Evaluation of Anti-Cancer Activity of Newly Synthesized Cobalt Complex of Coumarin Schiff Bases

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Abstract

Background: The present study was designed to evaluate the newly synthesized cobalt complex of coumarin schiff base of vanillin (CCV) against diethylnitrosamine (DEN) induced and phenobarbital promoted hepatocellular carcinoma in rats.

Material and Methods: Hepatocelluar carcinoma was induced by DEN at a single dose of 200 mg/kg intraperitoneally followed by administration of phenobarbital (0.05% w/v) daily from 2nd week through drinking water up to 16 successive weeks and synthesized CCV given orally at a dose of 10 mg/kg five days a week for the next 16 weeks. Cancer biomarker, oxidative stress and histopathological studies were carried out to assess the chemopreventive property of the compound.

Results: vanillin Coumarin-cobalt complex at a dose of 10 mg/kg decreased the elevated level of alpha fetoprotein, biochemical markers and increases the reduced level of antioxidant enzymes ($p<0.01$).

Discussion: The carcinogenic potential of DEN generates reactive oxygen species (ROS), which modify cellular functions such as signal transduction pathways and expression of genes related to generation of mitogenic signals. These pathways lead to conversion of normal cell into cancer cell. The antitumor activity of coumarin is believed to be its metabolic product 7-hydroxycoumarin. The studies showed that there is significant elevation of liver biochemical parameters in DEN+PB treated group. The vanillin coumarin-cobalt complex treated animals showed decreased the elevated levels of biochemical parameters and increase the decreased levels of antioxidant enzymes.

Conclusion: The present findings suggest that chemopreventive property of vanillin coumarin-cobalt complex could be due to decreased levels of cancer marker alpha fetoprotein and DNA content as well as scavenging of free radicals. Histopathological reports further confirms chemopreventive property.

Key words: Alfa – fetoprotein, Coumarin, Hepatocelluler carcinoma, Vanillin – cobalt complex.

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Introduction

Hepatocellular carcinoma (HCC) affects approximately half a million persons each year worldwide making it the fifth most common malignancy in male and the ninth most common in female. Hepatocarcinogenesis is a multistep process involving different genetic alterations that ultimately lead to malignant transformation of the hepatocytes.(1) *N*-Nitrosodiethylamine (DEN) is one of the most important environmental carcinogen and exposure of man to preformed nitrosamines occurs due to the use of tobacco products, cosmetics, pharmaceutical products and agricultural chemicals.(2) DEN known to cause cellular DNA damage that is involved in mutagenesis and the development of liver cancer. It is known to cause perturbations in the nuclear enzymes involved in deoxyribonucleic acid (DNA) repair/replication.(3) Its cytotoxic, mutagenic and carcinogenic activity is due to its capability of alkylating DNA structures and its bioactivation by cytochrome P450 enzymes to reactive electrophiles. Phenobarbital (PB) used as a tumour promoter that facilitates the preneoplastic cells by transforming them into foci.(4)

Modern treatment of cancer includes chemotherapy, hormone therapy, radiotherapy and surgery but they are associated with several adverse effects such as alopecia, fatigue and general weakening of the body's immune system due to bone marrow suppression.(5) Delivery of metal-based drugs to their targets poses one of the biggest challenges in cancer chemotherapy. For metal-based therapeutics, a prodrug approach for the inhibition of enzymes by cobalt complexes has been explored by Hambley et al.(6,7) Cisplatin was first synthesized in 1845 and known as Peyrone's chloride, accidentally discovered the anticancer activity of the platinum complex cisplatin.(8) Consequently, the mechanisms underlying resistance to cisplatin and to a lesser extent to carboplatin and oxaliplatin have been extensively investigated. Based on the limitations in the use of the platinum drugs, novel anticancer metal compounds have been designed with the aim of reducing side-effects or to synthesize drugs with less propensity to induce drug resistance.(9-11)

Schiff base, a splendid group ligand molecule that contains azomethine group (C=N) which is formed by the condensation of primary amines with aromatic aldehydes. Such schiff base ligands containing various donor atoms like O, N, S showed broad biological activities and they are bound to the metal ions of Cu (II), Ni (II), Zn (II), Co(II) and Cd(II).[11-14] The metal complexes of Schiff bases have a pivotal role in the field of coordination chemistry. Schiff bases are regarded as privileged ligands.[15-17] Metal complexes of Schiff bases showed biological activities including antibacterial and antifungal,[18] anti cancers,[20] anti inflammatory,[21] antitumor,[22] anti convulsant,[23] anti diabetic,[24] and herbicidal.[25,26] The Schiff base metal complexes and derivatives of coumarin triazoles so far reported as cytotoxic drugs,[27] anti HIV,[28] anti tubercular,[29] and effective therapies.[30]

Materials and Methods

Experimental design:[31]

Preparation of DEN: DEN (single dose - 200 mg/kg) was prepared in 0.9% NaCl.

Preparation of phenobarbital: Phenobarbital at the dose of 0.05 % w/v was finely ground with 0.2% w/v gum acacia and was given in drinking water for 16 weeks.

Dose fixation: From preliminary toxicity studies, it was observed that animals were found to be safe upto a maximum dose of 2000 mg/kg body weight. But there were few changes in the behavioral response like depression. Extensive review of literature[32,33] reveals that metal complexes were effect at low doses. Hence in the present study the 10 mg/kg b.w. was used to assess the pharmacological activities. Hence the present study was carried out by selecting dose of 10 mg/kg.

Collection of blood and organs: Rats were anaesthetized at the end of the study. Blood samples were collected by retro-orbital puncture in sterilized heparinized tubes. The plasma was separated and used for the evaluation of serum biochemical parameters and livers were dissected out for histopathological examination.

Pharmacological Screening

Experimental animals: Albino wistar rats (150-200 g) and albino mice (25-30 g) of either sex were obtained from KLE University's College of Pharmacy, Hubli, Karnataka. The animals were fed with standard pellet diet and water *ad libitum*. Animals were housed in polypropylene cages and were kept under alternate 12 hours of light/dark cycle at a constant temperature (25 ± 2ºC and 35-60% relative humidity). The animals were given 1 week time to get acclimatized with laboratory conditions. The animals were fasted atleast 12 hours before the experiment. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC No. KLEU's-011-IAEC.HBL-31/Aug 2013) after scrutinization.

Acute Toxicity[34]**:** The acute toxicity studies were performed as per the Organization for Economic Co-operation and Development OECD guidelines No. 423. Albino mice of either sex weighing 25-30g were selected and grouped into four groups of 6 animals each and starved for 12 h with water *ad libitum* prior to test. On the day of the experiment, cobalt complex of coumarin Schiff base compounds was administered to animals divided in different groups in an increasing dose of 50, 300, 1000 and 2000 mg/kg body weight orally. The animals were then observed continuously for 6h for general behavioral, neurological and autonomic profiles and then every 30min for next 3h and finally for next 24h or till death.

***In-vivo* Anti-Cancer Activity:** The animals were divided into four groups of ten animals each. Group 1 received 0.2% w/v gum acacia daily through drinking water for 16 weeks. Group 2 received DEN at a single dose of 200 mg/kg intraperitoneally on 1st week followed by administration of phenobarbital (0.05% w/v) daily from 2nd week through drinking water up to 16 successive weeks. Group 3 received DEN at a single dose of 200 mg/kg intraperitoneally on 1st week followed by administration of phenobarbital (0.05% w/v) daily from 2nd week through drinking water up to 16 successive weeks and synthesized CCV given orally at a dose of 10 mg/kg (body weight) five days a week for the next 16 weeks. Group 4 Synthesized drug was given orally from 2nd week at a dose of 10 mg/kg five days a week upto 16 successive weeks. After 16 weeks, the blood samples were collected and analyzed for various liver markers like ALT, AST, total serum bilirubin and direct serum bilirubin, liver cancer marker using commercial kits by ERBA diagnostics Mannheim GmbH. Antioxidant activity was assessed by measuring LPO,SOD, CAT, and GSH[35-38]. DNA was also estimated[39].

Histopathological Study: Histopathological study of the liver tissues was carried out using haematoxylin and eosin staining. All the slides were observed for changes in histopathological characteristics.

Statistical analysis: The results were expressed as Mean±SEM and statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's t-test and $p<0.05$ was considered as significant.

Results

Acute toxicity: No acute toxicity was observed upto a maximum dose of 2000 mg/kg body weight. However, there were few changes in the behavioral response like depression. Extensive review of literature reveals that metal complexes are effective at low doses. Hence, in the present study the 10mg/kg was used to assess the pharmacological activities.[40,41]

In-vivo **anti-cancer activity:** Hepatocarcinogenesis was intiated with DEN and promoted by PB. DEN+PB significantly enhanced the levels of SGPT, SGOT, total bilirubin and direct bilirubin in when compared with normal rats. CCV treated rats significantly decreased the elevated levels SGPT, SGOT, total bilirubin and direct bilirubin when compared with DEN+PB rats ($p<0.001$). (Fig. 1, 2, 3 and 4).

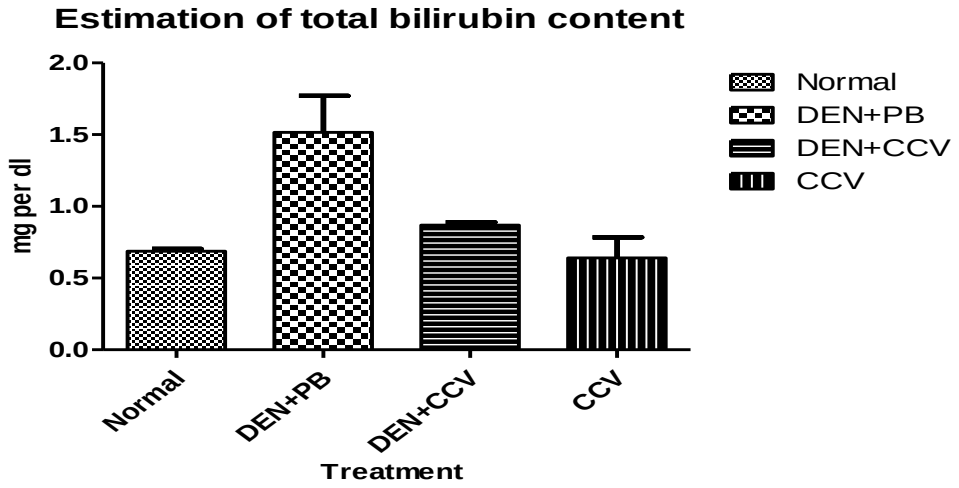


Fig. 1: Effect of metal complex of coumarin Schiff base on total bilirubin level (mg/dL) in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean±SEM . The statistical significance was analyzed by using One-way ANOVA followed by Dunnett test. * P< 0.05 when DEN+PB group compared with Normal group and *P< 0.05 when DEN+PB +CCV group compared with DEN+PB group.

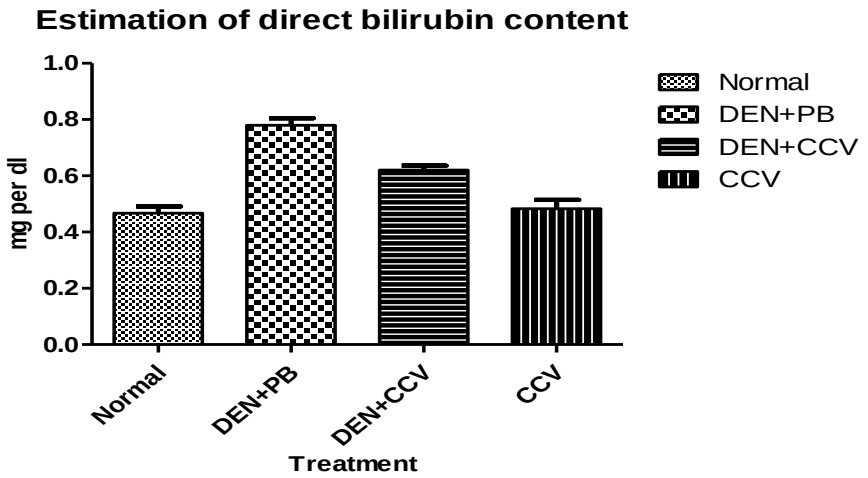


Fig. 2: Effect of metal complex of coumarin Schiff base on direct bilirubin level (mg/dl) in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean±SEM. The statistical significance was analyzed by using One-way ANOVA followed by Dunnett test. *P< 0.05 when DEN+PB group compared with Normal group and *P< 0.05 when DEN+PB +CCV group compared with DEN+PB group.

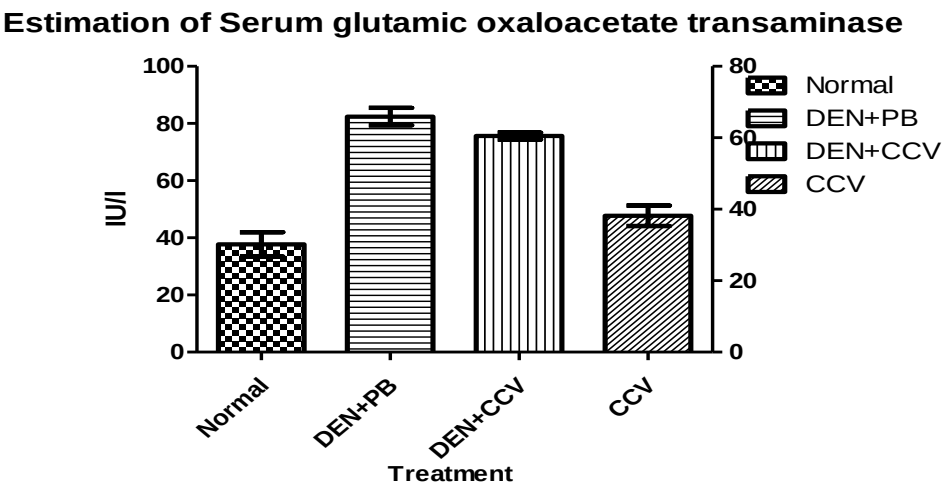


Fig. 3: Effect of metal complex of coumarin Schiff baseon serum SGOT level (IU/l) in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean±SEM. The statistical significance was analyzed by using One-way ANOVA followed by Dunnett test. *P< 0.05 when DEN+PB group compared with Normal group and *P< 0.05 when DEN+PB +CCV group compared with DEN+PB group.

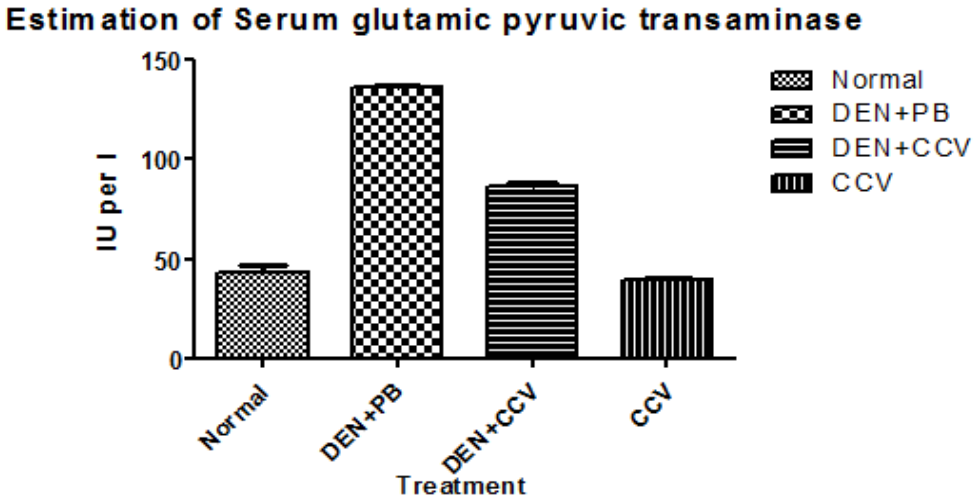


Fig. 4: Effect of metal complex of coumarin Schiff base on serum SGPT level (IU/l) in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean±SEM. The statistical significance was analyzed by using One-way ANOVA followed by Dunnett test. *P< 0.05 when DEN+PB group compared with Normal group and *P< 0.05 when DEN+PB +CCV group compared with DEN+PB group.

Effect of cobalt complex of coumarin Schiff baseon serum cancer biomarker alfa-fetoprotein (AFP): DEN treated animals showed significant increase in the AFP level($p<0.01$) indicating the presence of hepatocellular carcinoma (HCC). Similar rise in AFP level was observed in previous studies of DEN induced HCC. A cobalt complex of coumarin schiff bases of vanilla significantly reduced the rise of AFP level compared to DEN+PB treated group (Fig. 5).

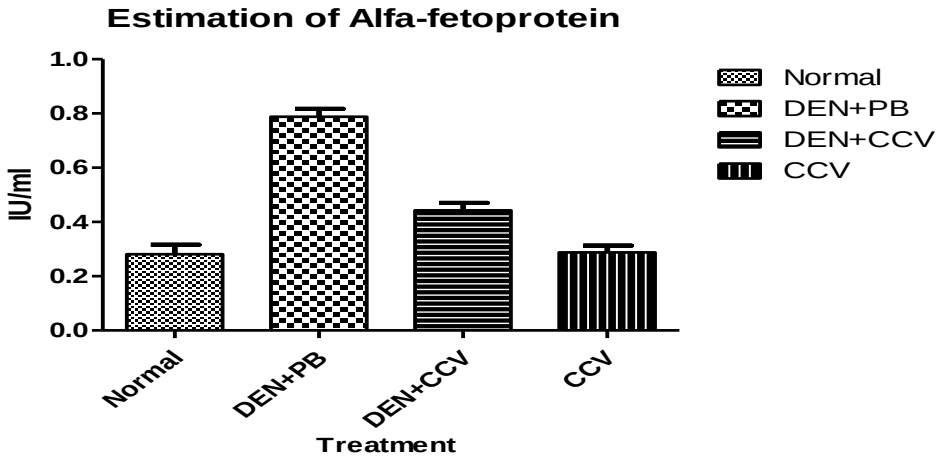


Fig. 5: Effect of metal complex of coumarin Schiff base on serum AFP (IU/l) in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean±SEM. The statistical significance was analyzed by using One-way ANOVA followed by Dunnett test. *P< 0.05 when DEN+PB group compared with Normal group and *P< 0.05 when DEN+PB +CCV group compared with DEN+PB group.

Effect of Cobalt complex of coumarin Schiff base on haemapoietic system: In the present study, the DEN administration produced a significant reduction in RBC count (p<0.001), Hb content (p<0.001), with simultaneous increase in the WBC count (p<0.001). Cobalt complex of coumarin schiff base of vanillin significantly increased the RBC and Hb count and decreased the WBC count(Fig. 6,7 and 8).

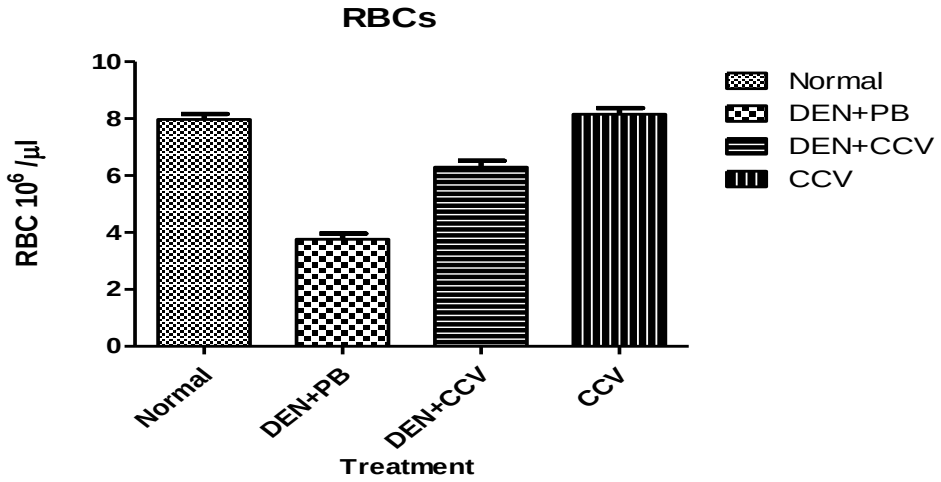


Fig. 6: Effect of metal complex of coumarin Schiff base on blood RBC level in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean±SEM. The statistical significance was analyzed by using One-way ANOVA followed by Dunnett test. *P< 0.05 when DEN+PB group compared with Normal group and *P< 0.05 when DEN+PB +CCV group compared with DEN+PB group.

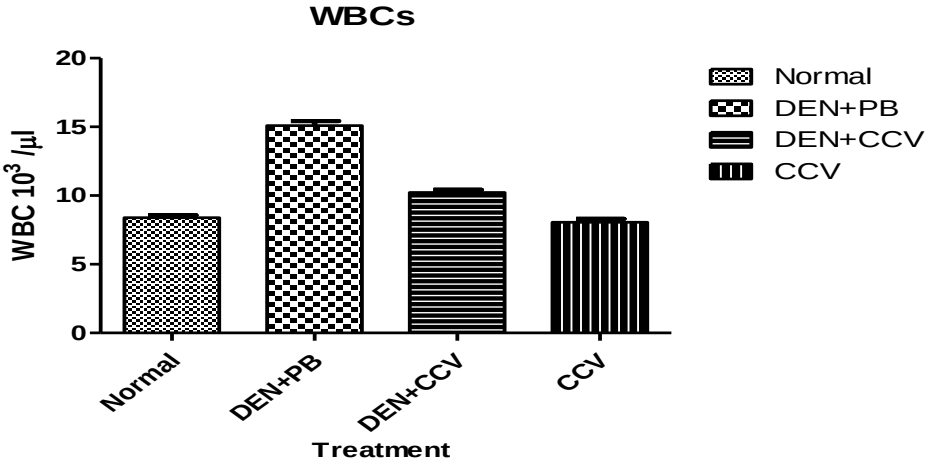


Fig. 7: Effect of metal complex of coumarin Schiff base on a Blood WBC level in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean±SEM. The statistical significance was analyzed by using One-way ANOVA followed by Dunnett test. *P< 0.05 when DEN+PB group compared with Normal group and *P< 0.05 when DEN+PB +CCV group compared with DEN+PB group.

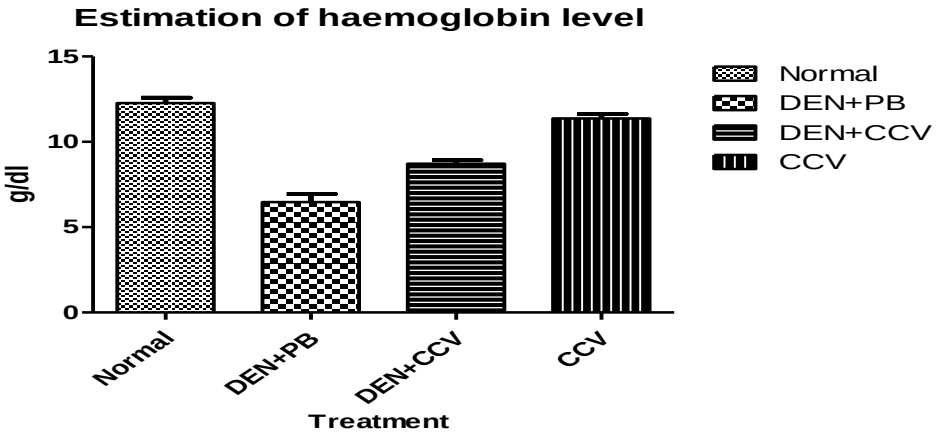


Fig. 8: Effect of metal complex of coumarin Schiff base on Blood Hb level in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean±SEM. The statistical significance was analyzed by using One-way ANOVA followed by Dunnett test. *P< 0.05 when DEN+PB group compared with Normal group and *P< 0.05 when DEN+PB +CCV group compared with DEN+PB group.

Effect of cobalt complex of coumarin Schiff base on free radicals:

The level of LPO was significantly ($p<0.001$) increased in DEN+PB treated rats compared with normal. Rats treated with 10 mg/kg ($p<0.01$) of CCV significantly decreased the level of lipid peroxidation (LPO) when compared with DEN+PB treated rats. A significant ($p<0.001$) decrease in non-enzymatic antioxidant, reduced glutathione (GSH) was observed in rats treated with DEN+PB treated rats compared to normal rats. Treatment with 10mg/kg ($p<0.001$) of CCV significantly elevated the GSH, and catalase (CAT) levels when compared to DEN+PB treated rats (Fig. 9-13).

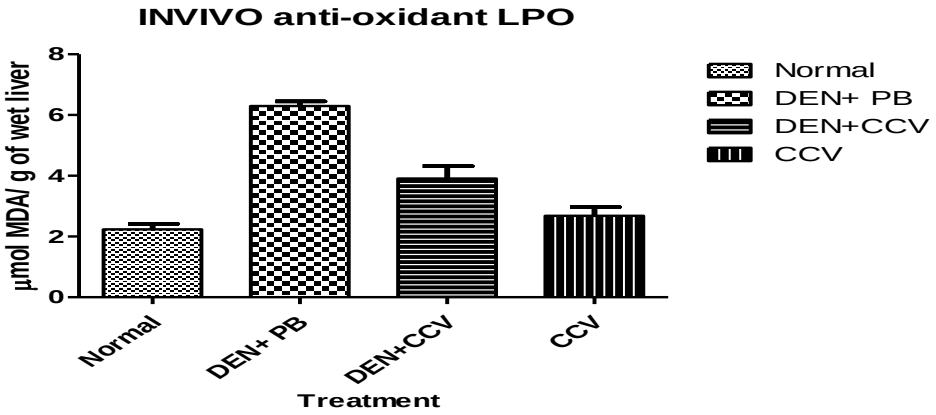


Figure 9: Effect of metal complex of coumarin Schiff base on *Invivo* LPO level in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean±SEM. The statistical significance was analyzed by using One-way ANOVA followed by Dunnett test.* P< 0.05 when DEN+PB group compared with Normal group and *P< 0.05 when DEN+PB +CCV group compared with DEN+PB group.

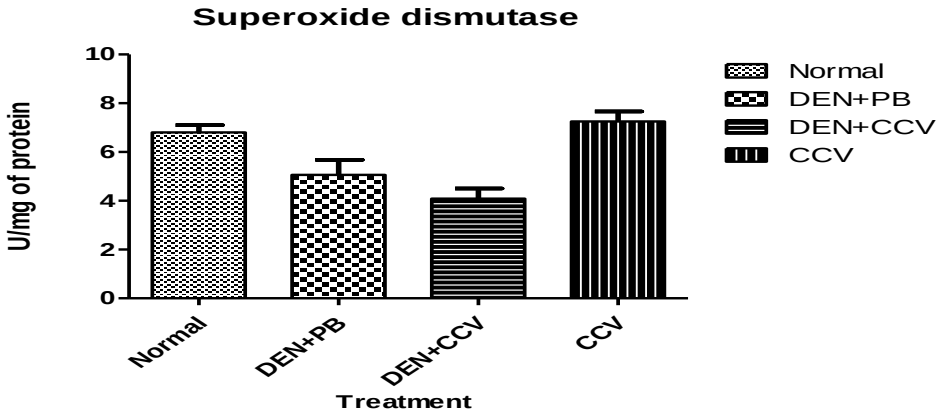


Fig. 10: Effect of metal complex of coumarin Schiff base on SOD level in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean±SEM. The statistical significance was analyzed by using One-way ANOVA followed by Dunnett test. *P< 0.05 when DEN+PB group compared with Normal group and *P< 0.05 when DEN+PB +CCV group compared with DEN+PB group.

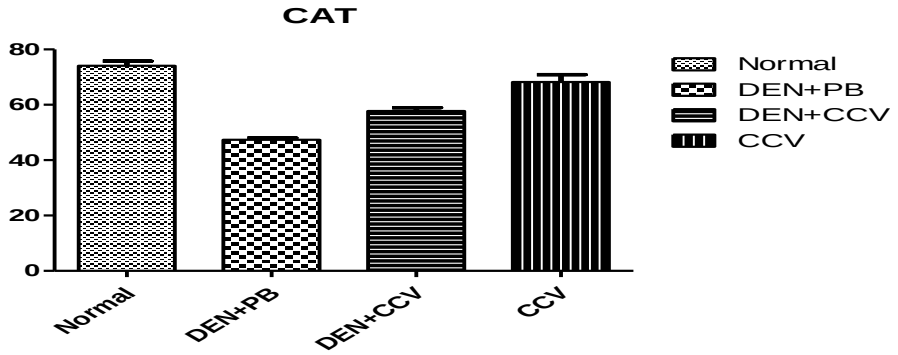


Fig. 11: Effect of metal complex of coumarin Schiff base on CAT level in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean±SEM. The statistical significance was analyzed by using One-way ANOVA followed by Dunnett test. *P< 0.05 when DEN+PB group compared with Normal group and *P< 0.05 when DEN+PB +CCV group compared with DEN+PB group.

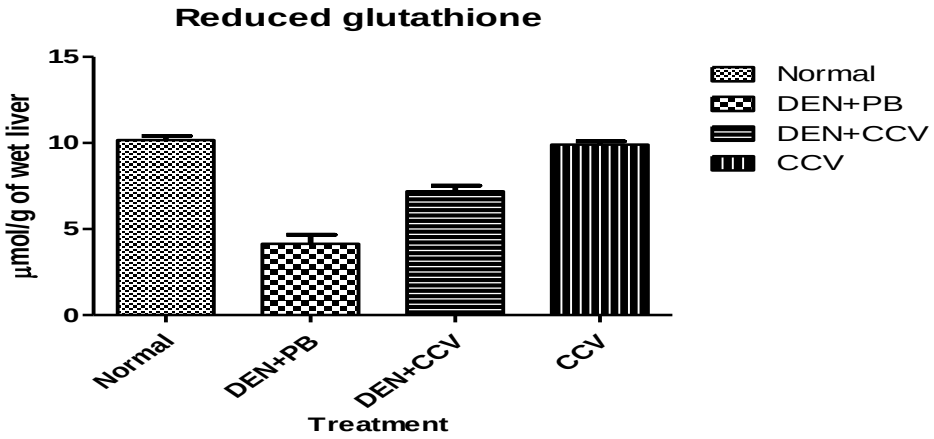


Fig. 12: Effect of metal complex of coumarin Schiff base on GSH level in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean±SEM. The statistical significance was analyzed by using One-way ANOVA followed by Dunnett test. *P< 0.05 when DEN+PB group compared with Normal group and *P< 0.05 when DEN+PB +CCV group compared with DEN+PB group.

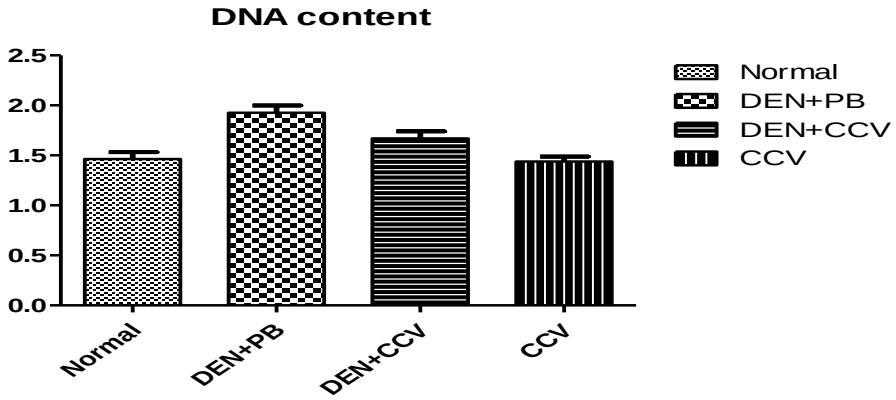
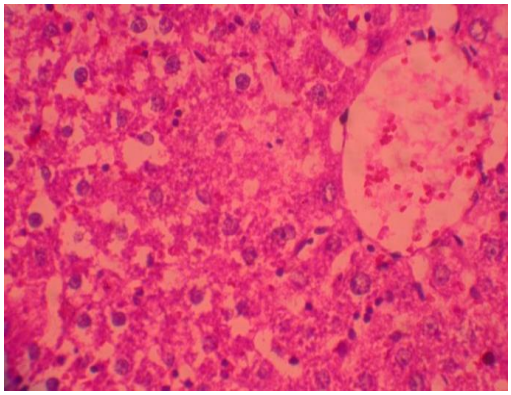


Fig. 13: Effect of metal complex of coumarin Schiff base on DNA level in rats exposed to DEN-induced hepatocellular carcinoma rats

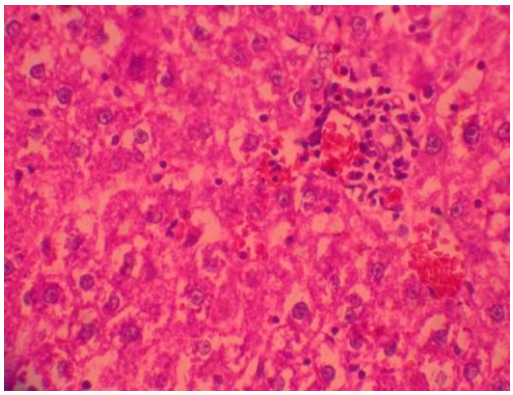
All values were expressed as mean±SEM. The statistical significance was analyzed by using One-way ANOVA followed by Dunnett test. *P< 0.05 when DEN+PB group compared with Normal group and *P< 0.05 when DEN+PB +CCV group compared with DEN+PB group.

Histopathological study: Histopathological study revealed the effect of DEN and cobalt complex of coumarin Schiff base of vanillin treated liver section in rats that are compared with normal rats.

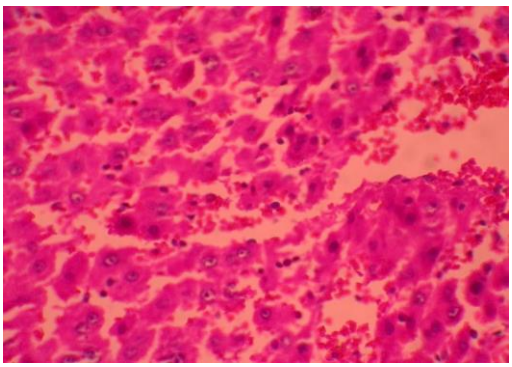
Histopathological study of cobalt complex of coumarin Schiff base on liver: Haematoxylin and Eosin staining:



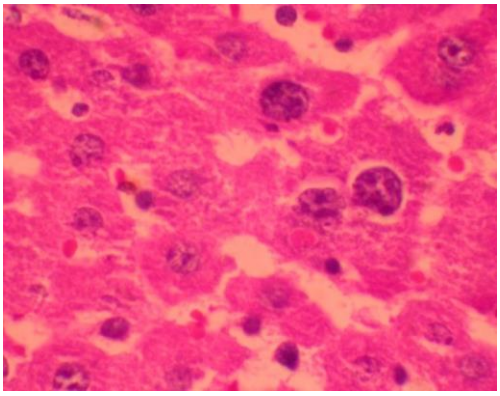
DEN+PB + Drug
A 40X; Mild congestion was observed and inflammation was seen with sinusoidal and central vein congestion.



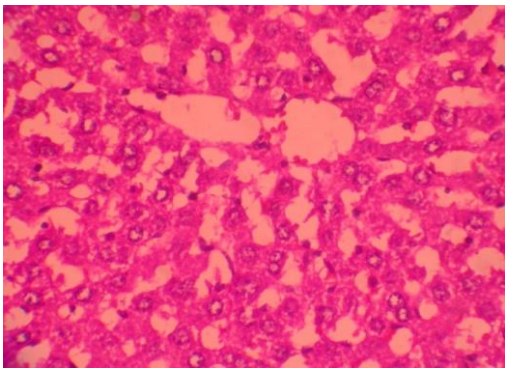
DEN+PB + Drug
A 40X; Portal triaditis with bile duct hyperplasia. In treatment group, mild portal triaditis and bile duct hyperplasia was seen. Mild inflammation occurred and **ballooning** hepatocytes were observed.



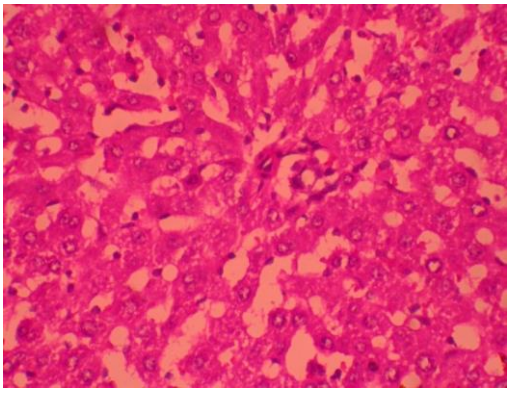
DEN+PB
B 40X Haemorrhage



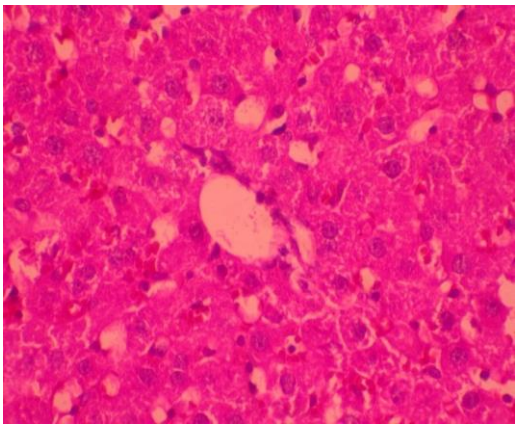
DEN+PB
B 100x Hepatocellular dysplasia
Inflammation, bile duct proliferation, focal haemorrhage was observed. Cystic hyperplasia and hepatocellular dysplasia. Spotty necrosis and zone one degeneration was seen.



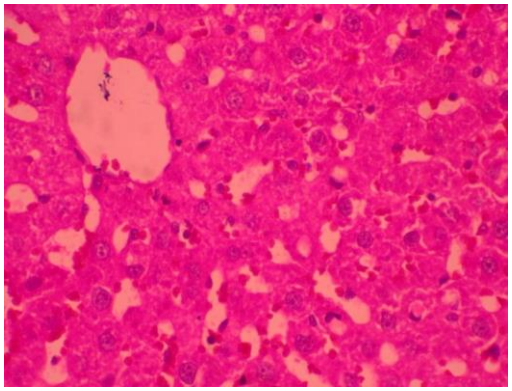
Drug only C 40x



Drug only C 40x
No spotty necrosis or zone one degeneration was observed,



Control group D 40x



Control group D 40x

In control group, there was no inflammation or focal haemorrhage, no bile duct proliferation or cystic hyperplasia was observed. The hepatocytes were found to be in normal condition.

Discussion

In the present study the chemoprevetive activity of cobalt complex of coumarin Schiff base was evaluated against diethyl nitrosamine (DEN) induced and phenobarbital (PB) promoted hepatocarcinogenesis (HCC). Diethyl nitrosamine is one of the principle chemical carcinogen, together with Phenobarbital, which initiates and promotes cancer in animals. The DEN induced hepatocarcinogenesis is evidenced by increased incidence of nodules, whereas the size and amount of hyperplastic nodules in the liver points to initiating and promoting activity.[42] DEN is known to be metabolized in liver by microsomal mixed function oxidase system to its active ethyl radical metabolites leading to the generation of reactive oxygen species (ROS) which interact with DNA to form adduct thereby producing mutation and tumor formation.[43,44] DEN generates reactive oxygen species (ROS), which in turn can use many pathways to modify cellular functions such as signal transduction pathways and expression of genes related to generation of mitogenic signals. These pathways lead to conversion of normal cell into cancer cell.[45] CCV possesss a chemopreventive role in hepatocelluar cancer by affect metabolism of carcinogen DEN by inducing enzymes/ or inhibit tumor promoter actions of phenobarbitone.[46] However, further study is required to confirm the same.

This may be due to liver damage with HCC lesions and parenchymal necrosis where these marker enzymes are released from the damaged hepatocytes into the blood stream since these are primarily localized in the liver.[47] Serum Aspartate aminotransferase (AST) is a tissue enzyme that catalyses the exchange of amino and keto groups between alpha amino acids and alpha keto acids. AST is located in the cytosol of liver. It is also found in the mitochondria and in many tissues of heart, liver, skeletal muscle and kidney. Injury to these tissues results in increase in the AST enzyme level into general circulation. Significant elevation in AST level has also been observed in DEN+PB treated group by earlier studies. This may be due to alteration in the membrane permeability and produce dearrangement in the transport of metabolites as this is membrane bound enzyme.[48,49] Significant elevation of total bilirubin level may be due to DEN, which is known to cause leakage of bilirubin into the circulatory system resulting from hepatocellular damage. This leads to permeability of liver membrane altering the buildup of unconjugated bilirubin in the blood.[50,51] These results suggest the chemopreventive potential of cobalt complex of coumarin Schiff base of vanillin. which is a sign of chemopreventive activity. Serum tumor biomarker diagnosis can be useful for determining the extent of cancer.[52]

Similarly rise in WBC and decreased RBC and Hb levels were found in DEN induced and phenobarbital promoted hepatocellular carcinoma in rats.[53] The reduced RBC count may be caused by destruction of erythrocytes or the consequences of adverse outcome of DEN on the erythropoietic tissue specifically the bone marrow. Reduced level of RBC count and Hb content can be correlated to anemic condition.

Histopathological observation shows the normal architecture with slight central vein and sinusoidal congestion in normal group and showed focal haemorrhage, inflammation, ballooning hepatocyte, centrilobular degeneration, centrilobular necrosis, bile duct proliferation and spotty necrosis in DEN induced group. Treatment with cobalt complex of coumarin Schiff base has found to reduce focal haemorrhage, inflammation and centrilobular necrosis.

Conclusion

In the present study concluded that the admistration of cobalt complex of coumarin schiff base of vanillin showed significant anti-cancer activity which is mediated by decreased level of cancer marker, improvement in various biochemical parameters and sacvening of free radicals. Further cellular and molecular studies is required to establish the exact mechanism of action.

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Conflict of interest: No conflict of interest

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