

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

Ashwini Mallikarjun Rayaji¹, AHM Vishwanathaswamy Agadihiremath^{2,*}

¹PhD Research Scholar, Dept. of Pharmacology chemistry,

²Associate Professor, Dept. of Pharamcology,

KLE University's College of Pharmacy, Vidyanagar, Hubli - 580031

*Corresponding Author

E-mail: vmhiremath2004@gmail.com

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: Hepatocellular 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, Hepatocellular carcinoma, Vanillin – cobalt complex.

Access this article online	
Quick Response Code:	Website: www.innovativepublication.com
	DOI: 10.5958/2394-2797.2016.00001.0

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 ± 2°C 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 initiated 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).

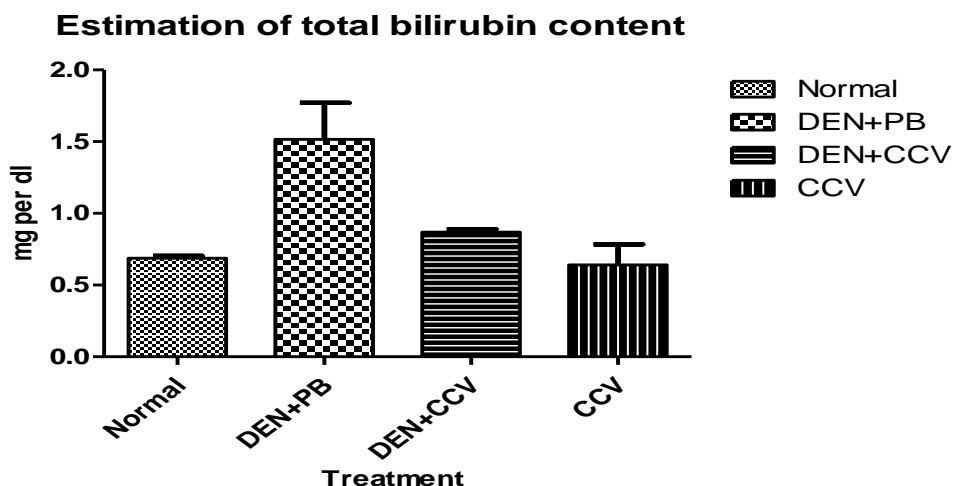


Fig. 1: Effect of metal complex of coumarin Schiff base on total bilirubin level (mg/dL) in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean \pm 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.

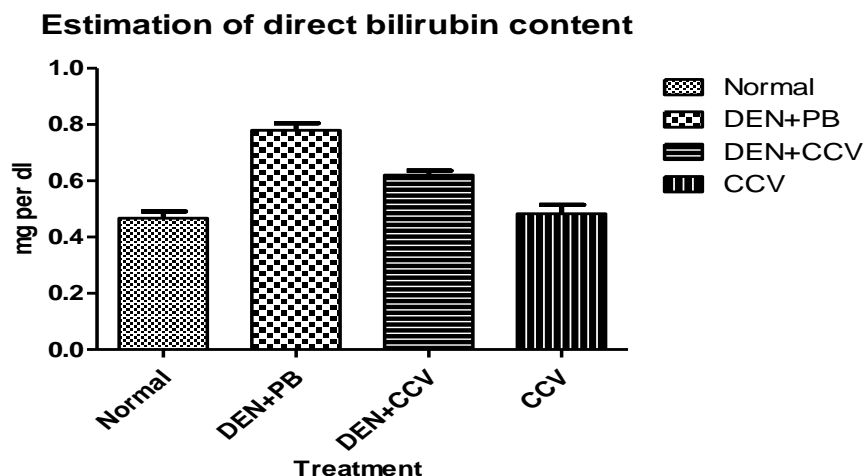


Fig. 2: Effect of metal complex of coumarin Schiff base on direct bilirubin level (mg/dl) in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean \pm 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.

Estimation of Serum glutamic oxaloacetate transaminase

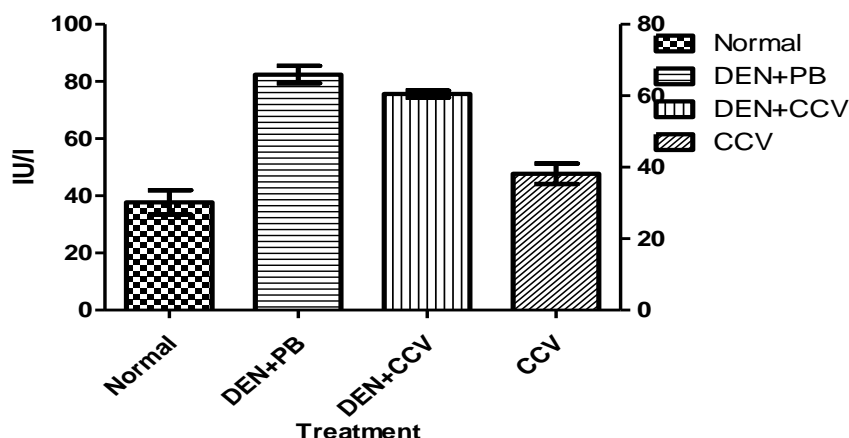


Fig. 3: Effect of metal complex of coumarin Schiff base on serum SGOT level (IU/l) in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean \pm 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.

Estimation of Serum glutamic pyruvic transaminase

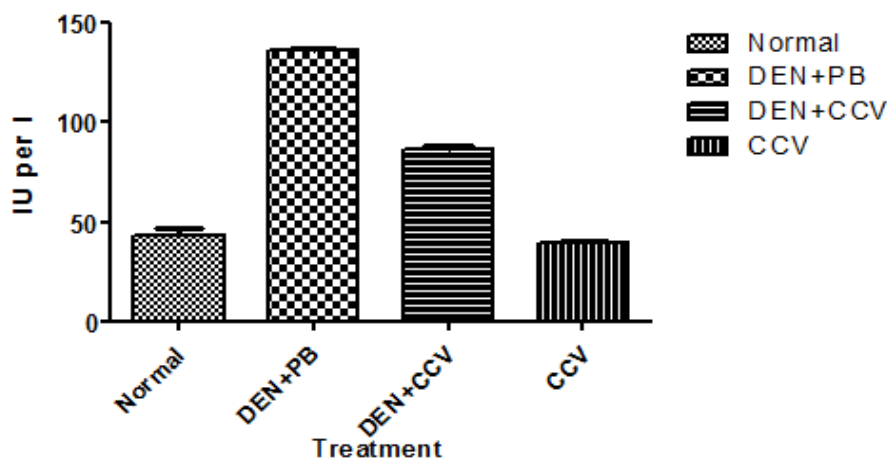


Fig. 4: Effect of metal complex of coumarin Schiff base on serum SGPT level (IU/l) in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean \pm 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 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).

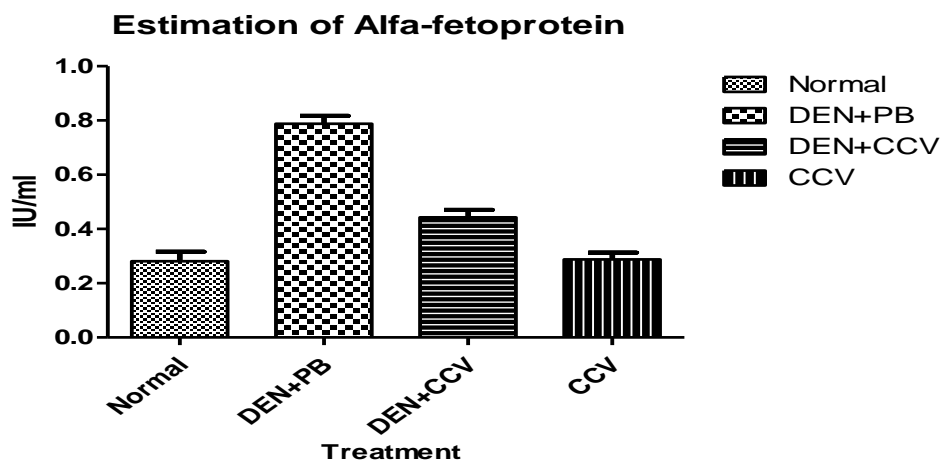


Fig. 5: Effect of metal complex of coumarin Schiff base on serum AFP (IU/l) in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean \pm 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 haemopoietic 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).

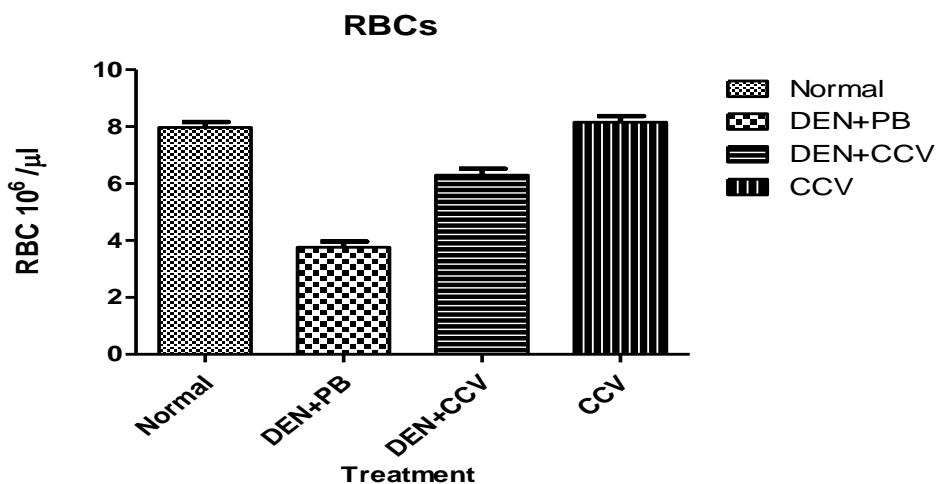


Fig. 6: Effect of metal complex of coumarin Schiff base on blood RBC level in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean \pm 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.

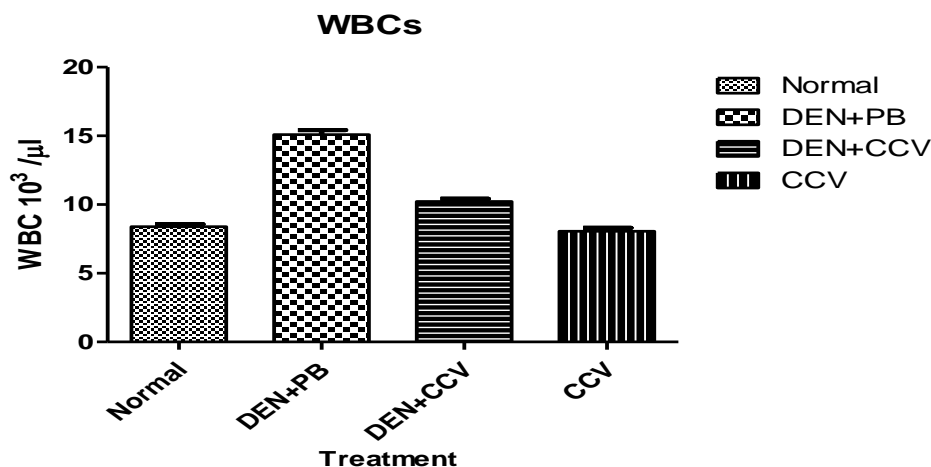


Fig. 7: Effect of metal complex of coumarin Schiff base on a Blood WBC level in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean \pm 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.

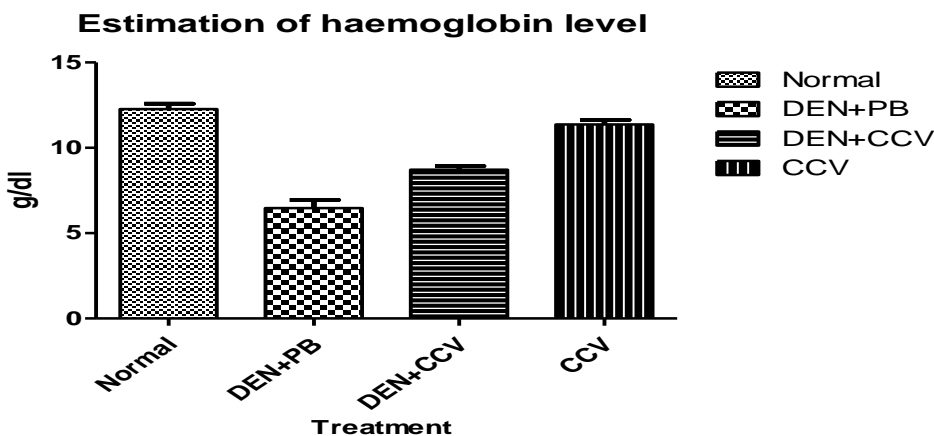


Fig. 8: Effect of metal complex of coumarin Schiff base on Blood Hb level in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean \pm 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).

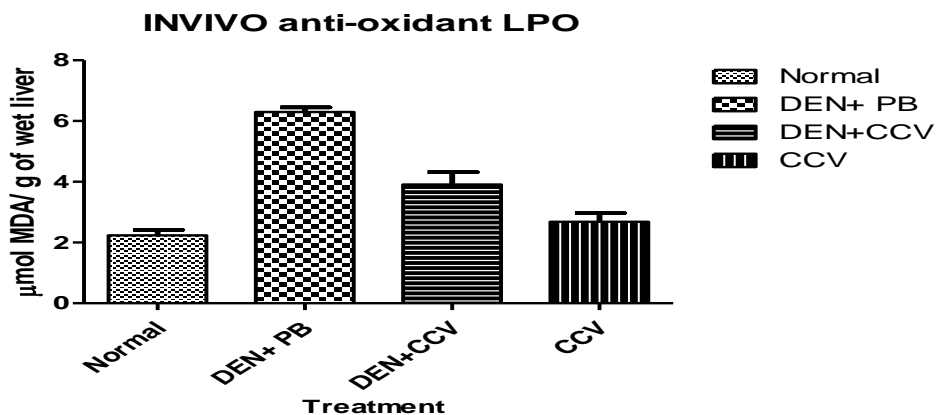


Figure 9: Effect of metal complex of coumarin Schiff base on *In vivo* LPO level in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean \pm 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.

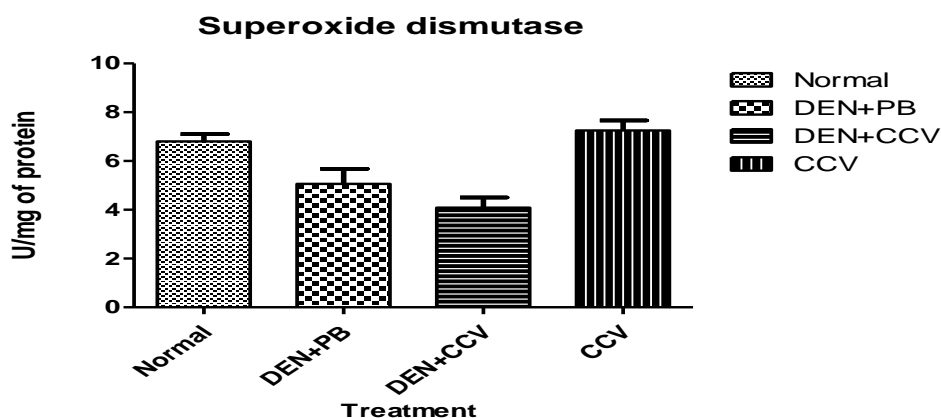


Fig. 10: Effect of metal complex of coumarin Schiff base on SOD level in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean \pm 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.

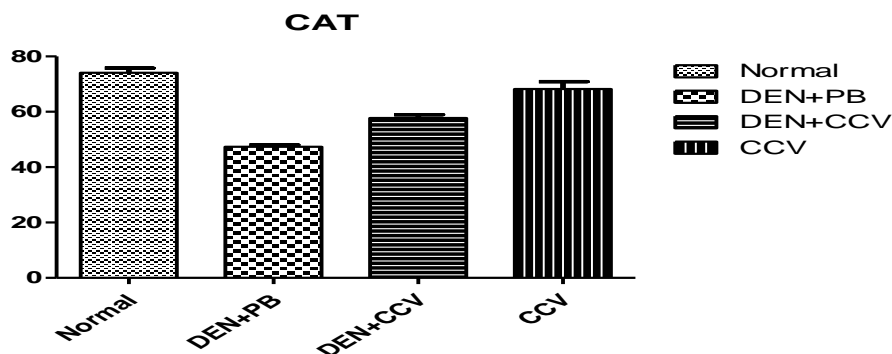


Fig. 11: Effect of metal complex of coumarin Schiff base on CAT level in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean \pm 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.

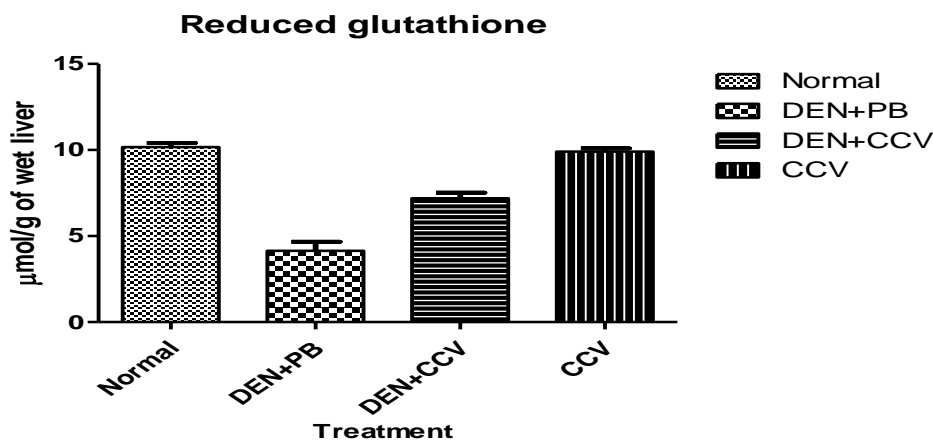


Fig. 12: Effect of metal complex of coumarin Schiff base on GSH level in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean \pm 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.

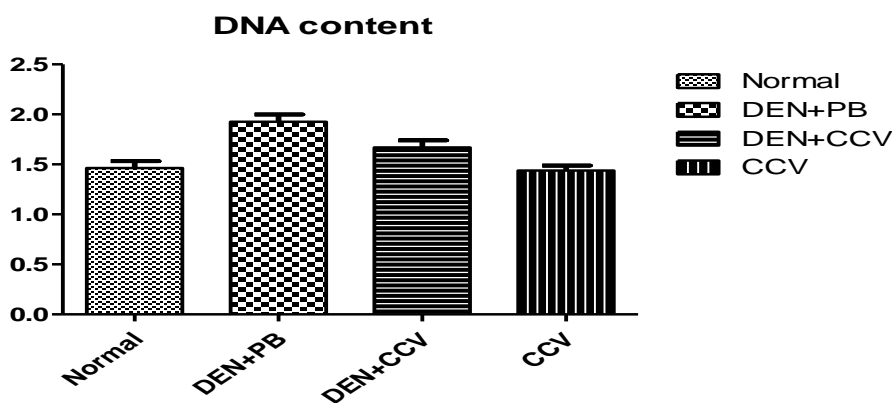
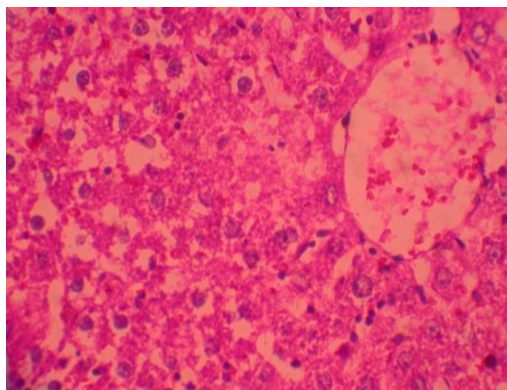


Fig. 13: Effect of metal complex of coumarin Schiff base on DNA level in rats exposed to DEN-induced hepatocellular carcinoma rats

All values were expressed as mean \pm 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.

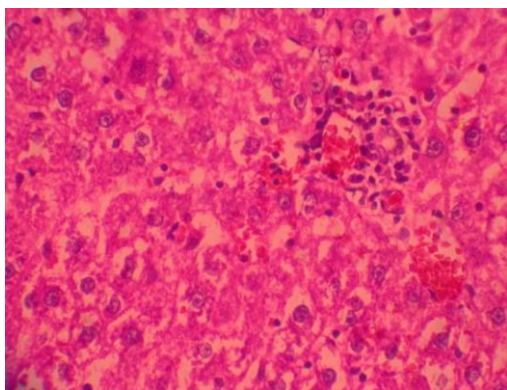
Histopathological study: Histopathological study revealed the effect of DEN and cobalt complex of coumarin Schiff base of vanillin treated liver section in rats that are compared with normal rats.

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



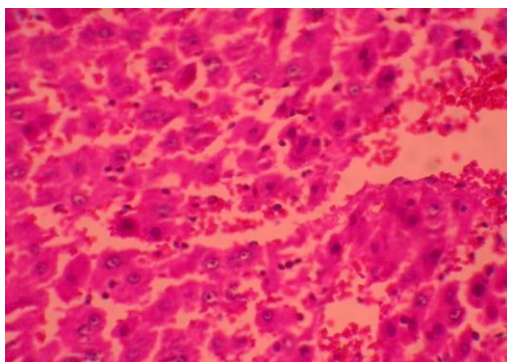
DEN+PB + Drug

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



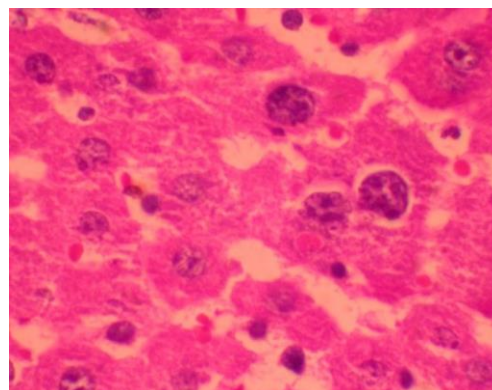
DEN+PB + Drug

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.



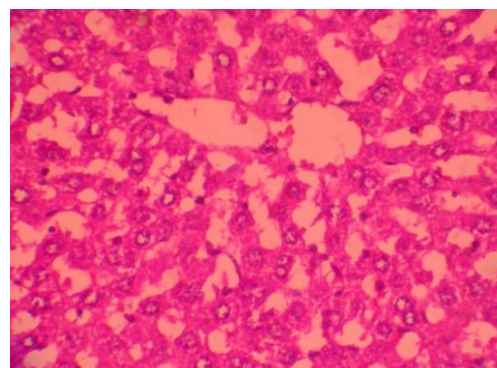
DEN+PB

B 40X Haemorrhage

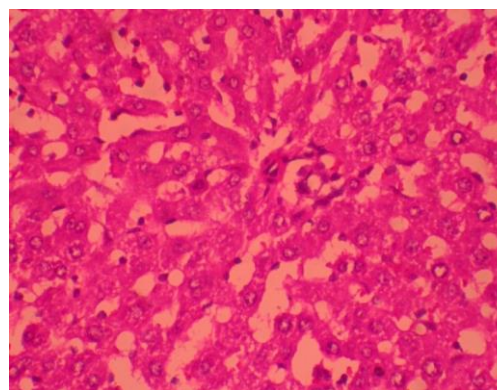


DEN+PB

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.

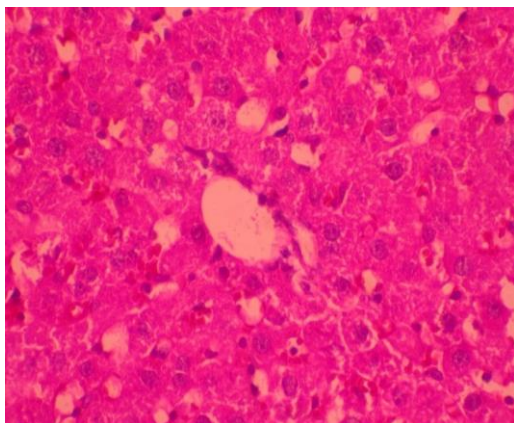


Drug only C 40x

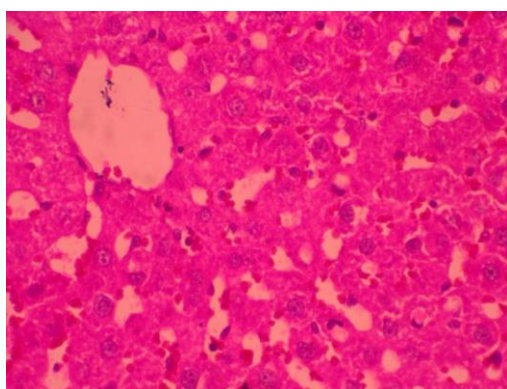


Drug only C 40x

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 chemopreventive 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 possess a chemopreventive role in hepatocellular 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 administration 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 scavenging of free radicals. Further cellular and molecular studies is required to establish the exact mechanism of action.

Acknowledgement: Authors are thankful to Principal, KLE University's College of Pharmacy, Vidyanagar, Hubli for providing necessary facilities to carry out research work.

Conflict of interest: No conflict of interest

Source of Support: Authors are thankful to KLE University Belgaum, Vision group of science and technology, Bangalore and AICTE, New Delhi for providing financial support.

References

- Heindryckx F, Colle I, Van Vlierberghe H. Experimental mouse models for hepatocellular carcinoma research. *International journal of experimental pathology*. 2009 Aug;90(4):367-86.
- Raj Kapoor B, Murugesh N, Chodon D, Sakthisekaran D. Chemoprevention of N-nitrosodiethylamine induced phenobarbital promoted liver tumors in rat by extract of *Indigofera aspalathoides*. *Biological & pharmaceutical bulletin*. 2005 Feb;28(2):364-6.
- Sadik NA, EL-Maraghy SA, Ismail MF. Diethylnitrosamine-induced hepatocarcinogenesis in rats: possible chemoprevention by blueberries. *Afr J Biochem Res*. 2008;2(3):81-7.
- Jennifer M. Phillips Jay I. Goodman. Multiple Genes Exhibit Phenobarbital-Induced Constitutive Active/Androstane Receptor-Mediated DNA Methylation Changes during Liver Tumorigenesis and in Liver Tumors *Toxicol Sci*. 2009;108(2):273-89.
- Love RR, Leventhal H, Easterling DV, Nerenz DR. Side effects and emotional distress during cancer chemotherapy. *Cancer*. 1989;63(3):604-12.
- Failes, T.W. et al. (2007) Studies of a cobalt(III) complex of the MMP inhibitor marimastat: a potential hypoxia-activated prodrug. *Chem. Eur. J*. 13,2974-2982.
- Farrer, N.J. and Sadler, P.J. (2008) Photochemotherapy: targeted activation of metal anticancer complexes. *Aust. J. Chem*. 61,669-674.
- Rosenberg, B., Vancamp, L., Krigas, T., 1965. Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode. *Nature* 205,698-699.
- Kelland, L., 2007b. The resurgence of platinum-based cancer chemotherapy. *Nat. Rev. Cancer* 7,573-584.
- Stewart, D.J., 2007. Mechanisms of resistance to cisplatin and carboplatin. *Crit. Rev. Oncol. Hematol*. 63,12-31.
- Stordal, B., Pavlakis, N., Davey, R., 2007. Oxaliplatin for the treatment of cisplatin-resistant cancer: a systematic review. *Cancer Treat. Rev*. 33,347-357.
- Belaïd S, Landreau A, Djebbar S, Benali-Baitich O, Bouet G, Bouchara JP. Synthesis, characterization and antifungal activity of a series of manganese (II) and copper (II) complexes with ligands derived from reduced N, N'-Ophenylenebis (salicylideneimine). *J. Inorg Biochem*. 2008;102:63-69.
- Rabie UM, Assran ASA, Abou-El-Wafa MHM. Unsymmetrical Schiff base functionalize as base bibasic tetradentate (ONNO) and monobasic trientate (NNO) ligands on complexation with some transition ions. *J MolStruct*. 2008;872:113-122.
- Yilmaz I, Temel H, Alp H. Synthesis, Electrochemistry and insituspectroelectrochemistry of a New Co (III) thio Schiff-base complex with N, N'-Bis(2-aminothiophenol)-1,4-bis (carboxylidene phenoxy) butane. *Polyhedron*. 2008;27:125-132.
- Salavati M, e Niasari, Sobhani A. Ship-in-a-bottle synthesis, characterization and catalytic oxidation of cyclohexane by host (nanopores of zeolite-Y)/ guest (Mn(II), Co(II), Ni(II) and Cu(II) complexes of bis(salicylaldehyde oxaloyldihydrazone) nanocomposite materials. *J Mol Catal (A)*. 2008;285:58-67.
- Z. H. Chohan, S. H. Sumrra, M. H. Youssoufi, and T. B. Hadda, "Metal based biologically active compounds: design, synthesis, and antibacterial/ antifungal/ cytotoxic properties of triazole-derived Schiff bases and their oxovanadium (IV) complexes," *European Journal of Medicinal Chemistry*, 2010;45(7):2739-2747.
- P. G. Cozzi, "Metal-Salen Schiff base complexes in catalysis: practical aspects," *Chemical Society Reviews*, 2004;33:410-421.
- Juan CL, Jie B, Ming MF, Xing LG. Oxidative carbonylation of aniline to N,N'-diphenyl urea catalyzed by cobalt (II) Schiff base complex/pyridine catalytic system. *Catal Commun*. 2008;9:658.
- Ziyadanogullari B, Cevizic D, Temel H, Gullari RZ. Synthesis, characterization and structure effects on pre concentration and extraction of N, N'-bis-(salicylaldehyde)-1,4-bis-(p-aminophenoxy) butane towards some divalent cations. *J Hazard Mater*. 2008;150:285-289.
- Sutar AK, Gupta KC. Catalytic activity of polymer anchored N, N'-bis (o-hydroxyacetophenone) ethylene diamine Schiff base complexes of Fe(III), Cu(II) and Zn(II) ions in oxidation of phenol. *Reactive Funct Polym*. 2008;68:12-26.
- Kalshetty BM, Gani RS, Karabasannavar SS, Kalashetti MB. Synthesis Structural characterization, spectral analysis and antimicrobial activities of Schiff base ligands and their metal complexes derived from 3-aldehydosalicylic acid. *Glob J Sci Frontier Res Chem*. 2013;13:29-37.
- Rudnicka W, Foks H, Jano M, Wiec, Zwolsk, Wiek K. Studies on antibacterial activities of Schiff base compounds. *ActaPol.Pharm*. 1986;43:523.
- Holla BS, Veerendra B, Shivanada MK, Poojary B. Synthesis characterization and anticancer activity studies on some Mannich bases derived from 1,2,4-triazoles. *Eur J Med Chem*. 2003;38:759-767.
- Mullican MD, Wilson MW, Connor DT, Kostlan CR, Schrier DJ, Dyer RD. Design of 5-(3,5-di-tert-butyl-4-hydroxyphenyl)- 1,3,4-thiadiazoles, -1,3,4-oxadiazoles, and -1,2,4-triazoles as orally-active, non-ulcerogenic anti-inflammatory agents. *J Med Chem*. 1993;36:1090-1099.
- Mir I, Siddiqui MT, Comrie. Antituberculosis agents: a-[5-(2-Furyl)-1,2,4-triazol-3-ylthio] acetylhydrazide and related compounds. *Tetrahedron*. 1970;26:5235-5238.
- Pujar GV, Purohit MN, Synesh. Synthesis and pharmacological activities of 1,2,4-triazol-3-yl thiols and related systems. *Indian J Heterocyclic Chem*. 2009;19:171.
- Yale HL, Piala JJ. Substituted s-triazoles and related compounds. *J Med Chem*. 1966 Jan;9:42e46.
- Hamilton DE, Drage RS, Zombecki A. Mechanistic studies on the cobalt(II) Schiff base catalyzed oxidation of olefins by O₂. *Am Chem Soc*. 1987;109:374-379.
- Kalshetty BM, Giraddi TP, Pattar RT, Gani Ramesh, Kalashetti MB. Synthesis, characterization and thermodynamic studies of some organometallic complexes of bidentate Schiff-bases having N- and S- donor system. *J Chem Bio PhysSci Section A*. 2012;2:1206-1217.
- Nawrot-Modranka J, Nowrot E, Graczik J. In vivo antitumor, in vitro antibacterial activity and alkylating properties of phosphorohydrazone derivatives of coumarin and chromone. *Eur J Med Chem*. 2006;41:1301-1309.

31. Yu D, Suzuki M, Xie I, Netschke SI, Lee KH. Recent progress in the development of coumarin derivatives as potent anti-HIV agents. *J. Med Res Rev.* 2003;23:322.
32. Korti N, Kecabalkanli A, GURSOYATES A. Studies on antitubercular activities of some Schiff base compounds. *Formica.* 2002;57:589.
33. Koster R, Anderson M, Debeer E, Acetic acid for analgesic screening. *Fed Proc Fed Am SocExpBiol* 1959;18:412-413.
34. Kostova I, Trendafilova N, Momekov G. Theoretical, spectral characterization and antineoplastic activity of new lanthanide complexes. *J Trace Elem Med Biol.* 2008;22:100-111.
35. Jiang ZY, Hunt JY, Wolff SP. Detection of lipid hydroperoxides using the fox method. *Anal Biochem* 1992;202:384-9.
36. Kakkar P, Das B, Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase. *Ind J Biochem Biophys* 1984;21:130-2.
37. Sinha AK. Colorimetric assay of catalase. *Anal Biochem* 1972;47:389-94.
38. Ellman GL. Tissue sulphhydryl groups. *Arch Biochem Biophys* 1959;82:70-7.
39. Burton K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochemical journal.* 1956;62(2):315.
40. Organisation for economic co-operation and development. Revised draft guidelines 423. *OECD Guidelines for testing of chemicals.* Revised document. October 2000. p:1-26.
41. Anticancer activity invivoYoshijil H, Nakael D, Kinugasal T, Matsuzaki M, Dendal A, Tsujii T, Konishi Y. Inhibitory effect of dietary iron deficiency on the induction of putative preneoplastic foci in rat liver initiated with diethylnitrosamine and promoted by phenobarbital. *British J cancer.* 1991;649:839-42.
42. Bispo W, Alexandre-Moreira MS, Anayive HB, Rebolledo P, Parrilha GL, Castellano EE, Barreiro EJ, Lima LM. Analgesic and Anti-Inflammatory Activities of Salicylaldehyde 2-Chlorobenzoyl Hydrazone (H₂LASSBio-466), Salicylaldehyde 4-Chlorobenzoyl Hydrazone (H₂LASSBio-1064) and Their Zinc(II) Complexes. *Molecules.* 2011;16:6902-6915.
43. Tamba BI, Jaba I, Ionescu D, Mungiu OC. Systemically administered cobalt- pharmacological data regarding an antinociceptive action. *Ther, PharmacolClinToxicol.* 2009;13:73-76.
44. Verna L, Whysner J, Williams GM. N-nitrosodiethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation. *Pharmacology & therapeutics.* 1996;71(1):57-81.
45. PitoSt HC, Campbell HA, Maronpot R, Bawa N, Rizvi TA, Xu YH, et al. Critical parameters in the quantitation of the stages of initiation, promotion, and progression in one model of hepatocarcinogenesis in the rat. *Toxicologic pathology.* 1989;17(4):594-611.
46. Archer MC. Mechanisms of action of N-nitroso compounds. *Cancer surveys.* 1989;8(2):241-50.
47. Nakae D, Kobayashi Y, Akai H, Andoh N, Satoh H, Ohashi K, et al. Involvement of 8-hydroxyguanine formation in the initiation of rat liver carcinogenesis by low dose levels of N-nitrosodiethylamine. *Cancer research.* 1997 Apr 1;57(7):1281-7.
48. Brown JP. A review of the genetic effects of naturally occurring flavonoids, anthraquinones and related compounds. *Mutation research.* 1980 May;75(3):243-77.
49. Ramakrishnan G, Augustine TA, Jagan S, Vinodhkumar R, Devaki T. Effect of silymarin on N-nitrosodiethylamine induced hepatocarcinogenesis in rats. *Experimental oncology.* 2007 Mar;29(1):39-44.
50. Nair KG, Deepadevi KV, Arun P, Kumar VM, Santhosh A, Lekshmi LR, et al. Toxic effect of systemic administration of low doses of the plasticizer di-(2-ethyl hexyl) phthalate [DEHP] in rats. *Indian journal of experimental biology.* 1998 Mar;36(3):264-72.
51. Rajkapoor B, Jayakar B, Muruges N, Sakthisekaran D. Chemoprevention and cytotoxic effect of Bauhinia variegata against N-nitrosodiethylamine induced liver tumors and human cancer cell lines. *Journal of ethnopharmacology.* 2006 Apr 6;104(3):407-9.
52. Taketa K. α -fetoprotein: Reevaluation in hepatology. *Hepatology.* 1990;12(6):1420-32.
53. Althaf Faimum D S. Influence of Vitex Leucoxylyon Linn on Oxidative Stress And Hepatocarcinogenesis Induced By Diethylnitrosamine And Phenobarbital In Rats. *International Journal of Toxicological and Pharmacological Research.* 2012-13;4(4):96-107.