Chemopreventive and Therapeutic Efficacy of Enhalus Acoroides Against Diethylnitrosamine Induced Hepatocellular Carcinoma in Wistar Albino Rats

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Research Article

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Abstract

Background: Hepatocellular carcinoma plays an inadequate mention in the second cause of death because of cancer worldwide. An alternative therapy with high rate of prognosis and also without side effects. Several data indicated the therapeutic efficacy of *Enhalus acoroides*. There were no scientific studies on chemopreventive and antioxidant potential of *Enhalus acoroides* against hepatocellular carcinoma.

Purpose: To investigate the hepatoprotective efficacy of ethanolic extract of *Enhalus acoroides* (EEEA) against DEN induced hepatocellular carcinoma using wistar albino rats.

Study design: Animals were divided into five groups each comprising six rats. Normal saline given to Group I- Control rats. By using DEN, liver cancer was induced to Group II, III, IV and V rats as single intraperitoneally injection (100 mg/kg body weight). At the beginning of 6^{th} week, Groups III rats received EEEA (200mg/kg body weight/day) upto 16 weeks. Group IV rats received EEEA for one week before the administration of DEN and continued till the 16^{th} week. After the administration of DEN, Group V positive control rats received Silymarin (100 mg/kg body weight) at the beginning of 6^{th} week and continued upto 16 weeks. The efficacy of *Enhalus acoroides* for its Hepatoprotective and antioxidant properties during its simultaneous treatment against DEN induced liver damage was evaluated in rats.

Methods: The hepatoprotective efficacy of EEEA (200 mg/kg) was investigated against DEN (100 mg/kg/b.w) induced hepatotoxicity, was measured by evaluating serum liver markers levels (ALT, AST, GGT and ALP), Kidney markers (Urea and Creatinine), Lipid profile (TG, HDL, LDL & Total cholesterol) and Serum tumor markers (DNA, RNA, AFP and CEA). EEEA-aided antioxidant defence against hepatotoxic insult of DEN was measured by evaluating various Antioxidant biomarkers (GSH, SOD, CAT, GPx, Vit C and Vit E) Morphometric gross analysis and Histopathological studies were done to support the outcomes of the present study.

Results: A significant increased antioxidant defence and reduced MDA levels in the serum of EEEA treated animals compared to the DEN induced animals. The resulting data showed that the administration of EEEA decreased the serum liver markers levels, kidney markers, Lipid profile and serum tumor markers when compared to the untreated rats. The histopathological anomalies were altered on administration of EEEA indicating its protective effects on hepatocytes when compared with untreated rats.

Conclusions: Our consequences established that crude ethanolic extract of *Enhalus acoroides* shown an effective impact against DEN-induced hepatocellular carcinoma, and serves as a better option for chemopreventive treatments.

Introduction

Cancer is a most common dreaded complication throughout the world. It is the second major cause of death besides cardiac problems and strikes one out of three people in the world [1]. Because of the long

duration of the disease and it is debilitating effects, it becomes a serious global burden to the patients and to the overall community. Among the different types of cancer, Hepatocellular carcinoma (HCC) plays an inadequate mention in the second cause of death because of cancer worldwide. Approximately 8 Lakhs new cases of HCC per year occur globally which makes HCC as fifth place among men and ninth among women, worldwide.

Liver is the most important organ in the body where it metabolizes the ingested material; it is more prone to carcinogenic in silt. As the tolerance level of liver is very high, hepatic carcinoma is seldom identified at the earlier stage and once identified; in most cases the treatment has a poor prognosis [2]. Apart from, the cancer treatments including radiation therapy, chemotherapy leads to various side effects. To overcome such problems alternative therapy is required for the overall prognosis of HCC. The improvement of such anticancer drugs provides hopeful evidence that herbal plants could be a resource of alternate therapy for finding the novel chemotherapeutics.

Marine components are said to be the strong source of medications. Some of the compounds derived from marine living beings have antioxidant property and anticancer activities, but they are to a great extent unexplored. Only, a few drugs based on marine source are found in commercially, including a powerful analgesic; antitumour agents; antiviral agents; and for treating hypertriglyceridemia [3]. Among the marine world, sea grass has been utilized for an assortment of medicinal reason. Sea grass is referred to deliver secondary metabolites as defence component under pressure conditions and these compounds are observed to be anti-oxidative in nature.

Thus, the prime goal of the present investigation is to choose a sea grass which has tremendous secondary metabolites that can be utilized as a compelling specialist in fighting against hepatic carcinoma. *Enhalus acoroides* (Linnaeus f.) Royle has a place with the Family Hydrocharitaceae is a profusely developing sea grass in coastal zones of Gulf of Mannar, submerged in shallow sea water along the coast and normally found in muddy soils. *E. acoroides* plays a significant role as cancer preventing agents [4]. Till now, no reports are accessible on the anticancer activities of *Enhalus acoroides* in wistar albino rats. Thus, this investigation was done to pick up knowledge into the anticancer and antioxidant capability of the ethanolic extract of *Enhalus acoroides* (EEEA) in wistar albino rats.

Diethylnitrosamine (DEN) is an N-nitroso alkyl compound, is one of the most major carcinogens, which is known to cause disruption in the enzymes of nucleus which is involved in repair of DNA or replication of DNA are generally used as a carcinogen to induce hepatic cancer in rodents [5].

Due to the antioxidant and hepatoprotective activities, Silymarin has been recommended in liver cirrhosis, although its clinical efficacy is continuously discussed [6]. Hence in this study, Silymarin is preferred as a standard hepatoprotective agent against experimentally induced hepatotocellular carcinoma in animal models.

There were no scientific evidences on antioxidant potential of EEEA and Silymarin against HCC with the anticancer drug induced hepatocellular carcinoma remains unexplored. Hence this study was done to

assess the liver markers; tumor markers, status of antioxidants and histopathological analysis of ethanolic extract of *Enhalus acoroides* on DEN induced HCC in Wistar albino rats.

Material And Methods

Plant Material

Enhalus acoroides was collected from Devipattinam, Ramanadhapuram District, Tamilnadu during the month of June 2016. The sea grass was authenticated in ICAR by Dr. N. Kaliaperumal M.Sc., Ph.D., Scientist-in-charge, CMFRI.

Preparation of Enhalus acoroides **Extract**

The leaves of *Enhalus acoroides* were collected, washed, shade dried and powdered mechanically and prepared. The grinded powder was initially soaked into 1:2 ratio ethanol with mild shaking for three days. Three days past, filter the macerate and concentrated in a rotary evaporator. Finally, the concentrated crude extract of *Enhalus acoroides* was lyophilized into paste and was taken for the *in vivo* study.

Experimental Rats and Diet

Wistar albino male rats approximately weighing 180-200g were taken for the study. Rats were procured from VISTAS, Chennai, India and kept in spacious polypropylene cages bedded with rice husk. The rodent room was well aerated and kept under standard experimental conditions (Temperature 27 ± 2ºC and 12 hrs dark / light cycle) throughout the procedure. All the rats were fed with standard pellet diet (VRK Nutritional, Maharashtra, India) and water *ad libitum*. They were acclimatized to the environment for 1 week prior to experimental use. The procedure was done according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Rats (CPCSEA), New Delhi, India **(IAEC No: XXI/VELS/PCOL/02/2000/CPCSEA/IAEC/01.12.2017)**.

Chemicals

From Sigma Chemical Company (St. Louis, MO, USA) Ethylene Diamine Tetraacetic Acid (EDTA), Diethylnitrosamine (DEN), Nitro Blue Tetrazolium (NBT), Trichloro Acetic Acid (TCA), Thiobarbituric Acid (TBA), 1-Chloro-2,4-Dintiro Benzene (CDNB), 5,5’-Dithio-Bis (2-Nitroben zoic Acid), Glutathione (reduced & oxidized) and ascorbic acid-L were purchased. Chemicals that are used for analytical grade were purchased from Glaxo Laboratories and Sisco Research Laboratories, Mumbai, India.

Dosage Fixation

The minimal effective dose 200mg/kg b.w. was fixed based on toxicity study carried in wistar albino rats with the Ethanolic extract of *Enhalus acoroides* [7]. Different doses of EEEA extract (100mg/kg b.w., 200mg/kg b.w., and 400mg/kg b.w.) were treated for 4 weeks in rats. The effective dose of EEEA was assessed based on the haematological parameters, biochemical markers and histopathological studies.

Supplementations of EEEA extract at doses of 200mg/kg b.w. and 400mg/kg b.w. for 4 weeks were seemed to be effective in rats. Among this, the minimal effective dose 200mg/kg b.w. was fixed as therapeutic dosage for the DEN induced Hepatocellular Carcinoma study in Wistar albino rats.

Experimental Design

Rats were divided into five groups each comprising six rats. One group served as the control while the remaining four groups were injected with Diethyl nitrosamine (100mg/kg/b.w.) as an intraperitoneal injection to induce tumor [8]. The ethanolic extract of *Enhalus acoroides* (EEEA) was given 200 mg/kg b.w. in rats. Silymarin (100 mg/kg b.w.) was used as a standard drug (Ramakrishnan *et al.*, 2006). The initial body weights of the rats were recorded. The efficacy of EEEA for its Hepatoprotective activity during its simultaneous treatment against DEN induced liver damage was evaluated in rats. The study protocol and the dosage schedule are given below:

GROUP I : Normal rats (n = 6, the rats were given normal saline only)

GROUP II : Hepatocarcinoma induced rats (n = 6, the rats were given DEN)

GROUP III : Post-treated rats (n = 6, the rats were given DEN + EEEA)

GROUP IV : Pre- treated rats (n = 6, the rats were given EEEA + DEN)

GROUP V : Drug control rats (n = 6, the rats were given DEN + Silymarin).

Treatment Protocol

Group I - Control rats received normal saline only. In Group II, III, IV and V rats, by using DEN hepatic cancer was induced as single intraperitoneally injection (100 mg/kg b. w). At the beginning of 6th week Groups III rats received EEEA (200mg/kg b.w) upto 16 weeks. Before the administration of DEN, Group IV rats received EEEA for one week and pursued till sixteen weeks. Group V rats received Silymarin (100 mg/kg b.w) at the beginning of sixth week after the administration of DEN and pursued till sixteen weeks and this group served as positive control.

Collection of Blood and Preparation of Serum Sample

After sixteen weeks of the procedure period, anesthetized the rats with diethyl ether followed by cervical decapitation. By the method of cardiac puncture, blood was collected into serum separator tubes. At room temperature the blood was allowed to clot for 30 minutes and refrigerated for another 30 minutes. At 3000 rpm for 10 minutes, the resultant clear part was centrifuged and then the serum was separated and refrigerated.

Haematology

For analysing the haematological parameters, the animals were fasted overnight prior to necropsy and by the method of orbital Sinus Venipuncture technique from retro orbital sinus of rats the blood samples were collected in the capillary tube. Within one hour, the samples were collected into tube containing

EDTA-2K (Merk Pvt. Ltd., Mumbai, India) for analysis. The haematological parameters including Haemoglobin (HB) concentration using Beacon Diagnostic Kit [9], red blood cell (RBC) count, white blood cells (WBC) count, Packed Cell Volume (PCV) count [10], mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) were measured.

Serum Biochemical Markers

For analysing the serum biochemical markers, blood was placed in tubes devoid of anticoagulant, allowed to clot at room temperature. After blood collection, within one-hour samples were centrifuged at 3000 rpm for 10 min, and then serum was separated. Serum biochemical parameters including Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) by Reitman and Frankel (1957)[11]; by Kind and King's (1954)[12] method for Alkaline phosphatase (ALP) [13] method for total protein (TP), Urea [14], Creatine [15], Total bilirubin [16], Albumin [17, 18] method for Albumin globulin ratio (A/G ratio) and Triglycerides (TG), High density lipoprotein (HDL) [19] and Total cholesterol; Low density lipoprotein (LDL) [20] were determined by Microlab-300 autoanalyzer (Merk Pvt. Ltd., Mumbai, India) were analysed.

Serum Antioxidants

For analysing the serum antioxidants, blood was placed in tubes devoid of anticoagulant, allowed to clot at room temperature. After blood collection, within one-hour samples were centrifuged at 3000 rpm for 10 min, and then serum was separated. Serum antioxidant parameters including Reduced Glutathione (GSH) [21], Catalase (CAT) [22], Superoxide dismutase activity (SOD) [23], Mitochondrial Glutathione Peroxidise (GPx) [24], Vitamin C [25] and Vitamin E [26] were measured.

Gross observation and organ weight

All the rats were sacrificed by cervical decapitation under anaesthesia at the end of the experiment period and examine carefully for macroscopic abnormalities. The complete and relative (organ-to-body weight ratios) weights of major organs including liver, kidney, spleen, heart and brain were measured.

Collection of Tissue Homogenate

After the collection of blood, immediately the rats were sacrificed by cervical dislocation, then the liver was dissected out and washed with ice-cold physiological saline. Using a Teflon homogenizer, the required amount of liver was weighed and homogenized. Tissue homogenate was prepared by using 0.1 M Tris Hcl buffer (pH 7.4) and used for evaluating the biochemical parameters.

Histopathological Examination

The liver, kidney, spleen, heart, and brain tissues were sliced to a thickness of 2.1mm each and fixed for 72 hrs in 10% normal saline. By using alcohol of graded concentration tissues were dehydrated. Tissues were further coated with paraffin wax and cast into blocks; sections of the tissues were cut on a microtome to 5 µm. Then the tissues were attached to a slide and then dried. By using photographic microscope, the slides were viewed to find out the histological changes [10].

Statistical Analysis

Values were expressed as mean ± SD for six rats in each group and statistically significant differences between mean values were determined by one way analysis of variance (ANOVA) followed by the individual comparison was obtained by Post-hoc Bonferroni test, a multiple comparison procedure by the SPSS software for Windows Version 20.0 (IBM Corp. Armonk, New York, NY, USA). A value of $p < 0.05$ was considered to indicate a significant difference between groups.

Results And Discussion

Effect of EEEA on Body Weight Changes in Control and Experimental Rats

Body weight changes have been used to evaluate the cause of the disease and response to drug therapies. Body weight gain by the animal is depending on the functional capacity of liver. Table 1 represents the initial and final body weight changes of rats from Group I – Group V. No significant change was found in the initial body weight among the five groups of rats. But the body weight was decreased significantly ($P < 0.05$) in DEN induced Group II rats when compared to the control Group I rats whereas in EEEA post treated Group III rats, it appeared near normal when compared with the Control Group I rats. This observation was in similar with Song *et al*., (2013) [27]. DEN brought a significant impairment in body growth. There were no significant ($P < 0.05$) changes observed among pre-treated Group IV and Standard treated Group V rats when compared with Control Group I rats. No death was observed in the experimental rats. This indicates the anticancerous effect of EEEA on hepatocellular carcinoma group.

Table 1
Effect of EEEA on Body Weight in Control and Experimental Animals

Organ (s)	Group I	Group II	Group III	Group IV	Group V
Weight of the rats before treatment (g)	180 ± 10.25	186 ± 11.74	188 ± 12.12	185 ± 11.02	190 ± 12.55
Weight of the rats after treatment (g)	228 ± 14.65^{ns}	202 ± 10.32*	230 ± 13.21^{ns}	227 ± 14.11^{ns}	234 ± 12.95^{ns}

Values are expressed as Mean ± SD for six rats

Data were analyzed by one-way ANOVA followed by the individual comparison was obtained by Post-hoc Bonferroni test, a multiple comparison procedure by the SPSS. Statistically significant variation was derived by comparing Group I versus Group II, Group III, Group IV and Group V, $*P = 0.001$, ns = Non-significant ($P < 0.05$).

Effect of EEEA on Organ weight changes in Control and Experimental Rats

Table 2 shows the liver, kidney, spleen, brain, and heart weights of control and experimental rats. Due to carcinogenesis, Group II rats were observed to have a significant increase ($P < 0.000$) in the organ weight when compared with Control Group I rats. The post treated Group III rats show significant increase in liver, kidney, and spleen ($P < 0.001$), brain ($P < 0.022$) and significant decrease in heart ($P < 0.014$,) weights when compared with the Control Group I rat. Observation of current study was similar to the findings of Furuta *et al*., (2008) [28] where he was described the gradual increase in liver weight. It was also studied by Mohammed *et al*., (2014) [29] that the liver weight in HCC induced rat showed slight increase when compared to the control rats. However, when compared with Group II rats, a gradual increase in the weights of the organs were observed in Post treated Group III rats indicating reduced proliferation of cells in these groups which indicates the effect of EEEA on HCC. Pre-treated Group IV and Silymarin treated Group V rats show non-significant ($P > 0.01$) weight in the organs when compared with the Control Group I rats.

Table 2
Effect of EEEA on Organ Weight in Control and Experimental Animals

Organ (s)	Group I	Group II	Group III	Group IV	Group V
Liver (g)	3.97 ± 0.24	6.63 ± 0.28 *	4.82 ± 0.13$^{\$}$	4.09 ± 0.28^{ns}	3.87 ± 0.10^{ns}
Kidney (g)	1.06 ± 0.03	1.83 ± 0.17 *	1.32 ± 0.16 $^{\$}$	1.09 ± 0.09^{ns}	1.11 ± 0.06 ^{ns}
Spleen (g)	0.32 ± 0.07	1.19 ± 0.06 *	0.68 ± 0.08 $^{\$}$	0.36 ± 0.08 ^{ns}	0.37 ± 0.09^{ns}
Brain (g)	1.49 ± 0.10	1.20 ± 0.05*	1.28 ± 0.02$^{\#}$	1.55 ± 0.14^{ns}	1.58 ± 0.11^{ns}
Heart (g)	0.66 ± 0.10	1.04 ± 0.11 *	0.81 ± 0.02$^{@}$	0.67 ± 0.06^{ns}	0.65 ± 0.07^{ns}

Values are expressed as Mean ± SD for six rats

Data were analysed by one-way ANOVA followed by the individual comparison was obtained by Post-hoc Bonferroni test, a multiple comparison procedure by the SPSS. Statistically significant variation was derived by comparing Group I versus Group II, Group III, Group IV and Group V, $^{*}P = 0.000$, $^{\$}P = 0.001$, $^{\#}P = 0.014$, $^{@}P = 0.022$ and ns = Non-significant ($P < 0.05$).

Measurement of Tumor Size

Tumor sizes for Group II rats were measured using vernier callipers on end of the experimental period which was depicted in the Table 3. An average size of tumor was found to be 7.32 mm at the end of the experiment (16th weeks) was observed in Group II DEN induced rats. Group III post treated rats, Group IV pre-treated rats and Group V Silymarin treated rats show reduced tumor size (0.25, 0.13 and 0.14) respectively, while Group I Control rats did not have tumor.

Table 3
Effect of EEEA on Tumor Size in Liver of Control and Experimental Animals

Liver	Group I	Group II	Group III	Group IV	Group V
Size of Tumor (mm)	0	7.31 ± 0.47*	0.20 ± 0.05^{ns}	0.13 ± 0.03^{ns}	0.14 ± 0.02^{ns}

Values are expressed as Mean ± SD for six rats (n = 6) and Data were analysed by Duncan's multiple range test (DMRT). Statistically significant variation was derived by comparing Group II versus Group III, Group IV and Group V. *P = 0.05, ns = Non-significant (P < 0.05).

Effect of EEEA on Serum Antioxidant Status in Control and Experimental Rats

Table 4 shows the levels of serum antioxidant enzymes in control and experimental rats. In carcinogenesis lipid peroxidation plays a major role [30], is the most studied biologically relevant free radical chain reaction and measured as Malonaldehyde (MDA). A significant (P < 0.000) increase in the level of MDA and significant (P < 0.000) decrease in the level of GSH, SOD, CAT, GPx, Vitamin C, and Vitamin E were noticeable in DEN induced Group II rats when compared with the Control Group I rat. Our result agrees with the previous study [31]. Antioxidant levels were reverted back to near normal levels in Pre-treated Group IV and Standard drug treated Group V rats when compared with DEN induced Group II rats. Thus, the drug EEEA restored the changes to near normal by its antioxidant efficiency. The control Group I rats exhibit a near-normal value of these enzymes whereas post treated Group III rats show significant(P < 0.000) increase in MDA and significant (P < 0.001) decrease in Catalase and Vitamin E levels and significant (P < 0.05) decrease in GSH, SOD, GPx and Vitamin C levels when compared with Control Group I rats. Along with Vitamin E and Glutathione, Vitamin C also scavenges and detoxifies free radicals [32]. However, when compared with Group II rats, a significant recovery in the antioxidant status were observed in Post treated Group III rats. EEEA has the ability to restore the levels of SOD, CAT, GPx, Vitamin C, Vitamin E and increased GSH content and also its ability to decrease the levels of lipid peroxidation.

Table 4
Effect of EEEA on Serum Antioxidant in Control and Experimental Animals

Parameters	Group I	Group II	Group III	Group IV	Group V
MDA (nmol of MDA formed/L)	7.38 ± 0.68	14.68 ± 1.83 *	11.12 ± 0.46 *	8.04 ± 0.45^{ns}	7.29 ± 0.56^{ns}
GSH (mg/ dl)	8.45 ± 0.87	5.50 ± 0.84 *	6.71 ± 0.38 $	7.94 ± 0.49^{ns}	8.14 ± 0.54^{ns}
SOD (U/ml)	4.76 ± 0.57	3.24 ± 0.21 *	3.88 ± 0.13 $	4.56 ± 0.22^{ns}	4.51 ± 0.38^{ns}
Catalase (U/ml)	9.43 ± 0.55	6.34 ± 0.51 *	7.56 ± 0.18 *	9.02 ± 0.51^{ns}	9.61 ± 0.83^{ns}
GPx (U/ml)	9.19 ± 0.71	6.54 ± 0.39 *	7.80 ± 0.21 $	8.83 ± 0.63^{ns}	9.03 ± 0.46^{ns}
Vit-C (µg/dl)	4.51 ± 0.37	2.18 ± 0.30 *	3.20 ± 0.66 $	4.41 ± 0.56 ns	4.61 ± 0.36^{ns}
Vit-E (µg/dl)	3.85 ± 0.26	2.16 ± 0.24 *	2.66 ± 0.19 *	3.46 ± 0.43^{ns}	3.60 ± 0.45^{ns}
Values are expressed as Mean ± SD for six rats					
Data were analysed by one-way ANOVA followed by post-hoc Bonferroni test. Statistically significant variation was derived by comparing Group I versus Group II, Group III, Group IV and Group V, $^{*}P$ = 0.000, $^{\$}P$ = 0.001 and NS = Non-significant (P < 0.05).					
SOD (U) = 50% of NBT reduction/min; Catalase (U) = µmol of H_2O_2 consumed/min;					
GPx (U) = µmole of GSH utilized/min					

Effect of EEEA on Antioxidant in Liver Tissues of Control and Experimental Rats

Table 5 shows the levels of liver tissue antioxidant in control and experimental rats. A significant (P < 0.000) increase in the level of MDA and significant (P < 0.000) decrease in the levels of GSH, SOD, CAT, GPx, Vitamin C, and Vitamin E were noticeable in DEN induced Group II rats when compared with the Control Group I rat. It leads to further production of free radicals overwhelming the cellular antioxidant defence [33]. The decreased levels of these antioxidant vitamins and GSH observed in Group II rats during DEN administration might be due to the excessive utilization of these vitamins in scavenging free radicals. A similar finding has been made in the seaweed *Acanthophora spicifera* [34]. Antioxidant levels were reverted back to near normal levels and non-significant (P < 0.05) changes in Pre-treated Group IV and Silymarin treated Group V rats when compared with DEN induced Group II rats. The Control Group I

rats exhibit a near-normal value of these enzymes whereas post treated Group III rats show significant ($P < 0.002$) increase in MDA and significant ($P < 0.002$) decrease in Catalase, GSH, SOD, GPx, Vitamin E, and Vitamin C levels when compared with Control Group I rats. In the antioxidant system, SOD is the first line of defence against the oxidative damage by superoxide radicals [35]. However, when compared with DEN induced Group II rats, a significant recovery in the antioxidant status were observed in Post treated Group III rats. The present investigation highlights the chemopreventive potential of *Enhalus acoroides* against DEN induced HCC by quenching lipid peroxidation and increasing the antioxidant status in the RBC through free radical scavenging and has the potential of protecting the endogenous enzymatic and non-enzymatic antioxidant activities.

Table 5
Effect of EEEA on Antioxidant in Liver Tissues of Control and Experimental Animals

Parameters	Group I	Group II	Group III	Group IV	Group V
MDA (nmol of MDA formed/L)	10.45 ± 0.45	15.63 ± 0.58 *	12.63 ± 0.65 $	9.51 ± 0.59 ns	10.28 ± 0.59 ns
GSH (mg/ dl)	6.52 ± 0.39	4.87 ± 0.43 *	5.66 ± 0.13 $	6.35 ± 0.25 ns	6.55 ± 0.38 ns
SOD (U/ml)	3.10 ± 0.37	2.02 ± 0.10 *	2.36 ± 0.09 $	2.94 ± 0.24 ns	3.11 ± 0.27 ns
Catalase (U/ml)	4.55 ± 0.42	2.29 ± 0.37 *	3.53 ± 0.38 $	4.21 ± 0.10 ns	4.54 ± 0.39 ns
GPx (U/ml)	8.03 ± 0.20	5.08 ± 0.26 *	6.79 ± 0.21 $	7.59 ± 0.46 ns	7.94 ± 0.35 ns
Vit-C (µg/dl)	5.41 ± 0.33	3.16 ± 0.24 *	3.85 ± 0.27 $	4.92 ± 0.52 ns	5.16 ± 0.25 ns
Vit-E (µg/dl)	4.20 ± 0.24	2.48 ± 0.36 *	3.45 ± 0.15 $	3.95 ± 0.15 ns	4.10 ± 0.34 ns

Values are expressed as Mean ± SD for six rats

Data were analysed by one-way ANOVA followed by the individual comparison was obtained by Post-hoc Bonferroni test, a multiple comparison procedure by the SPSS. Statistically significant variation was derived by comparing Group I versus Group II, Group III, Group IV and Group V, $^{*}P < 0.000$, $^{\$}P < 0.002$, and ns = Non-significant ($P < 0.05$).

3.6 Effect of EEEA on Tumor Markers in Control and Experimental Rats

Tumor markers produced by the tumor and when present in elevated levels, indicate the presence of carcinoma as intracellular substances in tissues or may be released into the circulation and found in serum [36]. AFP, DNA, RNA CEA and liver weight are considered to be most important references, broadly used in animal studies to diagnose and observe the development of hepatocellular carcinoma [37]. Table 6 indicates the effects of EEEA activity of Tumor markers such as DNA, RNA, AFP, and CEA of control and experimental rats. The levels of tumor markers were significantly ($P < 0.000$) elevated in DEN induced Group II rats compared with the Control Group I rat. AFP, a tumour associated fetal protein, has long been employed as a serum fetal tumour marker to monitor disease progression [38]. RNA levels were found to be increased in the cancerous condition as DNA and RNA are directly related to each other, an abnormally increased content of DNA may lead to an increased transcription, which in turn increased RNA content in tumor cells. Present findings are similar to the Pakkir *et al.*, (2011) [39] study. Tumor markers were reverted back to near normal levels and non-significant ($P < 0.05$) in pre-treated Group IV and Silymarin treated Group V rats when compared with Control Group I rats. Present findings are in concordance with Nermin *et al*., (2008) [40] study. Post treated Group III rats show significant ($P < 0.009$, $P < 0.003$, and $P < 0.002$) elevation in RNA, AFP, and CEA levels respectively when compared with the Control Group I rat. However, a significant decrease when compared with DEN induced Group II rats and significant increase when compared with the Control Group I rat in the levels of these tumor markers indicate a significant antitumor activity of ethanolic extract of *Enhalus acoroides.*

Table 6
Effect of EEEA on Tumor Markers in Control and Experimental Animals

Parameters	Group I	Group II	Group III	Group IV	Group V
DNA (mg/g wet tissue)	1.99 ± 0.20	3.14 ±. 0.56*	2.67 ± 0.19^{ns}	2.05 ± 0.12^{ns}	2.04 ± 0.99^{ns}
RNA (mg/g wet tissue)	2.32 ± 0.75	3.75 ± 0.14*	3.23 ± 0.29$^{\#}$	2.46 ± 0.36^{ns}	2.49 ± 0.29^{ns}
AFP (ng/ml)	0.09 ± 0.03	0.64 ± 0.15*	0.36 ± 0.07$^{\$}$	0.15 ± 0.06^{ns}	0.14 ± 0.05^{ns}
CEA (ng/ml)	0.05 ± 0.01	0.46 ± 0.13*	0.21 ± 0.05$^{@}$	0.07 ± 0.01^{ns}	0.06 ± 0.03^{ns}

Values are expressed as Mean ± SD for six rats

Data were analysed by one-way ANOVA followed by the individual comparison was obtained by Post-hoc Bonferroni test, a multiple comparison procedure by the SPSS. Statistically significant variation was

derived by comparing Group I versus Group II, Group III, Group IV and Group V, $^{*}P = 0.000$, $^{\$}P = 0.003$, $^{\#}P = 0.009$, $^{@}P = 0.002$ and ns = Non-significant ($P < 0.05$).

3.7 Effect of EEEA on Serum Liver Markers in Control and Experimental Rats

Table 7 indicates the effects of EEEA activity of the serum liver marker enzymes such as AST, ALT, ALP, GGT and non-enzymatic liver markers such as Bilirubin, Protein, Albumin, and Globulin of control and experimental rats. DEN induced Group II rats' exhibit significant change ($P > 0.000$) in the activity of these liver markers when compared to Control Group I rats. Rocchi *et al*., (1997) [41] stated that there was an elevation in the levels of transaminases in serum of HCC patients. In concurrent with the above reports an elevated serum aminotransferase were observed in Group II rats bearing HCC, whereas they appeared to be neutralized to near normal and non-significant ($P < 0.05$) in Group IV EEEA pre-treated rats. Due to the development of tumor, tissue gets damaged which leads to the elevation of ALP into circulation [42] and this enzyme level have been increased in serum of the tumor bearing rats and this elevation is significantly suppressed by the supplementation of EEEA. Standard drug treated Group V rats do not show noticeable changes in these parameters and non-significant ($P < 0.05$) when compared with the Control Group I rats. Post treated Group III rats show significant changes in GGT ($P < 0.000$), ALT, AST, ALP, Bilirubin, and protein ($P < 0.001$), albumin ($P < 0.038$) levels when compared with the Control Group I rat. The A/G ratio is primarily used to evaluate the liver function. The decrease in A/G ratio in post- treated Group III and pre-treated Group IV rats after the treatment was in similar pattern as that of control Groups I rats. This indicates the protective effect of EEEA over liver and improvement in its functional efficiency.

Table 7
Effect of EEEA on Serum Liver Markers in Control and Experimental Animals

Parameters	Group I	Group II	Group III	Group IV	Group V
ALT (IU/L).	28.18 ± 1.23	59.61 ± 1.39 *	37.67 ± 1.51$^{\$}$	25.58 ± 2.17^{ns}	27.24 ± 1.57^{ns}
AST (IU/L).	57.64 ± 1.42	82.61 ± 2.20 *	65.08 ± 1.86$^{\$}$	59.53 ± 1.83 ^{ns}	60.09 ± 1.77^{ns}
ALP(IU/L)	60.63 ± 1.91	71.67 ± 1.73 *	65.85 ± 0.88 $^{\$}$	59.98 ± 2.81^{ns}	59.12 ± 1.83^{ns}
GGT (IU/L)	19.03 ± 1.39	35.58 ± 2.43 *	29.24 ± 1.48 *	20.38 ± 1.59 ^{ns}	21.40 ± 1.46^{ns}
Bilirubin (mg/dl)	0.89 ± 0.16	1.86 ± 0.06 *	1.42 ± 0.15$^{\$}$	1.07 ± 0.19^{ns}	0.98 ± 0.04^{ns}
Protein (gm/dl)	7.38 ± 0.31	4.54 ± 0.30 *	6.08 ± 0.33$^{\$}$	6.98 ± 0.36^{ns}	7.33 ± 0.62^{ns}
Albumin (gm/dl)	3.79 ± 0.42	2.89 ± 0.31 *	3.18 ± 0.17 $^{@}$	3.78 ± 0.39^{ns}	4.03 ± 0.32^{ns}
Globulin (gm/dl)	3.59 ± 0.55	1.65 ± 0.43 *	3.04 ± 0.33^{ns}	3.20 ± 0.40^{ns}	3.30 ± 0.58^{ns}
A/G ratio	1.09 ± 0.27	1.75 ± 0.47*	1.12 ± 0.19^{ns}	1.00 ± 0.10^{ns}	1.25 ± 0. ^{ns}
Values are expressed as Mean ± SD for six rats					
Data were analysed by one-way ANOVA followed by the individual comparison was obtained by Post-hoc Bonferroni test, a multiple comparison procedure by the SPSS. Statistically significant variation was derived by comparing Group I versus Group II, Group III, Group IV and Group V, $^{*}P$ = 0.000, $^{\$}P$ = 0.001, $^{\#}P$ = 0.011, $^{@}P$ = 0.038 and ns = Non-significant (P < 0.05).					

Effect of EEEA on Serum Kidney Markers in Control and Experimental Rats

Table 8 indicates the effects of EEEA activity of the serum kidney marker such as Urea and Creatinine of control and experimental rats. Induction of oxidative stress by DEN altered the functions of kidney markers in rats. DEN induced hepatic rats showed impairment in kidney function which was indicated by the significantly increased levels of serum urea, and creatinine. In concurrent with the above statement, our results indicated that the exposure of rats to Group II DEN induced rats cause significant (P < 0.000) increase in the levels of urea and creatinine when compared with the Control Group I rat. Our results were similar to that of who showed that *Tabernaemontana coronaria* caused a marked reduction in the levels of blood urea and serum creatinine in DEN induced rats [43]. Kidney markers were reverted back to near normal levels and non-significant (P < 0.05) in pre-treated Group IV and Silymarin treated Group V rats when compared with Control Group I rats. Post treated Group III rats show significant (P < 0.001 and P <

0.041) increase in urea and creatinine levels respectively when compared with the Control Group I rat. However, a significant decrease when compared with DEN induced Group II rats and significant increase when compared with the Control Group I rat in the levels of these kidney marker enzymes shows the potential renal functions of EEEA.

Table 8
Effect of EEEA on Serum Kidney Markers in Control and Experimental Animals

Parameters	Group I	Group II	Group III	Group IV	Group V
Urea (mg/dl)	26.19 ± 1.85	40.71 ± 1.28 *	31.91 ± 1.18$^{\$}$	25.35 ± 1.98^{ns}	26.39 ± 1.68^{ns}
Creatinine (mg/dl)	0.70 ± 0.03	0.81 ± 0.02*	0.74 ± 0.02$^{\#}$	0.71 ± 0.02^{ns}	0.69 ± 0.03^{ns}

Values are expressed as Mean ± SD for six rats

Data were analysed by one-way ANOVA followed by the individual comparison was obtained by Post-hoc Bonferroni test, a multiple comparison procedure by the SPSS. Statistically significant variation was derived by comparing Group I versus Group II, Group III, Group IV and Group V, $^{*}P = 0.000$, $^{\$}P = 0.001$, $^{\#}P = 0.041$ and ns = Non-significant ($P < 0.05$).

Effect of EEEA on Serum Lipid Profile in Control and Experimental Rats

Table 9 indicates the effects of EEEA activity of Lipid parameters such as Triglycerides (TG), Total cholesterol (TC), HDL, and LDL of control and experimental rats. The levels of total cholesterol, triglycerides, and LDL in serum of control and experimental rats were significantly ($P < 0.001$) increased, and HDL level was significantly ($P < 0.001$) decreased in Group II DEN induced HCC rats when compared with the control Group I rats. The treatment with EEEA showed significantly altered the levels of lipid profile when compared with DEN induced Group II rats. EEEA post treated Group III rats showed significantly decreased levels of total cholesterol and triglycerides when compared with DEN induced rats. No significant ($P < 0.000$) changes were observed in EEEA pre-treated Group IV and Silymarin treated Group V rats compared to Control Group I rats indicated the effects of EEEA in maintaining the normal status of lipid profile in the serum.

Table 9
Effect of EEEA on Serum Lipid Profile in Control and Experimental Animals

Parameters	Group I	Group II	Group III	Group IV	Group V
Triglycerides (mg/dl)	109.34 ± 3.56	141.34 ± 2.91*	124.53 ± 3.15*	112.40 ± 2.38ns	110.83 ± 2.43ns
Total cholesterol (mg/dl)	89.90 ± 2.82	150.37 ± 2.40*	133.71 ± 2.43*	93.70 ± 1.76ns	91.46 ± 2.89ns
HDL (mg/dl)	35.31 ± 1.92	22.69 ± 1.31*	26.14 ± 1.82*	33.50 ± 2.17ns	31.21 ± 2.01ns
LDL (mg/dl)	32.72 ± 3.73	99.42 ± 2.60*	82.67 ± 2.35*	37.72 ± 1.74 ns	38.09 ± 4.68ns

Values are expressed as Mean ± SD for six rats

Data were analysed by one-way ANOVA followed by the individual comparison was obtained by Post-hoc Bonferroni test, a multiple comparison procedure by the SPSS. Statistically significant variation was derived by comparing Group I versus Group II, Group III, Group IV and Group V, $^{*}P = 0.000$ and ns = Non-significant ($P < 0.05$).

Effect of EEEA on Blood Haematological Profile in Control and Experimental Rats

Table 10 shows the levels of RBC, WBC, Hb, PCV, MCV, MCH, and MCHC in control and experimental rats. Mean value of haemoglobin in Group II rats decreased when compared to normal control Group I rats because of induction of hepatocellular carcinoma. This was similar with the reports of Ge *et al.*, (2011) [44] who also stated that significant decrease in haemoglobin in human patients with cancer in the GI system including hepatic carcinoma. Hassan *et al.*, (2018) [45] stated that DEN induced hepatic carcinoma rats was found to be slight decrease in the RBC count. Our finding was similar to the above reports that the haematological parameters were reverted back to near normal levels and non-significant ($P < 0.005$) in Pre-treated Group IV and standard drug treated Group V rats when compared with Group I control rats. Post treated Group III rats show significant ($P < 0.001$) changes (Hb and RBC levels were decreased with a concomitant increase in WBC) and significant ($P < 0.005$) increase in PCV and MCV were observed when compared with the Group I control rats. However, when compared with group II rats, a significant recovery in the haematological levels were observed in Group III rats shows the hepatoprotective potential of EEEA.

Table 10
Effect of EEEA on Blood Haematological Profile in Control and Experimental Animals

Parameters	Group I	Group II	Group III	Group IV	Group V
Haemoglobin (gm/dl)	14.62 ± 0.79	9.47 ± 0.27 *	11.49 ± 0.33 *	14.02 ± 0.34^{ns}	14.57 ± 0.38^{ns}
WBC(x10^3/L)	7.70 ± 0.26	12.55 ± 0.24 *	11.40 ± 0.40 *	8.05 ± 0.31^{ns}	7.55 ± 0.32^{ns}
RBC(x10^6/L)	6.35 ± 0.32	4.55 ± 0.39 *	5.05 ± 0.69 *	6.35 ± 0.31^{ns}	6.55 ± 0.45^{ns}
PCV (%)	46.31 ± 2.25	64.40 ± 1.95 *	51.20 ± 1.96 $	48.82 ± 1.26^{ns}	45.45 ± 1.36^{ns}
MCV (pg)	73.09 ± 5.17	142.25 ± 11.42 *	103.00 ± 14.89 $	77.06 ± 4.60^{ns}	69.72 ± 5.93^{ns}
MCH (fg)	23.05 ± 1.45	20.93 ± 1.82^{ns}	23.14 ± 3.39^{ns}	22.14 ± 1.53^{ns}	22.31 ± 1.16^{ns}
MCHC (%)	31.60 ± 1.73	14.71 ± 0.58 *	22.48 ± 1.12 $	28.73 ± 1.15^{ns}	32.08 ± 1.17^{ns}

Values are expressed as Mean ± SD for six rats

Data were analysed by one-way ANOVA followed by the individual comparison was obtained by Post-hoc Bonferroni test, a multiple comparison procedure by the SPSS. Statistically significant variation was derived by comparing Group I versus Group II, Group III, Group IV and Group V, $^*P = 0.000$, $^\$P = 0.001$, $^\#P = 0.010$, and ns = Non-significant ($P < 0.05$).

Mormphometric Analysis of Liver

The efficacy of any hepatic drug is essentially dependent on its ability in minimising the harmful effects or maintaining the normal hepatic physiology that has been distributed by a hepatotoxin. Figure 1 shows the effect of EEEA on morphological changes in liver of control and experimental rats. Gross liver pathology of the control Group I rats showed normal architecture with normal size and shape and brownish red liver with pale red tinge. Group II rats after DEN treatment showed grayish-white visible multi-nodules on the outer surface, about 1 mm in diameter. The liver showed a significant widening of intercellular spaces between hepatocytes, many invaginations of the cell membrane and irregularly shaped biliary canaliculi when compared with Control Group I rats. Our findings are similar to Hassan *et al.*, (2018) [45]. Post treated Group III rats showed a noticeable recovery in the liver architecture with normal size and shape when compared with DEN induced Group II rats. Pre-treated Group IV rats showed perceptible recovery in the liver architecture with normalizing of cell surface, cell membrane, reduced number of nodules, size and shape when compared to DEN induced Group II rats. No significant changes appeared between the Control Group I and Silymarin treated Group V rats.

Histopathological Studies

Liver

Figure 2 represent the photomicrographs of liver sections stained with hematoxylin and eosin (40X) from control and experimental rats of EEEA. Group I Control rats show normal liver tissue with hepatocytes, portal triad showing prominent central vein. DEN induced Group II rats show liver tissue with ballooning degeneration of the hepatocytes, nucleomegaly, kupffer cell activity, regular nuclear membrane and focal collection of inflammatory cells around portal triad with fibrosis. These observations were similar to the findings reported by Youssef *et al*., (2012) [46]. Mohammed *et al*., (2014) [28] who showed that treatment with DEN leads to vacuolated hepatocytes, dilated blood sinusoids, massive portal leukocyte infiltration and disordered arrangement of dysplastic hepatocytes. Post treated Group III rats show liver tissue with mild inflammation, degeneration and congested sinusoids. Pre-treated Group IV rats show liver tissue with normal architecture and central vein and Silymarin treated Group V rats show liver with normal histological arrangement and kupffer cell activity when compared with control rats. The present study confirmed the reliability of histopathological methods and biochemical indices in ascertaining liver integrity and functionality of the *Enhalus acoroides.*

Kidney

Plate showing histopathological observation (40X) of kidney tissue which shows Group I (a) Control rats show normal kidney with Bowman's capsules, Proximate convoluted Tubules (PCT), Distal convoluted Tubules (DCT) and Intersitium appears normal. The Group II (b) DEN induced rats distraction of bowman's capsules and glomeruli, congestion and sever degeneration of renal tubules when compared to control Group I. Group III (c) post treated rats show mild degeneration of bowman's capsules and glomeruli with congestion otherwise normal PCT and DCT and Group IV (d) Pre-treated rats shows normal Glomeruli with normal tubules. Group V (e) rats show kidney with normal Bowman's capsules and renal tubules.

Spleen

Plate showing histopathological observation (40X) of spleen tissue which shows Group I (a) Control rats show spleen with thin capsule, prominent red and white pulp. The Group II (b) DEN induced rats show spleen with congestion and moderate degeneration of red and white pulp when compared to control Group I. Group III (c) post treated rats show spleen with mild congestion and Group IV (d) Pre-treated rats' shows spleen with normal architecture. Group V (e) rats show spleen with normal histological structure.

Heart

Figure 5 depicted the photomicrographs of Heart sections stained with Hematoxylin and Eosin (40X) from control and experimental rats of EEEA. Group I Control rats' shows cardiac myocytes with normal striated muscle, homogenous sarcoplasm. DEN induced Group II rats show heart with broken cardiac

myocytes and irregular striated muscles. EEEA post treated Group III rats show heart with mild distraction of cardiac myocytes, intra muscular wall and centrally placed plump oval nuclei. EEEA pre-treated Group-IV rats show cardiac muscle with normal oval nuclei and Silymarin treated Group-V rats show heart with normal muscle fibres with normal nuclei when compared to the Control Group I rats.

Brain

Plate showing histopathological observation (40X) of brain tissue which shows Group I (a) Control rats show brain tissue with glial cells. The Group II (b) DEN induced rats show brain tissue with reactive gliosis, perivascular oedema and congestion when compared to control Group I. Group III (c) post treated rats show brain tissue with proliferation of neuroglial tissue, mild oedema, and prominent vessels and Group IV (d) Pre-treated rats' shows normal brain tissue. Group V (e) rats show brain with normal architecture of tissue with glial cells.

Conclusion

The present finding culminates that EEEA has the chemopreventive potential in DEN induced hepatic carcinoma which might be due to the antioxidant mechanisms. Decrease in the activity of transaminases and serum tumour markers which maintains the functional integrity because of the protective effect of EEEA. The biochemical markers and histopathological studies also explained the chemopreventive potential of EEEA. This proven anticancer activity of EEEA is mainly attributed to the presence of enriched therapeutic phytochemical constituents such as phenols, flavonoids, terpenoids. Further studies are needed to depict their mechanisms of action which is responsible for the inhibition of hepatic carcinoma. On the whole, our experimental studies suggest that ethanolic extract of *Enhalus acoroides* possess chemopreventive activity against DEN induced hepatocellular carcinoma in wistar albino rats.

Declarations

Submission declaration

The present work has not been published previously in any form and not under consideration for publication elsewhere.

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

Availability of Data and Material: If required will share the data.

Code Availability: Not applicable

Author's contributions: The corresponding author Dr.P.Amudha designed the protocol, assistance in animal euthanasia and supervised the research work. Dr.M.Jayalakshmi executed the experiment -

efficacy of EEEA in hepatocellular carcinoma using wistar albino rats, euthanasia of the animals. Dr.R.Vidya assistance in animal euthanasia and writing of the manuscript. All authors read and approved the final manuscript.

Ethics Approval: The procedure was done according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Rats (CPCSEA), New Delhi, India (IAEC No: XXI/VELS/PCOL/02/2000/CPCSEA/IAEC/01.12.2017).

Consent to Participate: Not applicable

Consent for Publication: Not applicable

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Figures

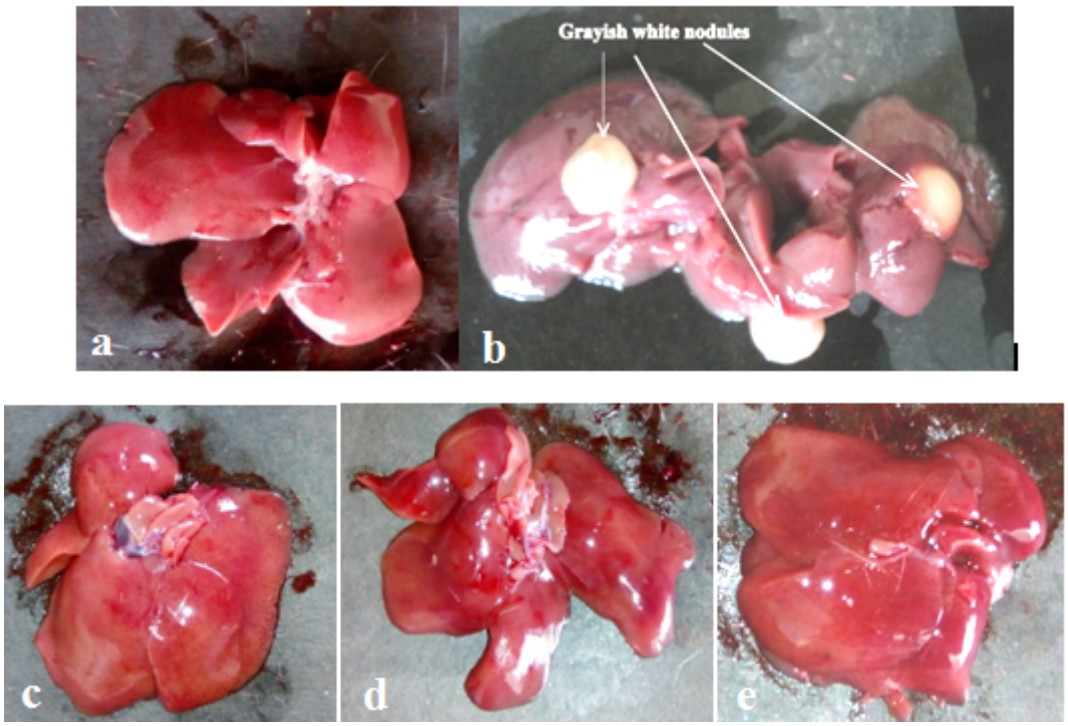

Figure 1

Gross Examination of Liver in Control and Experimental Animals: Plate showing the morphological changes in liver of control and experimental animals. The Group I (a) indicate the normal architecture with normal size and shape but Group II (b) showed a significant widening of intercellular spaces between hepatocytes, elongated microvilli over large regions of the cell surface, multiple nodules, size and shape, many invaginations of the cell membrane and irregularly shaped biliary canaliculi. In Group III (c) and IV (d) showed a noticeable recovery in the liver architecture with normal size and shape when

compared with Group II rats. No significant changes were observed in Group V (e) when compared to control Group I animals.

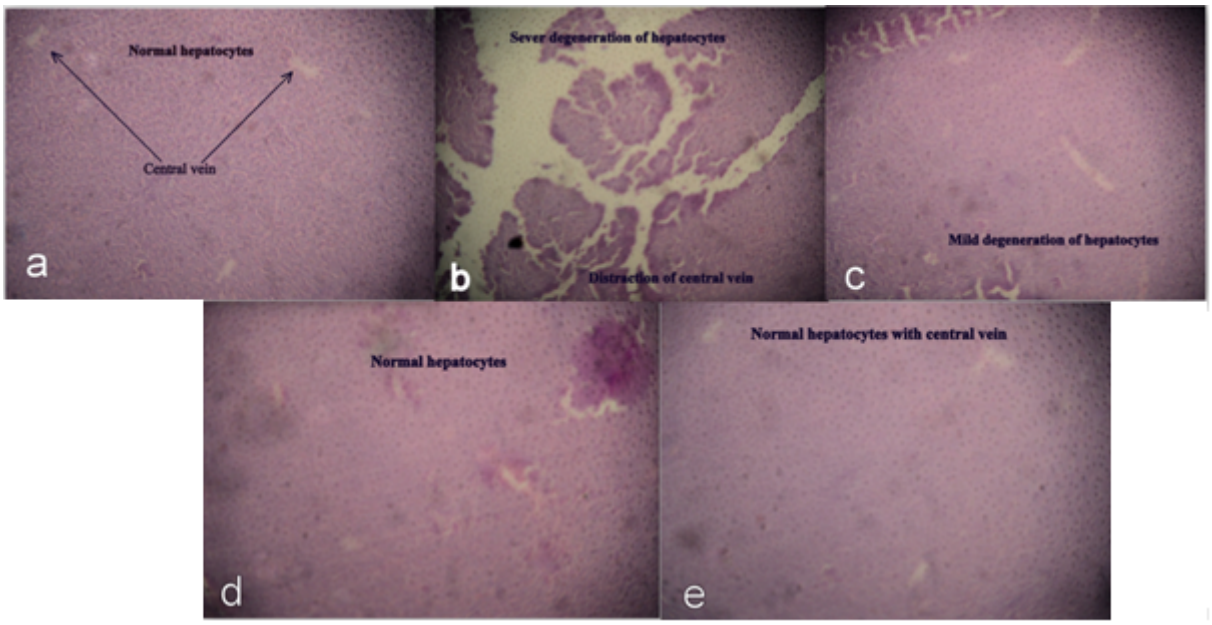

Figure 2

Liver Histopathology of Control and Experimental Animals: Plate showing histopathological observation (40X) of liver tissue which shows Group I (a) Control rats show normal liver tissue with hepatocytes, portal triad showing prominent central vein. The Group II (b) DEN induced rats show liver tissue with ballooning degeneration of the hepatocytes, nucleomegaly, kupffer cell activity, regular nuclear membrane and focal collection of inflammatory cells around portal triad with fibrosis when compared to control Group I. Group III (c) post treated rats show noticeable recovery of liver tissue with mild inflammation, degeneration and congested sinusoids and Group IV (d) Pre-treated rats shows liver tissue with normal architecture and central vein. Group V (e) rats show liver with normal histological arrangement and kupffer cell activity.

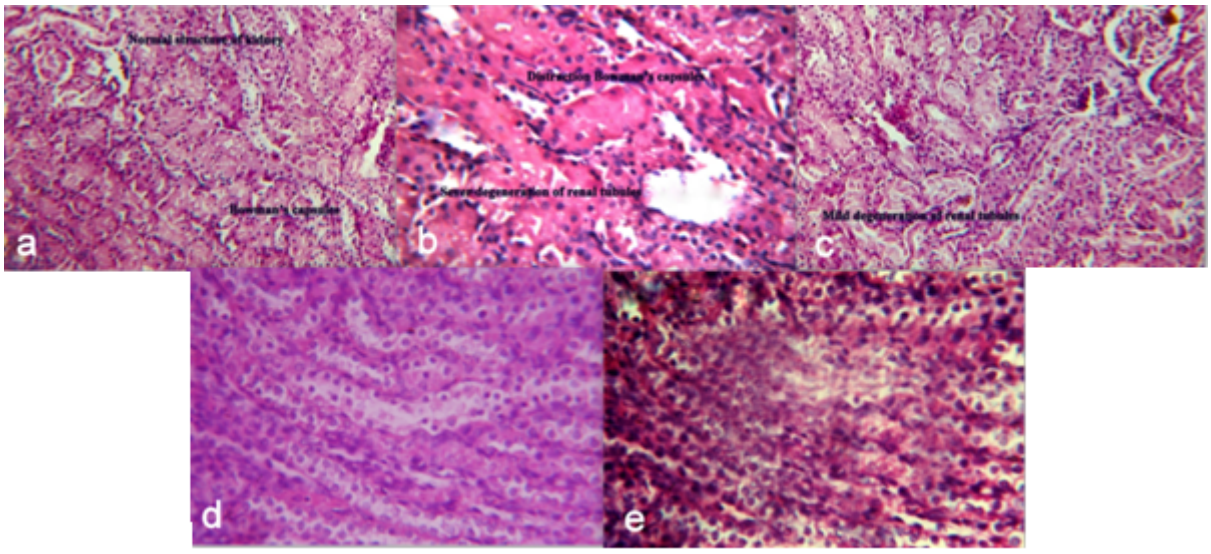

Figure 3

Kidney Histopathology of Control and Experimental Animals: Plate showing histopathological observation (40X) of kidney tissue which shows Group I (a) Control rats show normal kidney with Bowman's capsules, Proximate convoluted Tubules (PCT), Distal convoluted Tubules (DCT) and Intersitium appears normal. The Group II (b) DEN induced rats distraction of bowman's capsules and glomeruli, congestion and sever degeneration of renal tubules when compared to control Group I. Group III (c) post treated rats show mild degeneration of bowman's capsules and glomeruli with congestion otherwise normal PCT and DCT and Group IV (d) Pre-treated rats shows normal Glomeruli with normal tubules. Group V (e) rats show kidney with normal Bowman's capsules and renal tubules.

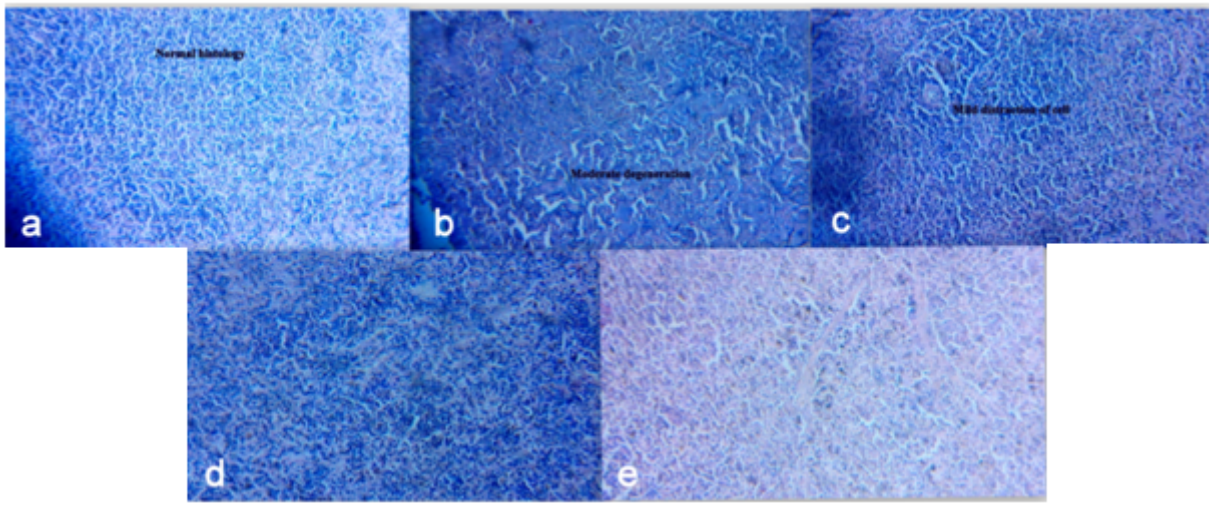

Figure 4

Spleen Histopathology of Control and Experimental Animals: Plate showing histopathological observation (40X) of spleen tissue which shows Group I (a) Control rats show spleen with thin capsule, prominent red and white pulp. The Group II (b) DEN induced rats show spleen with congestion and moderate degeneration of red and white pulp when compared to control Group I. Group III (c) post treated rats show spleen with mild congestion and Group IV (d) Pre-treated rats' shows spleen with normal architecture. Group V (e) rats show spleen with normal histological structure.

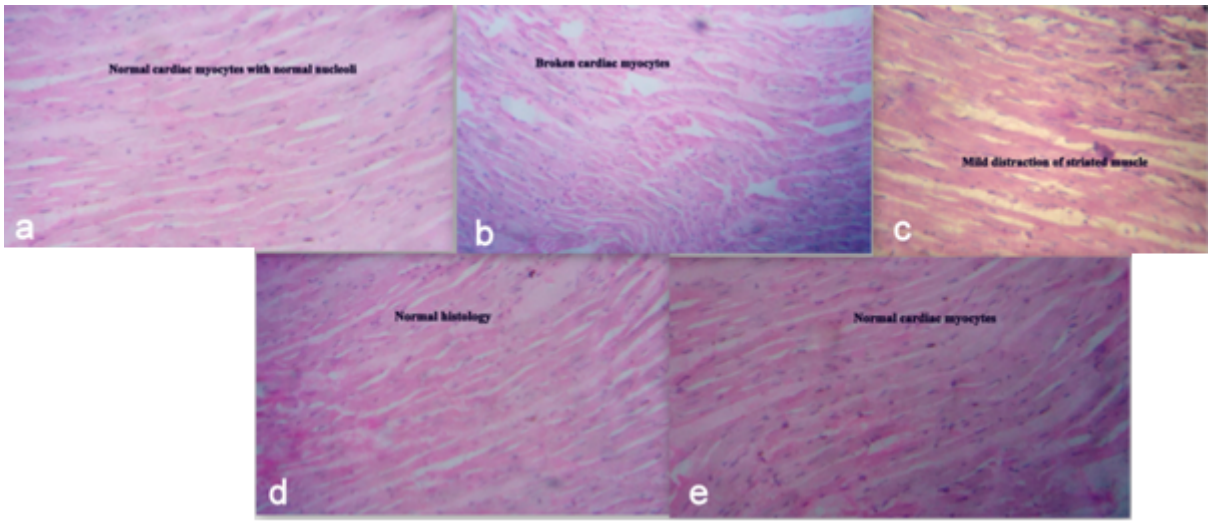

Figure 5

Heart Histopathology of Control and Experimental Animals: Plate showing histopathological observation (40X) of heart tissue which shows Group I (a) Control rats show cardiac myocytes with normal striated muscle, homogenous sarcoplasm. The Group II (b) DEN induced rats show heart with broken cardiac myocytes and irregular striated muscles when compared to control Group I. Group III (c) post treated rats show heart with mild distraction of cardiac myocytes, intra muscular wall and centrally placed plump oval nuclei and Group IV (d) Pre-treated rats' shows cardiac muscle with normal oval nuclei. Group V (e) rats show heart with normal muscle fibres with normal nuclei.

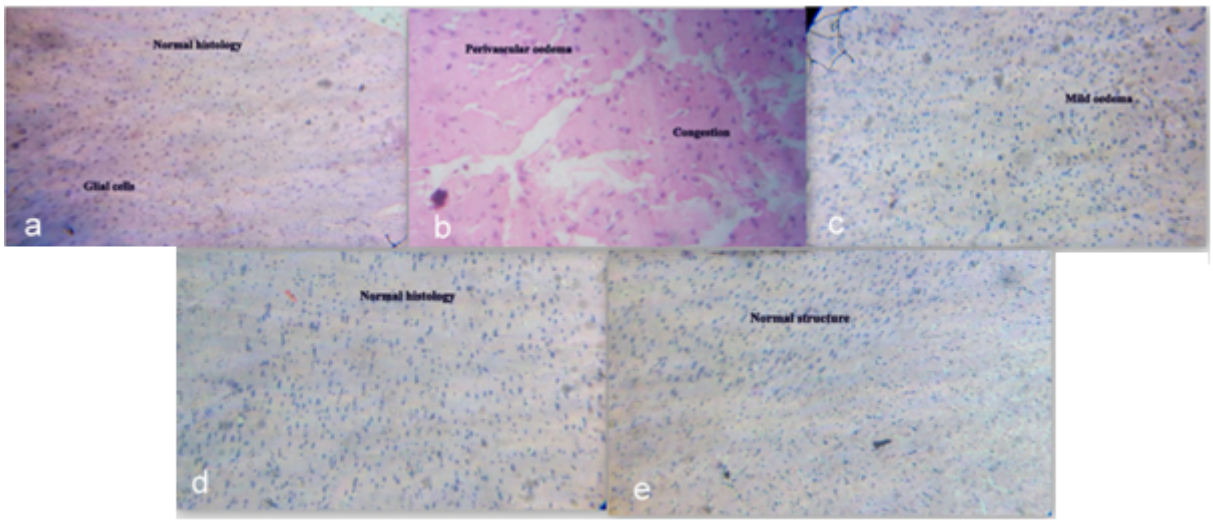

Figure 6

Brain Histopathology of Control and Experimental Animals: Plate showing histopathological observation (40X) of brain tissue which shows Group I (a) Control rats show brain tissue with glial cells. The Group II (b) DEN induced rats show brain tissue with reactive gliosis, perivascular oedema and congestion when compared to control Group I. Group III (c) post treated rats show brain tissue with proliferation of neuroglial tissue, mild oedema, and prominent vessels and Group IV (d) Pre-treated rats' shows normal brain tissue. Group V (e) rats show brain with normal architecture of tissue with glial cells.