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Research Article

Influence of *Vitex Leucoxylon* Linn on Oxidative Stress And Hepatocarcinogenesis Induced By Diethylnitrosamine And Phenobarbital In Rats

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ABSTRACT

A medium term bioassay of ethanolic extract of *Vitex leucoxylon* Linn (EVL) was evaluated for its anti-oxidant and anti-hepatocarcinogenic activity against Diethylnitrosamine (DEN) and Phenobarbital sodium (PB) against male Wister rats. The DEN given with single dose administration of 200mg/kg/i.p. and progression of cancer was promoted by PB at dose of 0.05% p.o./day in drinking water. After 6 weeks, on treatment with EVL at high dose showed significant increase in food intake, water intake, body weight and decrease in liver weight as that of 5- FU than low dose. Haematological parameters like Hb, Neutrophils, Monocyte, Lymphocyte, PCV showed significant increase with EVL at high dose but Total WBC and ESR showed significant decrease in EVL treated rats as that of 5- FU than low dose when compared to DEN+PB control. Serum biochemical parameters showed significant decrease in SGOT, SGPT, SALP, Urea, Creatinine, Bilirubin, Cholesterol, TGL and showed significant increase in Total protein, HDL in EVL treated rats as that of 5- FU than low dose when compared to DEN+PB control. Anti-oxidant study was performed in liver homogenate which showed decrease in LPO levels and increase in SOD, CAT, GPx, GR, GST, GSH in EVL treated rats as that of 5- FU than low dose when compared to DEN+PB control. Histopathological studies of rat liver in EVL treated liver showed protective effects in higher dose treated group with no much loss in cell architecture as that of standard than the lower dose treated group when compared to DEN+PB control rats. Hence it can be concluded that ethanolic extract of *Vitex leucoxylon* Linn possesses significant anti-hepatocacinogenic activity in rats.

Keywords: Ethanolic extract of *Vitex leucoxylon* Linn, Diethylnitrosamine, Phenobarbital, anti-oxidant, anti-hepatocarcinogenic.

INTRODUCTION

Hepatocellular carcinoma (HCC) constitutes about 85% of primary liver cancer [1]. Liver cancer is the fifth most common malignancy in men and the eighth in women [2] and around 440,000 new cases of HCC occur annually, accounting for around 5.5% of all human cancer incidence [3] with a high mortality rate [4]. Liver diseases often progress from subclinical icteric hepatitis to necroinflammatory hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma [5,6].

The major risk factors of HCC may be age, gender, hepatitis B [7], hepatitis C [8], alcohol consumption [9], hormone exposure, haemochromatosis [10], vinyl chloride, arsenic poisoning, aflatoxin B_1, obesity, diabetes [11], renal transplant patients, tobacco smoking and parasitic infections such as clonorchiasis and schistosomiasis [12]. These above risk factors may result to liver damage such as cirrhosis [13] which is major cause for HCC.

Natural products have been traditionally accepted as remedies due to popular belief that they present minor adverse effects. Therefore, understanding the potential beneficial or adverse influence of natural products used by human populations is important in implementing safety measures for public health. The over-the-counter medications like NSAIDs, acetaminophen leads to liver cirrhosis. This is the major cause for the development of HCC and so the herbal medicines are mostly used in the treatment of liver damage.

In general, hepatic chemical carcinogenesis is a multistep process in experimental animals [14]. DEN is a powerful environmental hepatocarcinogen that has been used as an initiating agent for hepatocarcinogenic activities [15]. It has been proven that metabolic bioactivation of cytochrome P_{450} enzymes to reactive electrophiles is required for initiation of their cytotoxic, mutagenic and carcinogenic activity [16] by the help of nitrosamines with hepatocyte proliferation following cell necrosis [17], hyperplasia, hyperbasophilia and tumour. The biotransformation of DEN resulting in oxidative stress [18] alters the structure of DNA and forming alkyl DNA adducts [19]. Chemical agents inducing hepatocarcinogenesis have been administrated either as DEN alone or in combination with acetylaminofluorene (AAF), ortic acid, phenobarbital, benzopyrene, N-amyl-N- methylnitrosamine and CCl4 [20]. Phenobarbital is an antiepileptic drug that promotes hepatocarcinogenesis in rodents when administered subsequent to an initiating carcinogen like DEN [21].

Vitex leucoxylon Linn commonly known as five-leaved chaste tree belongs to the family Verbenaceae which is an important medicinal plant generally found on the banks of

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river, streams and ponds throughout India. Although more studies are necessary, Vitex exhibits proven potential to become of important pharmacological interest [22]. The plant *Vitex leucoxylon* Linn was selected for this present study as it was used as folkloric medicine in India for the treatment of cancer [23]. The phyto-constituents such as Vitexin, agnoside, aucubin and beta- sitosterol, iridiod compounds A & B were present in the plant. The major compound such as vitexin which is a flavanoid is mainly responsible for the anti-cancer activity. These dietary constituents mostly act as anti-oxidants and may prevent from DNA damage.

Since the increase in the use of synthetic chemicals in cancer therapy has lead to many side effects and undesirable hazards, there is worldwide trend to go back to natural resources (medicinal plants) which are therapeutically effective, culturally acceptable and economically within the research of the poor people [24]. Hence the present study is an effort to ascertain the anti-hepatocarcinogenic and anti-oxidant potential of *Vitex leucoxylon* Linn against DEN (initiator) with PB (promoter) induced rats.

MATERIALS AND METHODS

Chemicals: The Diethylnitrosamine and Phenobarbital sodium was purchased from Sigma Chemicals, USA. All chemicals and reagents used were of analytical grade.

Plant Material: The fresh leaves of *Vitex leucoxylon* Linn was collected during the month of September 2010 from Tirunelveli district, Tamil Nadu, India. The plant was identified and authenticated by Botanist, V. Chelladurai, C.C.R.A.S.Govt. of India, Tirunelveli. A voucher specimen has been deposited at C.L.Baid Metha College of Pharmacy for future reference.

Preparation of Plant Extract: Fresh leaves were collected and shade dried. Dried leaves were crushed and powdered coarsely with an electronic blender and about 200g of this powder was macerated with 95% ethanol separately for 72h at room temperature with stirring for every 15min. The ethanolic extract of *Vitex leucoxylon* Linn (EVL) leaves was then evaporated on heating mantle at 60ºC till the semisolid mass was obtained and was stored in airtight containers in refrigerator below 10ºC and measured the yield of the extract. The percentage yield of EVL was found to be 14% w/v respectively. EVL extract showed the presence of various phytochemical constituents such as carbohydrate, flavonoid, flavones, protein, steroids, mucilage, phenol and glycoside. The ethanolic EVL was freshly suspended in distilled water before use for further studies.

Animals: Healthy male Wister rats (150-250g) were procured from C.L.Baid Metha College of Pharmacy, Chennai. The animals were acclimatized and divided into groups (six per cage) with rice husks for bedding and maintained standard laboratory conditions at a temperature of 25± 20º C and maintained on 12-h light: 12-h dark cycle. They were provided with regular rodent chow (Lipton India Ltd., Mumbai, India) and drinking water *ad libitum*. The animal care and experimental protocols were in accordance with Institutional Animal Ethical Committee (IAEC/XXX/10/CLBMCP- 2010-DATED 22-9-10).

Experimental Design

Induction of Carcinogens: The animals were randomly allocated into five groups with six animals in each group. GROUP I animals received normal saline 0.9% were referred as normal control. GROUP II, III, IV andV animals received i.p. a single dose administration of diethylnitrosamine (DEN) at 200 mg/kg b.w. After 2 weeks Phenobarbital (PB) 0.05% p.o was incorporated in drinking water daily for up to 4 weeks to GROUP II, III, IV and V rats. All rats were subjected to two-thirds partial hepatectomy at week 3. After administration of carcinogen up to 6 weeks the animals were administered with higher and lower doses of EVL extract 200 mg/kg p.o to GROUP IV rats and 400 mg/kg p.o to GROUP V rats until 2 weeks continuously. The efficacy of plant extracts was compared with 5-Fluorouracil (5-FU 20mg/kg/day i.p.) which is commonly used as an active anti cancer agent in vast series of preclinical and clinical studies [25]. 5-FU was administered to GROUP III animals up to 2 weeks similar to treatment groups. During the entire experimental period of 60 days, the food intake, water intake and body weight change of animals was recorded.

At the end of the experimental period, all the animals were sacrificed at the end of 8th week by cervical decapitation. The absolute and relative liver weights [26] of animals were recorded.

$$\text{RelativeLiver Weight} = \frac{\text{Absolute Liver weight(g)}}{\text{Body Weight of rat on sacrifice day (g)}} \times 100$$

GROUP I : Normal Control (0.9 % saline, p.o)

GROUP II : DEN+PB Control [DEN (200 mg/kg, i.p) single dose + PB (0.05% in drinking water, p.o, 4 weeks)]

GROUP III : Standard group [DEN + PB + 5-FU (20 mg/ kg, i.p for 2 weeks)]

GROUP IV : Low dose group [DEN + PB + Lower doses EVL (200 mg/kg, p.o for 2 weeks)]

GROUP V : High dose group [DEN + PB + Higher doses EVL (400 mg/kg, p.o for 2 weeks)]

Haematological activity: On the day of sacrifice blood was obtained from retro-orbital plexus and following parameters was estimated.

Biochemical Estimation: Blood samples were collected into clean non-heparinised bottles and allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2500 rpm at 30 ºC for 15 min. The liver was immediately excised, weighed and homogenised in 0.1M ice-cold Tris-HCl buffer (pH 7.4) to give 10% homogenate [27].

Anti-oxidant activity: After the collection of blood

Table 1: Results of Food intake, Water intake , Body and Liver weights of rats.

Groups	Food Intake (gm/day)		Water Intake (ml/day)		Body weight (g)		Liver weight (%)	
	Initial	Final	Initial	Final	Initial	Final	Absolute	Relative
Group I	19.27±0.08	21.26±0.69	23.47±0.80	25.22±0.31	216.25±6.88	228.25±6.88	8.25±0.01	3.82±0.12
Group II	17.78±0.09 a***	12.45±0.06 a***	17.65±0.63 a***	11.61±0.82 a***	172.50±3.22 a***	162.50±1.44 a***	4.40±0.16 a***	2.68±0.07 a***
Group III	18.28±0.07 a***	20.35±0.29 b***	17.9±0.75 a***	24.34±0.43 b***	181.25±4.26 a***	198.75±4.26 b***	7.78±0.06 a*b***	4.03±0.16 b***
Group IV	16.50±0.21 a***	18.30±0.15 a***	18.27±0.72 a***	19.72±0.68 a** b**	182.50±3.22 a***	177.50±3.22 a***b*	4.98±0.07 a***b**	2.80±0.06 a***
Group V	18.20±0.10 a***	20.28±0.23 b***	17.87±0.60 a***	22.66±0.43 a*b***	180±5.4 a***	190±2.04 b***	5.43±0.11 a***b***	2.85±0.09 a***

Table 2: Results of Haematological activity

Groups	Haematological Prameters							
	Hb (g/dL)	Total RBC (million cells/cu.mm)	Total WBC (thousand cells/cu.mm)	Neutrophil (%)	Lymphocyte (%)	Monocyte (%)	PCV (%)	ESR (mm/hr)
Group I	11.58±0.10	7.72±0.04	7.75±0.08	3.47±0.15	6.69±0.13	1.89±0.03	53.57±0.13	3.82±0.03
Group II	8.64±0.14 a***	4.21±0.04 a***	9.76±0.09 a***	1.63±0.09 a***	3.07±0.05 a***	0.29±0.02 a***	47.49±0.18 a***	8.27±0.02 a***
Group III	11.11±0.04 a*b***	6.90±0.05 a*b***	8.01±0.08 b***	3.06±0.04 a*b***	6.23±0.07 a**b***	1.60±0.07 a**b***	52.80±0.11 a*b***	3.99±0.04 a*b***
Group IV	9.08±0.13 a***b**	5.24±0.08 a***b**	9.15±0.05 a***b**	2.05±0.03 a***b**	4.89±0.05 a***b**	0.80±0.03 a***b**	48.54±0.12 a***b**	6.59±0.03 a***b**
Group V	10.44±0.10 a***b***	6.43±0.09 a***b***	8.14±0.06 a***b***	2.96±0.06 a***b***	6.03±0.04 a***b***	1.17±0.03 a***b***	50.44±0.09 a***b***	4.41±0.07 a***b***

The values are expressed as mean ± SEM. Statistical significance test for comparison was done by ANOVA, followed by Dunnet's t test. n=6

*a- Group II, III, IV, V are compared with Group I. *** p<0.001, ** p< 0.01, * p<0.05*

*b- Groups III, IV, V are compared with group II *** p<0.001, ** p< 0.01, * p<0.05*

samples, the rats were sacrificed. Then their liver was excised, rinsed in ice-cold normal saline followed by cold 0.15 M Tris-HCl (pH 7.4), blotted dry, and weighed. A 10% w/v homogenate was prepared in 0.15 M Tris-HCL buffer; a portion was utilized for the estimation of lipid peroxidation [28], and a second portion, after precipitating proteins with TCA, was used for the estimation of Glutathione Reductase [29], Glutathione Peroxidase [30], Reduced Glutathione [31]. The rest of the homogenate was centrifuged at 1500 rpm for 15 min at 4^0C. The supernatant thus obtained was used for the estimation of Superoxide Dimutase [32], Catalase [33].

Histopathological Studies: Liver tissues were excised, weighed and examined macroscopically on the surface cross section for gross visible persistent nodules. The persistent nodules were identified from reddish brown by their greyish white colour and sharp demarcation. Representative sections of right, left and caudate lobes of

Table 3: Results of Biochemical activity

Groups	Biochemical Prameters SGOT (IU/L)	SGPT (IU/L)	SALP (IU/L)	Serum Urea (mg/dL)	Serum Total Protein (g/dL)	Serum Creatinine (mg/dL)	Serum Bilirubin (mg/dL)	Total Cholesterol (mg/dL)	TGL (mg/dL)	HDL (mg/dL)
Group I	70.33±0.24	59.64±0.19	73.20±0.16	30.18±0.11	6.60±0.11	0.77±0.07	1.07±0.03	89.15±0.45	85.26±0.16	37.78±0.10
Group II	184.32±0.14 a***	142.08±0.10 a***	123.17±0.14 a***	46.56±0.52 a***	4.43±0.15 a***	1.81±0.06 a***	2.81±0.06 a***	131.98±0.35 a***	173.35±0.15 a***	24.43±0.12 a***
Group III	71.47±0.21 a**b***	60.61±0.31 b***	74.20±0.10 a*b***	29.40±0.05 b***	6.66±0.13 b***	0.75±0.04 b***	1.15±0.02 b***	92.44±0.45 a***b***	86.33±0.38 a*b***	36.52±0.11 a***b***
Group IV	113.30±0.15 a***b**	92.34±0.27 a***b**	95.33±0.16 a***b**	44.36±0.18 a***b**	5.41±0.15 a***b**	1.36±0.03 a***b**	1.75±0.05 a***b**	124.85±0.38 a***b**	129.37±0.18 a***b**	29.07±0.23 a***b**
Group V	83.32±0.17 a***b***	69.65±0.45 a***b***	78.16±0.38 a***b***	31.45±0.16 a***b***	6.51±0.08 b***	0.94±0.05 a*b***	1.29±0.01 a**b***	101.12±0.24 a***b***	95.78±0.13 a***b***	34.66±0.12 a***b***

RESULTS

Table 4: Results of antioxidant activity

Groups	Anti oxidant activity LPO (nmoles/mg protein)	SOD (U/mg protein)	CAT (units/min/mg protein)	GPx (µ/mg protein)	GR (units/min/mg protein)	GST (units/min/mg protein)	GSH (units/min/mg protein)
Group I	1.16±0.06	7.39±0.20	58.18±0.52	70.85±0.01	35.50±0.24	10.65±0.20	48.21±0.14
Group II	3.38±0.10 a***	3.54±0.23 a***	36.92±0.67 a***	40.57±0.36 a***	19.15±0.27 a***	6.39±0.16 a***	20.19±0.19 a***
Group III	1.04±0.08 b***	6.89±0.12 a**b***	53.49±0.42 a***b***	69.19±0.43 a*b***	32.28±0.24 a***b***	10.39±0.05 b***	47.35±0.09 a**b***
Group IV	1.77±0.09 a**b**	4.19±0.10 a***b*	43.79±0.28 a***b**	48.92±0.28 a***b**	25.64±0.24 a***b**	7.02±0.15 a***b**	29.48±0.18 a***b**
Group V	1.12±0.07 a**b***	5.46±0.13 a***b***	51.24±0.48 a***b***	61.09±0.40 a***b***	30.32±0.27 a***b***	9.21±0.05 a***b***	41.72±0.12 a***b***

The values are expressed as mean ± SEM. Statistical significance test for comparison was done by ANOVA, followed by Dunnet's t test. n=6

a- Group II, III, IV, V are compared with Group I. *** p<0.001, ** p< 0.01, * p<0.05

*b- Groups III, IV, V are compared with group II *** p<0.001, ** p< 0.01, * p<0.05*

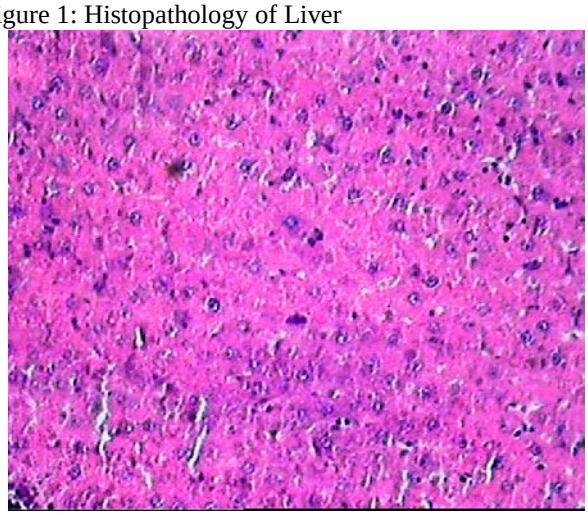
liver were fixed in formalin saline. Histological sections were processed using (H & E) haematoxylin and eosin stains. The following was seen

Tumour incidence: The nodules were round and were identified by their greyish white colour with sharp demarcation from the surrounding parenchyma which is reddish brown and non-nodular was noted [34].

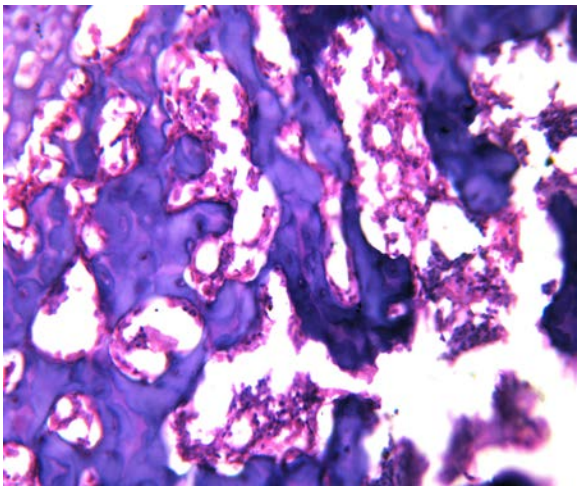
Tumour incidence = No. of rats with nodules / Total number of rats

The percentage yield HAVL and EVL were found to be 18 and 14% w/v respectively. The preliminary phytochemical analysis of leaves of HAVL extract showed presence of carbohydrates, steroids, flavanoids, flavones, mucilage and EVL extract showed the presence of various phytochemical constituents such as carbohydrate, flavonoid, flavones, protein, steroids, mucilage, phenol and glycoside.

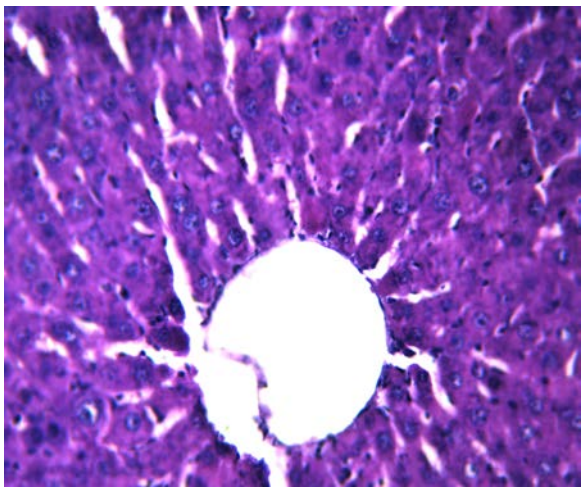
Figure 1: Histopathology of Liver



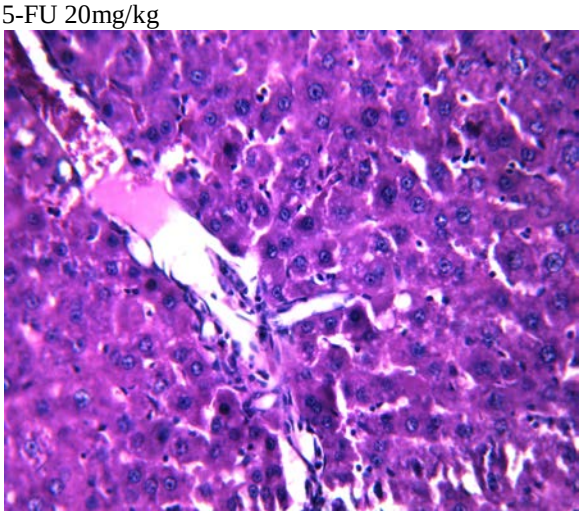
a. Normal Control



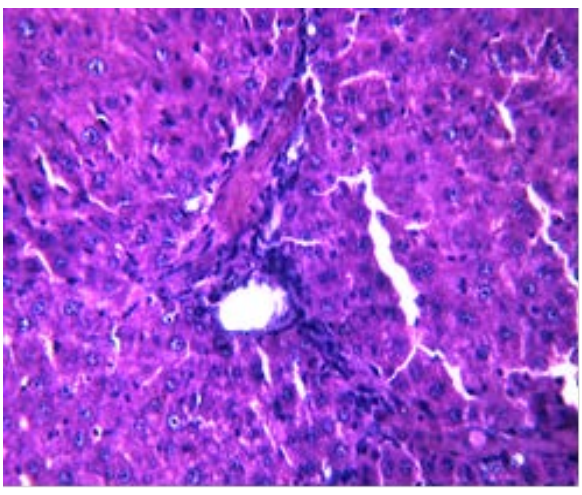
b. DEN+PB control



c. 5-FU 20mg/kg



d. EVL 200mg/kg



e. EVL 400mg/kg

The carcinogen treated group II rats (DEN+PB) showed significant decrease in food and water intake whereas group III (standard 5-FU) ($p<0.001$) and the low and high dose EVL treatment group IV($p<0.05$) and group V ($p<0.001$) showed significant increase.

Body and liver weights was significantly decreased in group II rats (DEN+PB) and treatment with group III (standard 5-FU) ($p<0.001$) and EVL at the doses of 200 and 400 mg/kg, group IV ($p<0.05$) and group V ($p<0.001$) significantly increased body and liver weight. (Table 1).

Table 2 shows the haematological conditions of blood collected on day of sacrifice in control and treated rats. The group II rats (DEN+PB) exhibited significant

decrease in Hb,RBC, differential WBC, lymphocytes, monocytes and PCV ($p<0.001$) and showed significant increase in total WBC and ESR ($p<0.001$). The group III (standard 5-FU) ($p<0.001$), group IV ($p<0.05$) and group V ($p<0.001$) exhibited significant increase in Hb,RBC, differential WBC, lymphocytes, monocytes and PCV. And showed significant decrease in total WBC and ESR.

Table 3 shows biochemical parameters of serum in control and treated animals. The group II (DEN+PB), group IV ($p<0.05$) and group V ($p<0.001$) rats exhibited significant increase in SGOT, SGPT, SALP, serum urea, serum creatinine, serum bilirubin, total cholesterol, TGL and significant decrease in HDL, total protein ($p<0.001$). The group III (standard 5-FU) ($p<0.001$) exhibited significant decrease in SGOT, SGPT, SALP, serum urea, serum creatinine, serum bilirubin, total cholesterol, TGL and significant increase in HDL, total protein.

Table 4 shows the antioxidant status of the haemolysate in control and experimental animals. The group II rats (DEN+PB) showed significant decrease in SOD, CAT, GPx, GR, GST, GSH and significant increase in LPO. The group III (standard 5-FU) ($p<0.001$), group IV ($p<0.05$) and group V ($p<0.001$) exhibited significant increase in SOD, CAT, GPx, GR, GST, GSH and significant decrease in LPO.

Histopathological examination of group I (normal) showed uniformly arranged liver plates with oval hepatocytes of uniform size (Fig 1a). The group II (DEN+PB) treated rats showed congestion, necrosis, and infiltration of inflammatory cells and loss of architecture due to cirrhosis (Fig 1b). The group III (standard 5-FU) Standard drug 5-FU showed protective effect with less damage of hepatocytes (Fig 1c). Treatment groups IV and V with EVL at the doses of 200 mg/kg showed moderate effect on damage liver and 400 mg/kg showed protective effect with less damage of hepatocytes (Fig 1d; Fig 1e). However, in some areas degenerating hepatic cells were detected. Tumour incidence was found in DEN+PB control rats 2 to 3 nodules in greyish white colour in 5 rats, with parenchyma which is reddish brown in colour and rough surface following demarcation found shows cirrhosis (Fig 2a). In EVL treated and standard rat liver showed no nodules with smooth outer surface (Fig 2b).

1 a. H & E stained sections show liver with normal hepatocyte cells. **1b.** H & E stained sections show liver with congestion, necrosis, and infiltration of inflammatory cells and loss of architecture due to cirrhosis. **1c.**H & E stained sections show liver with less infiltration of inflammatory cells, no evidence for congestion and necrosis, no loss of architecture. **1d.** H & E stained sections show liver with moderate infiltration of inflammatory cells, no evidence for congestion and necrosis, no loss of architecture. **1e.**H & E stained sections show liver with less infiltration of inflammatory cells, no evidence for congestion and necrosis, no loss of architecture

2a. The cancer induced liver showed presence of 2 to 3 nodules in greyish white colour, with parenchyma which is reddish brown in colour. Rough surface found shows cirrhosis.**2b.** EVL treated rat liver with nodules absent and shows smooth surface.

DISCUSSION

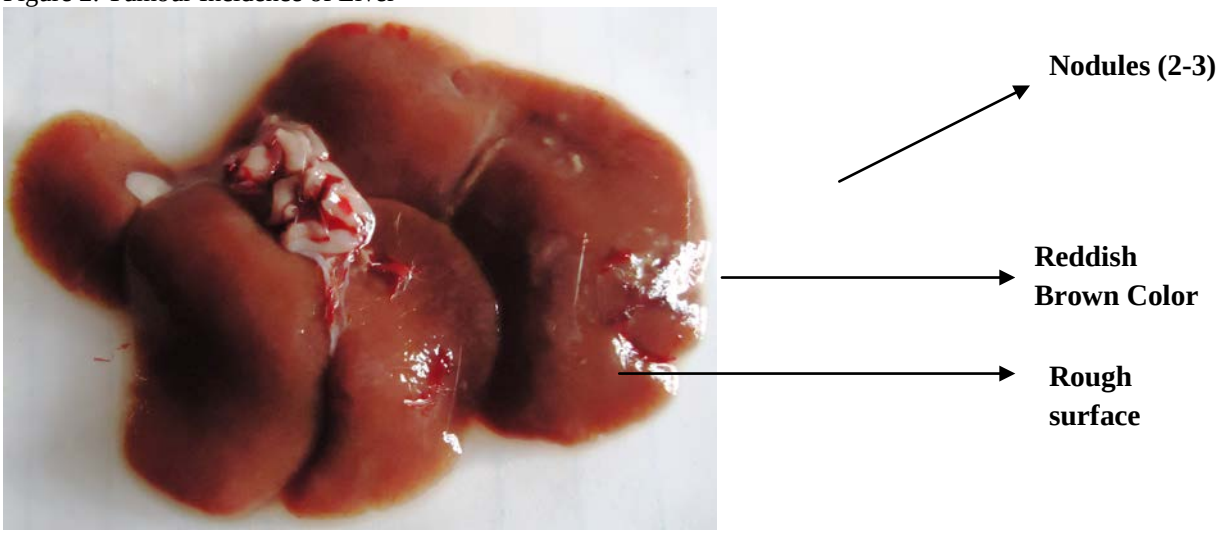
Hepatocellular carcinoma (HCC) constitutes about 85% of primary liver cancer. Liver diseases often progress from subclinical icteric hepatitis to necroinflammatory hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma [5,6]. Cirrhosis is the major cause of liver damage which proliferate further giving chance to the development of cancer. Cirrhosis mainly occurs due to alcohol consumption and also hepatitis virus infection leading to jaundice. By taking over-the-counter medications like NSAIDs etc. and also some herbs may lead to liver damage. There is no proper curative treatment for hepatocellular carcinoma and so it stands high in global cause of mortality. There are currently a few plant-derived drugs approved for its clinical use. This is largely because the chemical constituents of most herbal medicines are complex mixtures having diverse biological and pharmacological actions A huge number of herbal medicine i.e. herbs, formulations have been reported for their effective action in prevention and treatment of hepatocellular carcinoma.

The leaves of *Vitex leucoxylon* Linn were selected due to the presence of hepatoprotective activity [35] and also the plant represents as the folkoric medicine for cancer. The present study was carried out to investigate the anti-cancer and anti-oxidative activity of ethanolic extract of *Vitex leucoxylon* Linn. leaves in DEN and PB induced HCC in rats. Triterpinoids, flavonoids and saponins [22] are known to possess hepatoprotective activity in animals. Inhibition of tumour by EVL represents selective cytotoxic potential of flavonoids like vitexin, agnoside, aucubin and beta- sitosterol [36]. The EVL was found to inhibit the growth of cell proliferation in treated animals compared to untreated.

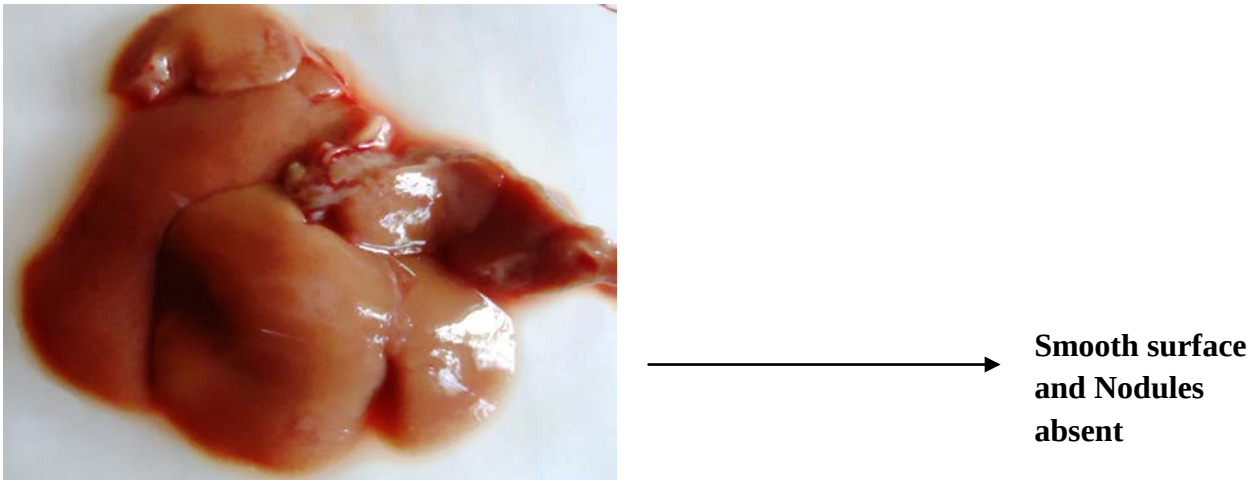
During toxicity studies, the administration of EVL on animals showed no mortality and no signs of toxicity. The gross behavioural parameters were observed showed some pharmacological effects such as decreased motor activity and relaxation after intake of EVL which proved that the EVL showed anti-depressant activity in rats, time being the rats became normal after 48 hours. In hepatotoxicity the most important mechanism of cell injury by DEN and PB involves the formation of reactive free radicals associated with many biochemical and molecular changes that induces oxidative stress leading to tumour promotion [37]. Most hepatocellular injuries involve the production of high- energy reactive metabolites by CYP450 system [38]. Liver injury occurs as these reactive metabolites forms covalent bonds with cellular proteins and nucleic acids leading to adduct formation and DNA damage which increases the cell proliferation.

Carcinogens initiate the process, which is followed by regeneration, growth and clonal proliferation, eventually leading to cancer [39]. A multistage process like cancer development is characterized by the combined action of multiple events occurring in a single cell. The initiation stage involving a non-lethal mutation in DNA is followed

Figure 2: Tumour Incidence of Liver



Group II (DEN+PB control)



Group IV and V (DEN+ PB+ EVL)

by the promotion stage, which is characterized by the clonal expansion of altered cells through induction of cell proliferation and/or initiation of apoptosis, resulting in the formation of identifiable focal lesions. Both of these stages are reversible and are followed by the progression stage, which is irreversible. This stage involves cellular and molecular changes, concomitant with cellular transformation from preneoplastic to neoplastic state and is characterized by the accumulation of additional genetic damage, leading to the transition of the cell from benign to malignant [40].

The early phase of rat liver carcinogenesis could be due to a modifying influence on the changes in phase I or II enzyme can result in either biotransformation/ detoxification of DEN, thus reducing its liver toxicity, mutagenicity, and carcinogenicity of the metabolites. CYP2E1 is known to be associated primarily with the biotransformation of a range of compounds, including DEN in the liver, but other P450 isozymes have been found to bioactivate as well [41]. Initiation arises through at least two events with the interaction of a carcinogen with the cell target molecules, particularly DNA and the fixation of the DNA damage [42].

In the rat, the DEN is metabolised primarily in the liver by cytochrome P-450 to α- hydroxy derivative (ethyl-acetoxyethyl-nitrosamine) by the mixed function oxidase system of class of phase I enzymes involved in the activation of chemical substances. This intermediate may be conjugated by the phase II enzymes to a non- toxic compound. Alternatively, the ethyl-acetoxyethyl-nitrosamine may spontaneously produce the ethyl-diazonium ion that is able to directly ethylate cellular macromolecules. The major biological effects are known to be caused by the ethyl-diazonium ion reacting with nucleophilic sites to generate adducts in the DNA [43].

It has been reported that their biotransformation produces the promutagenic adducts *O*6-ethyldeoxyguanosine, *O*4 and *O*6-ethyldeoxythymidine and 8-hydroxyguanine (8-OHG) that play a role in the initiation step of rat liver carcinogenesis [44,45,46]. Therefore, DNA adducts would have been formed in hepatocytes after DEN administration and enhanced post-necrotic compensatory

cell replication, which is necessary for the conversion of DNA adducts to mutation in daughter cells, and could have contributed to the appearance of preneoplastic hepatocyte observed after DEN exposure [47].

After DEN exposure, one hepatic response to DNA damage and centrilobular cytotoxicity/necrosis is characterized by regenerative cell proliferation [48]. The extent of DNA adduct formation will most often correlate with the extent of tumourigenesis, because it exceeds the ability of the cell to repair the carcinogen- induced DNA damage [43] which leads to DNA strand breaks and in turn hepatocellular carcinomas without cirrhosis through the development of putative preneoplastic focal lesions.

Further continuous administration of PB also results in the release of reactive oxygen species [49]. Free radicals play a significant role in the biotransformation of chemical carcinogens like activation of PB and promotion stage of cancer [50]. Non-genotoxic hepatocarcinogens such as PB have been proposed to function via a variety of mechanisms.

PB is known to induce the expression of several CYP and phase II enzymes, including CYP 2B1/2 and CYP 3A1/2 [51]. A causal relationship between induction of these CYP and promotion of liver tumours has been suggested [52,53]. However, the mechanism by which CYP induction is related to liver tumour promotion has not been resolved. The increased growth of preneoplastic lesions may also be attributed to increased cell proliferation and/or decreased apoptosis [54]. Chronic administration of promoting agent such as Phenobarbital has many effects on liver, including development of hyperplasia and hypertrophy without increasing cell death and has been shown to stimulate cell proliferation in carcinogen-challenged tissues. An uncompromised free radical generation in the liver overwhelms the antioxidant status and ultimately proceeds to oxidative stress paving way to carcinogenesis.

Nutritional deprivation causing loss in body mass might decrease tumour volume [55]. There was no nutritional deprivation occured on administration of EVL and 5-FU in rats, but inturn it increased the appetite and thirst o in EVL treated groups which lead to excess amount of food and water consumption in normal animals. This led to increased metabolism and body weight of the animals compared from the first day of experiment. The DEN+PB control induced cancer rats lead to anorexia. Hence the water intake was decreased which resembled as that of food intake. The liver weight increases in cancer induced rats due to rapid cell replication of damaged hepatocyte in enlarged liver [56]. This paved way to chronic cirrhotic liver conditions. The EVL administered rats in high dose group showed hepatoprotective activity as there was less liver damage than the lower dose animals. The standard group also showed much protective effect as that of normal rat liver. This proved the hepatoprotective effect on cancer induced rat liver.

In anaemia of cancer, multiple mechanisms can interfere with normal erythrocyte production. The cytokines tumour necrosis factor-alpha (TNF-), transforming growth factor-beta, interleukin (IL)-1, IL-6, and interferon-gamma are likely most prevalent as inhibitory mechanisms. This network of cytokines probably modulates iron metabolism, and the erythropoietin effect may be blunted by TNF- among others. Anaemia impairs virtually every organ and tissue of the body and causes multiple function disturbances, decreasing mental and physical performance capacity. One of the major symptoms of organ disturbance is fatigue. In oncology, this symptom ranks first among patient complaints [57], and parallels the haemoglobin level [58]. On average, over one third of patients become anaemic after three cycles of chemotherapy. The haemoglobin levels were decreased in DEN+PB control rats which confirms the anaemic condition in cancer rats and also the RBC, lymphocytes, monocytes, neutrophils and PCV levels also showed decrease in DEN+PB control cancer induced rats. There were changes in WBC and ESR levels which led to increase in haematological levels of DEN+PB induced rats. The treatment groups of EVL showed favourable effects in dose dependant manner and 5-FU which reduced the anaemic conditions of cancer induced rats.

Chemicals produce a wide variety of clinical and pathological hepatic injury. Biochemical markers (e.g. alanine transferase, alkaline phosphatase and bilirubin) are often used to indicate liver damage. Among the most sensitive and widely used liver enzymes are the aminotransferases. They include aspartate aminotransferase (AST or SGOT) and alanine aminotransferase (ALT or SGPT) and ALP or SALP. These enzymes are normally contained within liver cells. If the liver is injured or damaged, the liver cells spill these enzymes into the blood, raising the enzyme levels in blood and signalling the liver disease. The liver function tests mainly reduced the levels of bilirubin significant factor in measurement of liver enzymes [59,60]. Elevated levels of the serum enzymes (SGPT, SGOT and SALP) in the cancer control group reflect the significant alteration of liver function by DEN+PB induction. Treatment with EVL groups in dose dependant manner was found to be equipotent to the standard 5-FU in restoration of the elevated enzyme levels to normal, implying the normal functioning of liver. It is inferred that alteration in the plasma total protein is most often due to decrease in the quantity of albumin, which can be accompanied by a relative hyperglobulinaemia [61]. Affected liver functioning also resulted in the decreased protein synthesis, but showed increased creatinine, urea and bilirubin levels in DEN+PB induced rats while it was almost restored in the treated animals of EVL groups in dose dependant manner and in standard 5-FUgroup. Cholesterol is an essential lipid for mammalian life, but a high cholesterol level can almost guarantee the eventual onset of vascular diseases and, in some cases, can lead to death. It has been shown that there is a direct connection between high cholesterol levels and vascular diseases. Lipid plays an important role in the pathogenesis of complications involved with cancer. The elevated level of serum cholesterol, HDL and reduced level of serum TGL cholesterol in DEN+PB, poses to be a risk factor for developing microvascular complication leading to cancer and further leads to

cardiovascular diseases like coronary heart disease. Treated groups with EVL showed much better activity in dose dependant manner and in 5- FU group.

In DEN+PB induced cancer animals, oxidative stress imposed by DEN and chronic PB administration was reflected from significant increases in the levels of LPO of liver containing neoplasia. However, treatment with EVL high dose than the lower dose and 5-FU exhibited significantly low levels of LPO which shows the anti-lipid peroxidative role of EVL through the oxidative pathways and is probably mediated by its ability to inhibit superoxide radical generation. SOD has been reported as one of the most important enzymes in the enzymic antioxidant defence system. It scavenges the superoxide anion to form hydrogen peroxide, hence diminishing the toxic effect caused by this radical [62] inducing oxidative damage to liver Catalase. In the present study, there was a significant decrease in the levels of SOD in DEN+PB control rats is a sensitive index in hepato cellular damage and is the most sensitive enzymatic index in liver injury which may be due to the utilisation of the enzyme to scavenge H_2O_2 radicals and increased in treatment with EVL extracts in dose dependant manner and 5-FU respectively. CAT is an enzymatic antioxidant widely distributed in all animal tissues, and the highest activity is found in the red cells and liver that decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radicals [63] and it is thought to be the first line of defence against oxidative damage caused by hydrogen peroxide and other radicals induced by carcinogen. CAT shows decreased enzyme levels in DEN+PB control rats and increased in treatment with EVL extracts in dose dependant manner and 5-FU respectively.

GSH is a well known non enzymic antioxidant defence system of cells. It has been shown to provide protection against super oxides as well as H_2O_2 [64] and it contributes to membrane stability. It is said to be involved in many cellular processes including detoxification of endogenous and exogenous compounds [65]. Reduced cell GSH has been reported in patients with liver diseases of alcoholic, non-alcoholic, viral and other etiology [66]. DEN, an electrophilic carcinogen may interact with the large nucleophilic pool of GSH thereby reducing the macromolecules and carcinogen interaction [67]. Glutathione is a potent inhibitor of the neoplastic process, plays an important role in the endogenous anti-oxidant systems. The enzyme levels of GSH, GST, GPx, GR in cancer induced rats also showed significant decrease in DEN+PB control rats as they are involved in the defence mechanism against oxidative damage, it reduces the H2O2 and hydroperoxide levels. Treatment with EVL showed favourable effects with increase in enzyme levels in dose dependant manner and 5-FU bringing nearby to normal levels.

Histopthological studies in normal rat liver showed uniformly arranged liver plates with oval hepatocyte of uniform size. In DEN+PB control cancer induced rats showed irregularly formed cell plates because it showed congestion, necrosis, and infiltration of inflammatory cells and loss of architecture due to liver cirrhosis. This showed the presence of chronic hepatotoxicity due to the scattered masses of necrotic tissues which were detected in most of the areas in cancer induced rat liver. Enlarged nuclei were also spotted in DEN+PB treated rats. The treatment with EVL lower dose groups showed moderate infiltration of inflammatory cells followed by congestion, necrosis of liver. Rats in preventive and curative groups administered with EVL higher dose and with standard 5-FU showed less infiltration of inflammatory cells with no congestion, necrosis of liver and no loss of architecture. However, in some areas degenerating hepatic cells were detected. Tumour incidence was found in DEN+PB cancer control rats 2 to 3 nodules in greyish white colour, with parenchyma which is reddish brown in colour. Rough surface found shows cirrhosis and no nodules in normal rats. Hence EVL treatment with high dose showed increase protective effect as that of standard than the low dose of EVL extract.

Thus, the *Vitex leucoxylon* deserves additional evaluation as a provider of hepatoprotective agents. Indeed, there is a current need for availability of new plant-derived bioactive molecules; thus genus *Vitex.* may be a great natural source for the development of new drugs and may provide a cost-effective mean of treating diseases in the developing world.

REFERENCE

1. Sarveswaran Sivalokanathan, Muthaiyan Ilayaraja and Maruthaiveeran Periyasamy Balasubramanian. Antioxidant activity of Terminalia arjuna bark extract on N-nitrosodiethylamine induced hepatocellular carcinoma in rats. Molecular and Cellular Biochemistry 2006; 281: 87–93.
2. Bosch FX, Ribes J, Díaz M, Cléries R. Primary liver cancer: worldwide incidence and trends. Gastroenterology, 2004; 127:S5-S16.
3. Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of twenty-five major cancers in 1990. Int J Cancer 1999; 80: 827–841.
4. Ping Wu, Jean Jacques Dugoua, Oghenowede Eyawo and Edward J Mills. Traditional Chinese medicines in the treatment of hepatocellular cancers: a systematic review and meta-analysis. Journal of Experimental & Clinical Cancer Research 2009, 28:112.
5. Cristovao, F.L., Manuel, F.F., Cristina, P.W. Drinking of Salvia officinalis tea increases CCl4-induced hepatotoxicity in mice. Food Chem. Toxicol 2007; 45: 456– 464.
6. Vitaglione, P., Morisco, F., Caporaso, N., Fogliano, V. Dietary antioxidant compounds and liver health. Crit. Rev. Food Sci. Nutr. 2004; 44: 575–586.
7. Mortel CG. The liver. In: Holland JF, Frei E III, eds. Cancer medicine. Philadelphia: Lea & Feiger 1973; 1541-1547.
8. Zala G, Havelka J, Altorfer J et al. Hepatitis C and Hepatoma. Schweiz Med Wochenschr 1992; 122:194-197.
9. Seitz HK, Poschl G, Simanowski UA. Alcohol and cancer. Recent Dev Alcohol 1998; 14:67-95.

10. Jenny Heathcote, Anna Lok. Hepatocellular carcinoma- Screening, Diagnosis and Management 2004; pp 147- 156.
11. Richard A. Helms, David J. Quan, Eric T. Herfindal, Dick R. Gourley. Textbook of Therapeutics. Drug and Disease Management 2003. 8th Edition.
12. Harsh Mohan. Text Book of Pathology Feb 2005. 5th Edition.
13. Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology. 2004 Nov;127(5 Suppl 1):S35-50.
14. Bannasch P, Mayer D, Hacker HJ. Hepatocellular glycogenesis and hepatocarcinogenesis. Biochim Biophys Acta 1980; 605: 217–245.
15. Dyroff MC, Richardson FC, Popp JA. Correlation of O4- ethyldeoxythymidine accumulation, hepatic initiation and hepatocellular carcinoma induction in rats continuously administrated diethylnitrosamine. Carcinogenesis 1986; 7: 241–246.
16. Balasubramanian Rajkapoor, Narayanan Murugesh, Dechen Chodon, Dhanapal Sakthisekaran. Chemoprevention of N-Nitrosodiethylamine Induced Phenobarbitol Promoted Liver Tumors in Rat by Extract of Indigofera aspalathoides. Biol. Pharm. Bull 2005; 28(2): 364—366.
17. L.F. Barbisan, M. Miyamoto, C. Scolastici, D.M.F. Salvadori, L.R. Ribeiro, A.F. Eira, Joa˜o Lauro Viana de Camargo. Influence of aqueous extract of Agaricus blazei on rat liver toxicity induced by different doses of diethylnitrosamine. Journal of Ethnopharmacology 2002; 83: 25- 32.
18. Brahma N. Singha, Braj R. Singh, B.K. Sarma, H.B. Singh. Potential chemoprevention of N-nitrosodiethylamine-induced hepatocarcinogenesis by polyphenolics from Acacia nilotica bark. Chemico-Biological Interactions 2009; 181: 20–28.
19. Amit Singh Yadav, D. Bhatnagar. Chemo-preventive effect of Star anise in N-nitrosodiethylamine initiated and phenobarbital promoted hepato-carcinogenesis. Chemico-Biological Interactions 2007; 169: 207–214.
20. Woo-song Ha, Chi-kyeong Kim, Seung-hee Song and Chung-boo Kang. Study on mechanism of multistep hepatotumorigenesis in rat: development of hepatotumorigenesis. J. Vet. Sci., 2001; 2(1): 53–58.
21. Loppen S, Schneider D, Gaunitz F, Gebhardt R, Kurek R, Buchmann A, et al,. Cancer Res 2001; 62:5685.
22. A K Meena, Uttam Singh, A K Yadav, B Singh, M M Rao. International Journal of Pharmaceutical and Clinical Research 2010; 2(1): 01-09.
23. Madhava chety K., K. shivaji, K.Tulasi Rao. Flowering plants of chittoor district Andhra Pradesh, India 2001.
24. Fauziah O.P, Hanachi S, Yogespriya and R. Asmah. Reducing effect of Strobilanthes crispus leaf extract in hepatocarcinogenesis rats. Int J Cancer Res 2005; 1: 109-112.
25. Tomlinson S.K., Melin S.A., Higgs V., White D.R., Savage P., Case D., Blockstock A.W., Schedule selective biochemical modulation of 5-fluorouracil in advanced colorectal cancer - a phase II study , BMC Cancer 1990; 2: 9.
26. Fernando Salvador Moreno, Luciana Passos Toledo , Aline de Conti , Renato Heidor , Alceu Jord˜ao Jr. , H´elio Vannucchi , Monica Testoni Cardozo , Thomas Prates Ong. Chemico-Biological Interactions 2007; 168 : 221–228

A. A. Adedapo, O. M. Mogbojuri and B. O. Emikpe. Safety evaluations of the aqueous extract of the leaves of Moringa oleifera in rats. Journal of Medicinal Plants Research, August 2009; 3(8): 586-591.

27. Ohkawa, H., Ohishi, N., Yagi, K. Assay for lipid peroxidation in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry 1979; 95: 351 – 358.
28. Staal, G.E., et al. Purification and properties of an abnormal Glutathione reductase from human erythrocytes. Biochim. Biophys. Acta 1969; 185: 63-69.
29. Rotruck, J.T., Pope, A.L., Ganther, H.E. Selenium. Biochemical role as a component of glutathione peroxidase purification and assay. Science 1973; 179: 588 – 590.
30. Moron, M.S., DePierre, J.W., Manerwik, K.B. Levels of glutathione, glutathione reductase and glutathione-S-transferase activities in rat lung and liver. Biochimica Biophysica ACTA 1979; 582: 67 – 68.
31. Marklund, S., Marklund, G. Involvement of superoxide anion radical in the autooxidation of pyrogallol and a convenient assay for superoxide dismutase. European Journal of Biochemistry 1974; 47: 469 – 474.
32. Sinha, A.K. Colorimetric assay of catalase. Analytical Biochemistry 1972; 47: 389 – 394.
33. M. Shahjahan, G. Vani, C.S. Shyamaladevi et al. Effect of Solanum trilobatum on the antioxidant status during diethyl nitrosamine induced and phenobarbital promoted hepatocarcinogenesis in rat. Chemico-Biological Interactions 2005; 156: 113–123.
34. R.v. Krishna rao, Ranjit jena, and P. Mallikarjuna rao. Studies on hepatoprotective activity of vitex leucoxylon l. Anc sci life. 1997 oct-dec; 17(2): 128–134.
35. Prabodh shukla. Preliminary phytochemical investigation & pharmacological evaluation of Vitex leucoxylon linn for anti-inflammatory activities. Ethnobotanical Leaflets Jan 2009; 13: 956-61.
36. Trush, M.A, and Kensler, T.W. An overview of thr relationship between oxidative stress and chemical carcinogenesis. Free Radic. Biol. Med. 1991; 10: 201-209.
37. Lee W. Drug induced hepatotoxicity. N Engl J Med 2003; 349:474-485.
38. Goldworthy TL, Pitot HC. An approach to the development of a shortterm whole animal bioassay to

distinguish initiating agents (incomplete carcinogens), promoting agents, complete carcinogens, and noncarcinogens in rat liver. J Toxicol Environ Health 1985; 16: 389–402.
39. Valko, M., C.J. Rhodes, J. Moncol and M.M. Izakovic. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Mini-review. Chem. Biol. Interact 2006; 160: 1-40.
40. Marcos C. Dias, Maria A.M. Rodrigues, Maria C.H. Reimberg, Luıs F. Barbisan. Protective effects of Ginkgo biloba against rat liver carcinogenesis. Chemico-Biological Interactions 2008; 173:32–42.
41. Muzio G., B. Marengo, R. Salvo, A. Semeraro, R. A. Canuto, and L. Tessitore. Liver cancer is induced by a subnecrogenic dose of DENA when associated with fasting/refeeding: role of Glutathione-transferase and lipid peroxidation. Free Radical Biology & Medicine 1999; 26: 1314–1320.
42. Hemminki, K. DNA adducts, mutations and cancer. Carcinogenesis 1993; 14**:**2007–2012.
43. Dragan, Y.D., Hully, J.R., Nakamura, J., Mass, M.J., Swenberg, J.A., Pitot, H.C. Biochemical events during initiation of rat hepatocarcinogenesis. Carcinogenesis 1994; 15: 1451-1458.
44. Swenberg, J.A., Hoel, D.G., Magee, P.N. Mechanistic and statistical insight to the large carcinogenesis bioassays on Nnitrosodiethylamine and N-nitrosodimethylamine. Cancer Research 1991; 51: 6409- 6414.
45. Verna, L., Whysner, J., Williams, G.M. N-Nitrodiethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation. Pharmacology and Therapy 1996; 71: 57- 81.
46. Barbisan L.F., M. Miyamoto , C. Scolastici , D.M.F. Salvadori , L.R. Ribeiro , A.F. Eira , Joa o Lauro Viana de Camargo. Influence of aqueous extract of Agaricus blazei on rat liver toxicity induced by different doses of diethylnitrosamine. Journal of Ethnopharmacology 2002; 83: 25-32.
47. Williams G.M., M.J. Iatropoulos, C.X. Wang. Diethylnitrosamine exposure-responses for DNA damage, centrilobular cytotoxicity, cell proliferation and carcinogenesis in rat liver exhibits some non-linearities, Carcinogenesis 1996; 17: 2253–2258.
48. Scholz W., K. Schutze, W. Kunz, M. Schwarz. Phenobarbital enhances the formation of reactive oxygen in neoplastic rat liver nodules, Cancer Res. 1990; 50:7015–7022.
49. Gey K.F. Prospects for the prevention of free radical disease regarding cancer and cardiovascular disease, Br. Med. Bull. 1993; 49: 679–699.
50. Waxman, D.J., Azaroff, L. Phenobarbital induction of cytochrome P-450 gene expression. Biochemical Journal 1992; 281: 577–592.
51. Kitano, M., Ichihara, T., Matsuda, T., Wanibuchi, H., Tamano, S., Hagiwara, A., Imaoka, S., Funae, Y., Shirai, T., Fukushima, S. Presence of a threshold for promoting effects of Phenobarbital on diethylnitrosamine-induced hepatic foci in the rat. Carcinogenesis 1998; 19: 1475–1480.
52. Lubet, R.A., Nims, R.W., Ward, J.M., Rice, J.M., Diwan, B.A. Induction of cytochrome P450b and its relationship to liver tumor promotion. Journal of the American College of Toxicology 1989; 8: 259– 268.
53. Guyonnet D., R. Berg_es , M.H. Siess , M.F. Pinnert , M.C. Chagnon , M. Suschetet , A.M. Le Bon. Post-initiation modulating effects of allyl sulfides in rat Hepatocarcinogenesis. Food and Chemical Toxicology 2004; 42: 1479–1485.
54. Weitzberg AB, Corvese D. Translocation of chromosomes 16 and 18 in oxygen radical-transformed human lung fibroblasts. Biochem Biophys Res Commun 1990; 169:70-74.
55. Orton T.C., S.E. Doughty, A.E. Kalinowski, P.G. Lord and P.F. Wadsworth. Life Sciences & Medicine, Carcinogenesis 1996; 17(5): 973-981.
56. Curt GA, Breitbart W, Cella D et al. Impact of cancer-related fatigue on the lives of patients: new findings from the Fatigue Coalition. The Oncologist 2000; 5: 353–360.
57. Littlewood TJ, Bajetta E, Nortier JW et al. Effects of epoetin alfa on hematologic parameters and quality of life in cancer patients receiving nonplatinum chemotherapy: results of a randomized, double-blind, placebo-controlled trial. J Clin Oncol 2001; 19:2865–2874.
58. Mumoli N, Cei M, Cosimi A. "Drug-related hepatotoxicity". N. Engl. J. Med. 2006; 354 (20): 2191–3.
59. Benichou C. "Criteria of drug-induced liver disorders. Report of an international consensus meeting". J. Hepatol. 1990; 11 (2): 272–6.
60. Duncan, J.R., Prasse, K.W. and Mahaffey, E.A. Veterinary Laboratory Medicine: Clinical Pathology 1994, 3rd (ed.), Lowa State, university press, Ames, Lowa.
61. Y Y Soon, B K H Tan. Evaluation of the Hypoglycemic and AntiOxidant Activities of Morinda officinalis in Streptozotocin-induced Diabetic Rats. Singapore Med J 2002; 43(2) : 077-085.
62. F. Annie Felicia and M. Muthulingam. Antihepatotoxic Efficacy of Methanolic Extract of Indigofera tinctoria (Linn.) on Paracetamol – Induced Liver Damage in Rats. J. Nat. Prod. Plant Resour., 2012; 2 (2):244-250.
63. Clemens MR, Walker HD. Lipid peroxidation in erythrocytes. Chem Phys Lipids 1987; 45: 251–268.
64. Yu BP. Cellular defence against damage from reactive oxygen species. Physiological Reviews 1994; 74: 136–162.
65. Czuczejko, J., Zachara, B. A., Staubach-Topczewska, E., Halota, W. & Kedziora J. Selenium, glutathione and glutathione peroxidases in blood of patients with chronic liver diseases. Acta Biochimica Polinca 2003; 50: 1147-1154.
66. Ketterer B., D.J. Meyer. Glutathione-S-transferases: a possible role in the detoxification and repair of

DNA and lipid hydroperoxides, Mutat. Res. 1989; 45:1–8.