way analysis of variance (ANOVA) followed by Bonferroni post hoc correction for multiple comparisons. Statistical computations were done using Excel 2010 and Statistical significance was considered with probability value of p <0.05.

**Results**

**Hepatoprotective effect of vitamin D either alone or in combination with 5-fluorouracil in HCC induced by TAA in rats**

HCC group showed a marked increase in the activity of serum AST, ALT, ALP and GGT enzymes, marked decrease in albumin level and marked increase in bilirubin level (p<0.05) as compared to control group (Table 2). On other hand, 5-FU and Vit D groups exhibited significant reduction of serum activities of ALT, AST, ALP and GGT enzymes, marked increase in albumin level and reduction in bilirubin level (P<0.05) as compared to HCC group. Interestingly, 5-FU+Vit D group showed further improvement in liver functions (P<0.05) as compared to either 5-FU or Vit D groups (Table 2).
Vitamin D enhances the anti-tumor activity of 5-FU in HCC-induced in rats

Liver sections from different groups were stained with H&E. Control group showed normal liver with normal central vein (CV) and hepatic parenchyma (Fig. 1A). HCC group showed tumor tissue with dysplastic hepatocytes with more than two cell thick plates (Fig. 1B). 5-FU treated group and Vit D treated group showed regression of dysplastic changes but with slightly thickened fibrosed portal tracts (PT) (Fig. 1C and D). Interestingly, 5-FU+Vit D group showed improvement in hepatic tissue with complete regression of dysplastic and fibrotic changes as compared to normal control group (Fig. 1E).

Moreover, AFP concentrations in rats of the HCC group were significantly increased as compared to the control group. On the other hand, AFP level was significantly decreased in Vit D and 5-FU groups (P<0.05) as compared to HCC group that was further reduced in the group treated with 5-FU+Vit D combination (P<0.05) (Fig. 2).

Combined treatment of 5-FU+Vit D downregulates both hepatic TGF-β1, NrF2 and upregulates caspase-3 gene expression levels

TAA induction of HCC significantly upregulated TGF-β1 gene expression (p<0.05) compared with control group. However, treatment with either 5-FU or Vit D significantly decreased TGF-β1 gene expression compared with HCC group. Moreover, combination of Vit D and 5-FU showed further reduction of TGF-β1 as compared to group treated with 5-FU or Vit D alone (Fig. 3).

Regarding NrF2 gene expression, it increased in HCC group compared to control group; however, this increase was not statistically significant. All treated groups showed a marked decrease in NrF2 gene expression levels as compared to HCC
group that was only significant in groups treated with Vit D either alone or in combination with 5-FU (Fig. 4).

Regarding caspase-3 gene expression level, HCC group showed significant downregulation in caspase-3 gene expression as compared to control group (p<0.05). On the other hand, groups treated with Vit D, 5-FU and 5-FU+Vit D showed a significant increase in caspase-3 gene expression when compared to HCC group (p<0.05) (Fig. 5).

**Immunohistochemistry for TGF-β1 and caspase-3 expression**

The expression of TGF-β1 in liver tissues of HCC group was significantly (p<0.05) high when compared to control group. However, 5-FU and Vit D significantly decreased (p<0.05) the cytoplasmic TGF-β1 expression compared to HCC group. Moreover, 5-FU+Vit D combination treatment significantly decreased TGF-β1 expression compared to each treatment alone (p<0.05) (Fig. 6).

Although HCC group showed clearly active apoptotic pathway as indicated by strong brown staining compared to control group (p<0.05) (Fig.7), all treated groups showed a marked activation in apoptosis that was significantly high in the group treated with 5-FU+Vit D combination as compared to HCC group (P<0.05) (Fig. 7).

**Discussion**

HCC is one of the most common aggressive cancers worldwide, accounting for more than two-thirds of all primary liver cancers (Sherman 2010). The HCC incidence is grim since most patients are diagnosed at end stages (Marrero and Lok 2004). The current study evaluate the effects of Vit D treatment alone and in combination with 5-FU in TAA-induced HCC in rats. HCC induced by TAA was indicated by increased serum AFP level. Moreover, stained liver sections showed
tumor tissue with dysplastic hepatocytes more than two cell thick plates. Indeed, vitamin D insufficiency has been related with the development of an elevated risk of HCC and HCC mortality (Lange et al. 2013; Colombo and Sangiovanni 2014; Fedirko et al. 2014; Finkelmeier et al. 2014). Moreover, role of vitamin D in the prevention and therapy of many tumors have been suggested by several studies (Garland et al. 2007; Pereira et al. 2012; Refaat et al. 2015). Although, Vit D enhanced anti-cancer effects in various tumor (Zugmaier et al. 1996; Nakagawa et al. 2005; Zhang et al. 2005), to our knowledge, we report in the current study for the first time, the ability of Vit D to enhance anti-tumor activity of 5-FU in TAA-induced HCC rats.

Our data indicated that both Vit D and 5-FU groups showed regression of dysplastic hepatocytes changes and improvement of liver functions. In addition, Vit D and 5-FU were able to decrease serum AFP level, the important diagnostic for HCC. Interestingly Vit D treatment enhanced antitumor activity of 5-FU as indicated by decreased serum AFP concentration as compared to 5-FU group. Moreover, Histopathological examination revealed more improvement in 5-FU+Vit D group as compared to either Vit D or 5-FU groups.

TGF β1, a multifunctional cytokine, is related to cell proliferation, cell differentiation and formation of extracellular matrix. Its function in tumor development is complex, and can stimulate as well as inhibit tumor growth (Shi and Massagué 2003). TGF-β acts as a tumor suppressor in the early stage of cancer development, whereas in late stage it can take on role of tumor promoter, favoring of invasion and metastasis (Mishra et al. 2005; Padua and Massagué 2009). Moreover, TGF-β1 expression in HCC cells is highly upregulated (Kim et al. 2002). Also, there is a very close relationship between high expression of TGF-β1 and development, metastasis, and prognosis of HCC (Xu et al. 2003). Regarding to
our results, expression of TGF-β1 was significantly greater in HCC group than control group. Moreover, Vit D and 5-FU treatment downregulated the gene expression TGF-β1, as well as reducing its protein level that was in agreement with a previous study (Motawi et al. 2016; Wahsh et al. 2016). The most significant reduction in TGF β1 gene expression and protein level was detected in 5-FU+Vit D combined treatment group in comparison with HCC group. The reduction in TGF-β1 expression after treatment with vitamin D if alone or combination can be related to the antifibrotic effects of Vit D (Abdelghany et al. 2016). For most solid tumor, angiogenesis is a key regulator of tumor growth and metastasis (Hanahan and Folkman 1996; Folkman 2001). TGF-β1 plays another role in the pathogenesis of HCC by promoting angiogenesis. In addition, it promotes colon cancer cell growth, migration, invasion and metastasis (Lampropoulos et al. 2012; Neuzillet et al. 2015). This TGF-β1 role might clarify its upregulation behavior in most HCC cases and goes in accordance with its overexpression showed in the present study on HCC rat model (Ito et al. 1991; Vinal and Ponyssegur 2001). The blockage of the angiogenic effect of TGF-β by the anti-angiogenic agent Vit D could provide a possible mechanism for the tumor suppressive effect of Vit D (Ma et al. 2011).

TGF β1 mRNA is a more reliable marker for diagnosis of HCC and that amplification of TGF-β1 mRNA by means of PCR is a sensitive method for detection of HCC (Teama¹ et al. 2016). This confirm the role of Vit D for improving the efficacy of 5-FU.

Apoptosis is a physiological process that plays an essential role during liver development and regeneration (Guicciardi and Gores 2005). However, the dysregulation of programmed cell death is an essential reason for HCC progression (Fabregat 2009). Apoptosis is controlled by several signaling pathway including caspase protease (Yuan et al. 2017). Tumor cells with complete loss or
downregulation of caspase-3 expression is commonly accompanying with resistance to apoptosis as well as chemotherapy in several kinds of tumors (Kolenko et al. 1999). Killing of tumor cells by anticancer drugs has been mediated through induction of apoptosis in target cells (Hickman 1992). Most signaling pathways activated by anticancer drugs ultimately result in activation of caspases (Slee et al. 1999). In our study, caspase-3 gene expression was significantly upregulated in 5-FU+ Vit D group compared to HCC and 5-FU group. This supporting the apoptosis-inducing effect of vitamin D. On other hand, 5-FU+Vit D stimulated the intrinsic pathway, caspase-3, was showed from immunohistochemistry result in 5-FU+Vit D group compared to control and HCC groups this result agrees with Zaghloul et al. (2017).

NrF2 is an important factor in modulation of response against oxidative stress and important in regulating a network of genes involved in maintaining redox states and host defense mechanisms and critical for liver functions (Cheng et al. 2015). High expression of NrF2 has been identified in several types of cancers including HCC, which may be associated with cancer cell proliferation, invasion, and chemoresistance (Teng et al. 2017). Many chemical compounds and natural plant extracts have been identified to inhibit of NrF2 such as vitamin C (Tarumoto et al. 2004), falavonoids as (luteolin and apigenin) (Gao et al. 2013; Zhang et al. 2014), isoniazid (Verma et al. 2015) and camptothecin (Chen et al. 2017). In the current study, Vit D either alone or in combination with 5-FU showed significant decrease in NrF2 gene expression compared to HCC group. The result is inconsistent with Nakai et al (2013). Our result of Vit D as an Nrf2 inhibitor may have significant implications for cancer therapy, particularly for HCC with High expression of NrF2.

Conclusion
In the current study Vit D treatment potentiate the efficacy of 5-FU in HCC induced by TAA through: (1) downregulating TGF-β1 expression at gene and protein level (2) induce apoptosis by upregulation of caspase-3 expression (3) decrease NrF2 expression.

**Conflict of interest:** The authors declare that there is no conflict of interest.

**Reference**


**Table 1: The primer sequence for real-time PCR analysis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5'-TGC CAC TCA GAA GAC TGT GG-3'</td>
<td>5'-GGA TGC AGG GAT GAT GTT CT-3'</td>
</tr>
<tr>
<td>TGF β 1</td>
<td>5'-CCT GCA AGA CCA TCG ACA TG-3'</td>
<td>5'-GCG AGC CTT AGT TTG GAC AG-3'</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>5'-GGC CGA CTT CCT GTA TGC TT-3'</td>
<td>5'-CGT ACA GTT TCA GCA TGG CG-3'</td>
</tr>
<tr>
<td>NrF2</td>
<td>5'-CAG TCT TCA CCA CCC CTG AT-3'</td>
<td>5'-TTG CTC CAT GTC CTG CTG TA-3'</td>
</tr>
</tbody>
</table>

Table 2: Effect of 5-FU, Vit D and 5-FU+Vit D on liver functions in thioacetamide (TAA) induced HCC in rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control n=10</th>
<th>HCC n=6</th>
<th>5-FU n=7</th>
<th>Vit D n=7</th>
<th>5-FU+Vit D n=8</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>38.5±4.92</td>
<td>520.16±20.7*</td>
<td>55.43±9.6#</td>
<td>50.86±5.39#</td>
<td>43.37±4.83#</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>64.75±6.18</td>
<td>144.16±16.5*</td>
<td>99.57±7.69#</td>
<td>93.5±6.52#</td>
<td>67±4.2#*#@</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>74.37±12.65</td>
<td>254±41.7*</td>
<td>192.28±50.2#</td>
<td>176.71±32.07#</td>
<td>79.75±31.61#*#@</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>8.25±3.35</td>
<td>37.33±6.18*</td>
<td>27.7±5.64#</td>
<td>23.43±4.11#</td>
<td>18.87±3.94#*#@</td>
</tr>
<tr>
<td>Albumin (mg/dl)</td>
<td>4.52±0.30</td>
<td>2.66±0.16*</td>
<td>3.12±0.17#</td>
<td>3.42±0.22#</td>
<td>4.26±0.21#*#@</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>0.26±0.16</td>
<td>1.25±0.18*</td>
<td>0.61±0.21#</td>
<td>0.40±0.11#</td>
<td>0.31±0.11#*#@</td>
</tr>
</tbody>
</table>

Results are represented as (mean ±SD). *Significant against control group at p<0.05. #Significant against HCC group at p<0.05. $Significant against Vit D group at p<0.05. @Significant against 5-FU group at p<0.05.
HCC, hepatocellular carcinoma; 5-FU, 5-flourouracil; Vit D, vitamin D.

Figure Captions

Fig. 1: Representative images of liver sections stained with hematoxylin-Eosin (H&E).
Single treatment with 5-FU or Vit D showed regression of dysplastic changes with slightly thickened fibrosed portal tracts. Combined treatment showed complete regression of dysplastic and fibrotic changes compared to control rats. HCC, hepatocellular carcinoma; 5-FU, 5-flourouracil; Vit D, vitamin D, CV; central vein, PT; portal tract.

Fig. 2: Effect of 5-FU, Vit D and 5-FU+Vit D on serum alpha-fetoprotein concentration in thioacetamide (TAA) induced HCC in rats.
Results are represented as (mean ± SD). *Significant against control group at p<0.05. #Significant against HCC group at p<0.05. $Significant against Vit D group at p<0.05. @Significant against 5-FU group at p<0.05.

Fig. 3: Effect of 5-FU, Vit D and 5-FU+Vit D on hepatic transforming growth factor beta 1 (TGF-β1) gene expression in thioacetamide (TAA) induced HCC in rats.
Result are represented as (mean ± SD).*Significance against control group at p<0.05. #Significance against HCC group at p<0.05.

Fig. 4: Effect of 5-FU, Vit D, 5-FU+Vit D on nuclear factor erythroid-2 related factor-2 (NrF2) gene expression in thioacetamide (TAA) induced HCC in rats.
Results are expressed as (mean ± SD). *Significance against control group at p<0.05. #Significance against HCC group at p<0.05.

Fig. 5: Effect of 5-FU, Vit D and 5-FU+Vit D on hepatic caspase-3 gene expression in thioacetamide (TAA) induced HCC in rats.
Results are expressed as (mean ± SD). *Significance against control group (p<0.05). #Significance against HCC group (p<0.05).
Fig. 6: Representative images of immunohistochemically stained liver sections isolated from rats showing expression of TGF-β1.

Values are represented as (mean ± SD).*Significance against control group (p<0.05).  
#Significance against HCC group (p<0.05).

Fig. 7: Representative images of immunohistochemically stained liver sections isolated from rats showing expression of caspase-3.

Values are represented as (mean ± SD).*Significance against control group (p<0.05).  
#Significance against HCC group (p<0.05).
Fig. 1

189x38mm (300 x 300 DPI)
Fig 2

118x70mm (300 x 300 DPI)
Fig 3

118x70mm (300 x 300 DPI)
Fig 4

Hepatic NrF2 mRNA expression level

Control   HCC   5-FU   Vit D   5-FU+Vit D

119x70mm (300 x 300 DPI)
Fig 5

Hepatic caspase-3 mRNA expression level

control  HCC  5-FU  Vit D  5FU+Vit D

*  #  #  #

114x69mm (300 x 300 DPI)
(A)

Control  HCC  5-FU  Vit D  5-FU+ Vit D

(B)

Number of TGF-β1 +ve cells / 10 HPF

control  HCC  5-FU  Vit D  5-FU+Vit D

Fig 6

151x155mm (300 x 300 DPI)
**Fig 7**

153x159mm (300 x 300 DPI)