Chemopreventive and Therapeutic Efficacy of *Enhalus acoroides* against Diethylnitrosamine Induced Hepatocellular Carcinoma in Wistar Albino Rats

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Abstract

Hepatocellular carcinoma is the second most cause of death among the various cancers worldwide. Recent research searching an alternative therapy for cancer treatment without or less side effects. Many studies indicated the beneficial effects of *Enhalus acoroides*. There has been no scientific validation on antioxidant and chemopreventive potential of ethanolic extract *E. acoroides* against hepatoma. To assess the hepatoprotective activity of *E. acoroides* (EEEA) against DEN-induced hepatoma using Wistar albino rats. Animals were distributed into five groups, each containing six rats. To Group I — control rats — normal saline given. Groups II, III, IV and V rats were injection of DEN at a dose of 100 mg/kg body weight i.p. to induce liver cancer. At the commencement of 6th week, Group III rats supplemented with EEEA at a dose of 200 mg/kg body weight/day upto 16 weeks. Group IV rats supplemented with EEEA for 1 week before the administration of DEN and continued till the sixteenth week. Group V supplementation of silymarin at a dose of 100 mg/kg body weight at the beginning of 6th week after the injection of DEN and continued upto 16 weeks and considered as positive control rats. The efficiency of *E. acoroides* for its antioxidant hepatoprotective and activity evaluated in rats against DEN-induced liver damage. The hepatoprotective ability of EEEA at a dose of 200 mg/kg was examined against DEN at a dose of 100 mg/kg/b.w. induced hepatotoxicity and analysed by evaluating serum liver and kidney marker levels, lipid profile (TG, HDL, LDL and total cholesterol) and serum tumour markers (DNA, RNA, AFP and CEA). Supplementation of EEEA to DEN treated rats was determined by evaluating various antioxidant biomarkers (SOD, CAT, GPx, GSH, Vit E and Vit C). Histopathological studies and morphometric gross analysis were also support the consequences of this study. A significant improvement of antioxidant defence and declined MDA levels within the serum of EEEA treated animals compared to the DEN-induced hepatoma. The supplementation of EEEA declined the serum liver, kidney and serum tumour marker levels and lipid profile as comparatively to Group I rats. The histopathological changes were changed on supplementation of EEEA demonstrating its protecting effects on hepatocytes as comparatively to Group I rats. Our significances recognized that crude extract (ethanol) of *E. acoroides* revealed a potential impact against DEN-induced hepatoma and assists as a superior choice for chemopreventive treatments.

Extended author information available on the last page of the article
Keywords  Hepatocellular carcinoma · Diethylnitrosamine · Enhalus acoroides · Tumour markers · Histopathology

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) is the second most cause of death among the various cancers worldwide. Approximately eight lakhs fresh cases of HCC every year occur worldwide which makes HCC as fifth place and ninth among women and among men globally.

The liver is the vital organ of the body where it metabolizes the ingested material and it is more prone to carcinogenic in silt. As the tolerance level of the liver is very high, hepatic carcinoma is seldom identified at the earlier stage, and once identified in most cases, the management has a humble prediction [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 hepatoma. Enhalus acoroides (Linnaeus f.) belong to the family of Hydrocharitaceae is an abundantly emerging sea grass in shoreline zones of Gulf of Mannar and normally found in muddy soils with submerged in shallow sea water along the coast. E. acoroides plays a significant role as cancer preventing agents [4]. The anticancer activities of E. acoroides in albino rats are not reported till now. Thus, the present study was undertaken to investigate the anticancer and antioxidant capability of the EEEA in 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, and is generally used as a carcinogen to induce hepatic cancer in rodents [5]. Silymarin has been recommended in liver cirrhosis, although its scientific efficiency is constantly deliberated due to its antioxidant and hepatoprotective activities [6]. Hence in this study, silymarin is commonly used standard for hepatoprotective agent against experimentally induced hepatotocellular carcinoma in animal models.

There were no scientific evidences on antioxidant efficiency of EEEA and silymarin against hepatoma remains unexplored. Hence, this study was done to assess the liver...
markers, tumour markers, status of antioxidants and histopathological analysis of extract (ethanol) of *E. 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 fine powder was soaked into 1:2 ratio ethanol with mild shaking for 3 days. Three days past, filter the macerate and reduced in a rotary evaporator. Lastly, the concentrated crude extract of *E. acoroides* was lyophilized into paste and was taken for the in vivo study.

**Experimental Rats and Diet**

Wistar albino male rats approximately weighing 180–200 g were taken for the study. Rats were procured from VISTAS, Chennai, India and kept in spacious cages (polypropylene) bedded with rice husk. The rodent room was well aerated and kept under standard experimental conditions (temperature 27 ± 2 °C and 12 h dark/light cycle) throughout the procedure. All the rats were served with normal pellet diet (VRK Nutritional, Maharashtra, India) and water ad libitum. They were acclimatized to the atmosphere 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-nitrobenzoic acid), glutathione (reduced and 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 200 mg/kg b.w. was fixed based on toxicity study carried in wistar albino rats with the extract of *Enhalus acoroides* [7]. Different doses of EEEA extract at the dose of 100 mg/kg b.w., 200 mg/kg b.w. and 400 mg/kg b.w. were treated for 4 weeks in rats. The effective dose of EEEA was assessed based on the biochemical
markers, haematological parameters and histopathological studies. Supplementation of EEEA extract at the doses of 200 mg/kg b.w. and 400 mg/kg b.w. for 4 weeks were seemed to be effective in rats. Among this, the minimal effective dose as 200 mg/kg b.w. was fixed as therapeutic dosage for the DEN-induced hepatoma study in Wistar albino rats.

**Experimental Design**

Rats were separated into five groups, each containing six rats. One group served as the control while the remaining four groups were injected with diethyl nitrosamine at a dose of 100 mg/kg/b.w. as an intraperitoneal injection to induce tumour [8]. The ethanolic extract of *Enhalus acoroides* (EEEA-200 mg/kg b.w.) was given in rats. Standard drug as silymarin at a dose of 100 mg/kg b.w. was used (Ramakrishnan et al., 2006). The initial body weights of the rats were recorded. The efficacy of EEEA for its hepatoprotective activity against DEN-induced liver damage was assessed 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 Groups II, III, IV and V, hepatic cancer was induced to rats using DEN injection at a dose of 100 mg/kg b.w. At the beginning of 6th week, Group III rats received EEEA at a dose of 200 mg/kg b.w. upto 16 weeks, Group IV rats received EEEA for 1 week and pursued till 16 weeks before the administration of DEN. Group V rats received silymarin at a dose of 100 mg/kg b.w. at the beginning of sixth week after the administration of DEN and pursued till 16 weeks and this group served as positive control.

**Collection of Blood and Preparation of Serum Sample**

After 16 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 min and refrigerated for another 30 min, the resultant clear part was centrifuged after 3000 rpm for 10 min and then the serum was separated and refrigerated.

**Haematology**

For analysing the haematological parameters, the animals were starved overnight and sacrificed and the blood samples were collected in the capillary tube. The haematological parameters including haemoglobin (Hb) concentration using Beacon Diagnostic Kit [9], white blood cells (WBC) count, red blood cell (RBC) count, packed cell volume (PCV) count [10], mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) were measured.
Serum Biochemical Markers

For analysing the serum biochemical markers, blood was collected and allowed to clot at room temperature. The blood samples were centrifuged at 3000 rpm for 10 min and then serum was separated. Serum biochemical parameters including aspartate and alanine aminotransferase (AST and ALT) by Reitman and Frankel (1957) method [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; and low density lipoprotein (LDL) [20] were determined by Microlab-300 autoanalyser (Merk Pvt. Ltd., Mumbai, India) and were analysed.

Serum Antioxidants

For analysing the serum antioxidants, blood was collected and allowed to clot at room temperature. The blood 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

At the end of the experiment periods, all the rats were sacrificed by cervical decapitation under anaesthesia and examine carefully for macroscopic abnormalities. The complete and relative (organ-to-body weight ratios) weights of major organs including the 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 the 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, brain and heart tissues were sliced to a thickness of 2.1 mm each and fixed for 72 h 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 6 rats in each group and statistically significant differences between mean values were determined by one-way analysis of variance.
(ANOVA) followed by post-hoc Bonferroni test. The Bonferroni test is a type of multiple comparison test used in statistical analysis 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**

**Impact of EEEA on Body Weight Changes in Experimental and Control 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 the liver. Table 1 represents the initial and final body weight changes of rats from Group I to Group V. No significant change was found in the initial body weight among the five groups of rats. Group II rats showed the body weight was decreased significantly ($P < 0.05$) in DEN treated as comparatively to Group I rats whereas in Group III rats post treated with EEEA, it appeared near normal as comparatively to Group I rats. This observation was in similar with Song et al. (2013) [27]. DEN brought a significant impairment in body growth. Pre-treated Group IV and standard-treated Group V rats were non-significant ($P < 0.05$) changes observed as comparatively to Group I rats. No death was observed in the experimental rats. This indicates the anticancerous effect of EEEA supplementation on hepatocellular carcinoma group.

**Impact of EEEA Supplementation on Body Weight in Experimental and Control Animals**

Table 2 shows the kidney, liver, brain, spleen and heart weights of experimental and control rats. Group II carcinogenesis rats were observed to have a significant increase ($P < 0.000$) in the organ weight as comparatively to Group I rats. The post-treated Group III rats show significant increase in the kidney, liver and spleen ($P < 0.001$), the brain ($P < 0.022$) and significant decline in heart ($P < 0.014$) weights as comparatively to Group I rats. This observation was similar to the findings of Furuta et al. (2008) [28] where he reported the slow develop in liver weight. It was also studied by Mohammed et al. (2014) [29] that the

| 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  | 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 analysed by one-way ANOVA followed by post-hoc Bonferroni test. The Bonferroni test is a type of multiple comparison test used in statistical analysis. 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 ($P < 0.05$)
liver weight in HCC-induced rat showed slight increase as comparatively to Group I rats. However, as compared with Group II rats, a gradual increase in the weights of the organs were detected in post-treated Group III rats indicating reduced proliferation of cells in these groups which shows the effect of EEEA supplementation on HCC. Pre-treated Group IV and silymarin-treated Group V rats show non-significant (P > 0.01) weight in the organs as comparatively to Group I rats.

Measurement of Tumour Size

Tumour sizes for Group II rats were measured using vernier callipers on the end of the experimental period which is depicted in the Table 3. An average size of tumour 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 tumour size (0.25, 0.13 and 0.14) respectively, while Group I control rats did not have tumour.

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

Table 4 shows the levels of serum antioxidant enzymes in experimental and control rats. In carcinogenesis, lipid peroxidation plays a major role [30], and it 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

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

Data were analysed by one-way ANOVA followed by post-hoc Bonferroni test. The Bonferroni test is a type of multiple comparison test used in statistical analysis. 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)

| Liver | Group I | Group II | Group III | Group IV | Group V |
|-------|---------|----------|-----------|----------|---------|
| Size of tumour (mm) | 0 | 7.31 ± 0.47* | 0.20 ± 0.05 ns | 0.13 ± 0.03* | 0.14 ± 0.02 ns |

Values are expressed as mean ± SD for six rats (n = 6) and data were analysed by one-way ANOVA followed by post-hoc Bonferroni test. The Bonferroni test is a type of multiple comparison test used in statistical analysis. 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)
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 Group I rats. 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 as compared with DEN-induced Group II rats. Thus, the drug EEEA restored the changes to near normal by its antioxidant efficiency. The Group I rats control exhibit a near-normal value of these enzymes whereas post-treated Group III rats show significant ($P < 0.000$) increase in MDA and significant ($P < 0.000$) decrease in the levels of GSH, SOD, CAT, GPx, vitamin C and vitamin E when compared with control Group I rats. Along with vitamin E and glutathione, vitamin C also scavenges and detoxifies free radicals [32]. However, as compared with Group II rats, a significant recovery in the antioxidant status was 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 supplementation on serum antioxidant in experimental and control 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 ± 0.45ns | 7.29 ± 0.56ns |
| GSH (mg/ dl)       | 8.45 ± 0.87 | 5.50 ± 0.84$^*$ | 6.71 ± 0.38$^s$ | 7.94 ± 0.49$^s$ | 8.14 ± 0.54$^s$ |
| SOD (U/ml)         | 4.76 ± 0.57 | 3.24 ± 0.21$^t$ | 3.88 ± 0.13$^s$ | 4.56 ± 0.22$^s$ | 4.51 ± 0.38$^s$ |
| Catalase (U/ml)    | 9.43 ± 0.55 | 6.34 ± 0.51$^*$ | 7.56 ± 0.18$^*$ | 9.02 ± 0.51$^s$ | 9.61 ± 0.83$^s$ |
| GPx (U/ml)         | 9.19 ± 0.71 | 6.54 ± 0.39$^*$ | 7.80 ± 0.21$^s$ | 8.83 ± 0.63$^s$ | 9.03 ± 0.46$^s$ |
| Vit-C (µg/dl)      | 4.51 ± 0.37 | 2.18 ± 0.30$^t$ | 3.20 ± 0.66$^s$ | 4.41 ± 0.56$^s$ | 4.61 ± 0.36$^s$ |
| Vit-E (µg/dl)      | 3.85 ± 0.26 | 2.16 ± 0.24$^*$ | 2.66 ± 0.19$^*$ | 3.46 ± 0.43ns | 3.60 ± 0.45ns |

Values are expressed as mean ± SD for six rats

Data were analysed by one-way ANOVA followed by post-hoc Bonferroni test. The Bonferroni test is a type of multiple comparison test used in statistical analysis. Statistically significant variation was derived by comparing Group I versus Group II, Group III, Group IV and Group V, $^tP < 0.000$, $^sP < 0.001$ and ns non-significant ($P < 0.05$)

SOD (U), 50% of NBT reduction/min; Catalase (U), µmol of H$_2$O$_2$ consumed/min; GPx (U), µmole of GSH utilized/min

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

Table 5 shows the levels of liver tissue antioxidant in experimental and control 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 as comparatively to Group I rats. 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 as compared with DEN-induced Group II rats. The Group I rats control 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 as comparatively to Group I rats. In the antioxidant system, SOD is the first line of defence against the oxidative damage by superoxide radicals [35]. However, as compared with DEN-induced Group II rats, a significant recovery in the antioxidant status was observed in post-treated Group III rats. The present investigation highlights the chemo preventive 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.

### Effect of EEEA Supplementation on Tumour Markers in Experimental and Control Rats

Tumour markers produced by the tumour 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 tumour markers such as DNA, RNA, AFP, and CEA of experimental and control rats. The levels of tumour markers were significantly ($P < 0.000$) elevated in DEN-induced Group II rats compared with the control Group I rats. 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 tumour cells. Present findings are similar to the Pakkir et al. (2011) [39] study. Tumour 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 as comparatively to Group I rats. Present findings are in concordance with Nermin et al. (2008) [40] study. Post-treated Group III rats show significant

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

Values are expressed as mean±SD for six rats

Data were analysed by one-way ANOVA followed by post-hoc Bonferroni test. The Bonferroni test is a type of multiple comparison test used in statistical analysis. 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 ($P < 0.05$)


(P < 0.009, P < 0.003, and P < 0.002) elevation in RNA, AFP, and CEA levels respectively as compared with the control Group I rats. However, a significant decrease as compared with DEN-induced Group II rats and significant increase as comparatively to Group I rats in the levels of these tumour markers indicate a significant antitumour activity of ethanolic extract of Enhalus acoroides.

**Effect of EEEA Supplementation on Serum Liver Markers in Experimental and Control 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 experimental and control rats. DEN-induced Group II rats exhibit significant change (P > 0.000) in the activity of these liver markers as comparatively

**Table 6** Effect of EEEA supplementation on tumour markers in experimental and control 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 post-hoc Bonferroni test. The Bonferroni test is a type of multiple comparison test used in statistical analysis. 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)

**Table 7** Effect of EEEA supplementation on serum liver markers in experimental and control 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 post-hoc Bonferroni test. The Bonferroni test is a type of multiple comparison test used in statistical analysis. Statistically significant variation was derived by comparing Group I versus Group II, Group III, Group IV and Group V, *P = 0.000, $P = 0.001, #P = 0.011, @P = 0.038 and ns non-significant (P < 0.05)
to Group I rat. Rocchi et al. (1997) [41] stated that there was an elevation in the levels of transaminases in serum of HCC patients. In concurrent with the above reports, an elevated serum aminotransferase was observed in Group II rats bearing HCC, whereas they appeared to be neutralized to near normal and non-significant ($P < 0.05$) in Group IV EEEA pre-treated rats. Due to the development of tumour, tissue gets damaged which leads to the elevation of ALP into circulation [42] and this enzyme level have been increased in serum of the tumour-bearing rats and this elevation is significantly suppressed by the supplementation of EEEA. Standard drug-treated Group V rats do not show noticeable changes in these parameters and non-significant ($P < 0.05$) as comparatively to Group I rats. Post-treated Group III rats show significant changes in GGT ($P < 0.001$), ALT, AST, ALP, bilirubin and protein ($P < 0.001$), albumin ($P < 0.038$) levels as comparatively to Group I rats. 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 Group I rats. This indicates the protective effect of EEEA supplementation over liver and improvement in its functional efficiency.

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

Table 8 indicates the effects of EEEA activity of the serum kidney marker such as urea and creatinine of experimental and control 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 causes significant ($P < 0.000$) increase in the levels of urea and creatinine as comparatively to Group I rats. 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 as comparatively to Group I rats. Post-treated Group III rats show significant ($P < 0.001$ and $P < 0.041$) increase in urea and creatinine levels respectively as comparatively to Group I rats. However, a significant decrease as compared with DEN-induced Group II rats and significant increase as comparatively to Group I rats in the levels of these kidney marker enzymes shows the potential renal functions of EEEA.

Table 8 Effect of EEEA supplementation on serum kidney markers in experimental and control 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 post-hoc Bonferroni test. The Bonferroni test is a type of multiple comparison test used in statistical analysis. 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 Supplementation on Serum Lipid Profile in Experimental and Control Rats

Table 9 indicates the effects of EEEA activity of lipid parameters such as triglycerides (TG), total cholesterol (TC), HDL and LDL in serum of experimental and control rats. The levels of total cholesterol, triglycerides and LDL in serum of experimental and control rats were significantly \((P < 0.001)\) increased, and HDL level was significantly \((P < 0.001)\) decreased in Group II DEN-induced HCC rats as comparatively to Group I rats. The treatment with EEEA showed significantly altered the levels of lipid profile as compared with DEN-induced Group II rats. EEEA post-treated Group III rats showed significantly decreased levels of total cholesterol and triglycerides as 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 Group I rats control indicated the effects of EEEA in maintaining the normal status of lipid profile in the serum.

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

Table 10 shows the levels of RBC, WBC, Hb, PCV, MCV, MCH and MCHC in experimental and control rats. Mean value of haemoglobin in Group II rats decreased as compared to normal Group I rats control 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 as comparatively to Group I 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 was observed as comparatively to Group I rats. However, as compared with Group II rats, a significant recovery in the haematological levels was observed in Group III rats which show the hepatoprotective potential of EEEA.

### Table 9 Effect of EEEA supplementation on serum lipid profile in experimental and control 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 ± 2.38 | 110.83 ± 2.43 |
| Total cholesterol (mg/dl) | 89.90 ± 2.82  | 150.37 ± 2.40 | 133.71 ± 2.43 | 93.70 ± 1.76  | 91.46 ± 2.89  |
| HDL (mg/dl)      | 35.31 ± 1.92  | 22.69 ± 1.31  | 26.14 ± 1.82  | 33.50 ± 2.17  | 31.21 ± 2.01  |
| LDL (mg/dl)      | 32.72 ± 3.73  | 99.42 ± 2.60  | 82.67 ± 2.35  | 37.72 ± 1.74  | 38.09 ± 4.68  |

Values are expressed as mean ± SD for six rats

Data were analysed by one-way ANOVA followed by post-hoc Bonferroni test. The Bonferroni test is a type of multiple comparison test used in statistical analysis. 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 \((P<0.05)\)

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Morphometric Analysis of Liver

The efficacy of any hepatic drug is essentially dependent on its ability in minimizing the harmful effects or maintaining the normal hepatic physiology that has been distributed by a hepatotoxin. Figure 1 shows the effect of EEEA supplementation on morphological changes in the liver of experimental and control rats. Gross liver pathology of the Group I rats control showed regular architecture with normal shape, size and brownish red liver with pale red tinge. Group II rats after DEN treatment displayed grayish-white visible multi-nodules on the outer surface about 1 mm in diameter. The liver showed a noteworthy widening of intercellular spaces between hepatocytes, irregularly shaped biliary canaliculi and many invaginations of the cell membrane as comparatively to Group I rats. Our findings are similar to Hassan et al. (2018) [45]. Post-treated Group III rats exhibited a noticeable retrieval in the liver architecture with normal shape and size as 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 as 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 represents the photomicrographs of liver sections stained with eosin and haematoxylin (40X) from experimental and control rats of EEEA. Group I control rats showed 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 as compared to Group I rats. The present study confirmed the reliability of histopathological methods and biochemical indices in ascertaining liver integrity and functionality of the *Enhalus acoroides*.

**Kidney**

Figure 3 represents the photomicrographs of kidney sections stained with eosin and haematoxylin (40X) from experimental and control rats of EEEA. Group-I control rats show normal kidney with Bowman’s capsules, proximal convoluted tubules (PCTs), distal convoluted tubules (DCTs) and interstitium appears normal (indicates arrow mark). DEN-induced Group II rats show distraction of Bowman’s capsules and glomeruli, congestion and severe degeneration of renal tubules (indicates arrow mark). EEEA post-treated Group III rats show mild degeneration of Bowman’s capsules and glomeruli with congestion otherwise normal PCT and DCT (indicates arrow mark). Pre-treated Group IV rats show normal glomeruli with normal tubules. Silymarin-treated Group V rats show kidney with normal Bowman’s capsules and renal tubules (indicates arrow mark).
Fig. 2  Liver histopathology of experimental and control 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 (indicates arrow mark) as comparatively to Group I rats (indicates arrow mark). Group III (c) post-treated rats show noticeable recovery of liver tissue with mild inflammation, degeneration and congested sinusoids (indicates arrow mark) and Group IV (d) pre-treated rats show liver tissue with normal architecture and central vein. Group V (e) rats show liver with normal histological arrangement and kupffer cell activity (indicates arrow mark).

Fig. 3  Kidney histopathology of experimental and control animals. Plate showing histopathological observation (40X) of kidney tissue which shows Group I (a) control rats show normal kidney with Bowman’s capsules, proximal convoluted tubules (PCTs), distal convoluted tubules (DCTs) and interstitium appears normal. The Group II (b) DEN-induced rats distraction of Bowman’s capsules and glomeruli, congestion and sever degeneration of renal tubules as comparatively to Group I rats. 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 show normal glomeruli with normal tubules. Group V (e) rats show kidney with normal Bowman’s capsules and renal tubules.
Spleen

Figure 4 represents the photomicrographs of spleen sections stained with eosin and haematoxylin (40X) from experimental and control rats of EEEA. Group I control rats show spleen with thin capsule, prominent red and white pulp. DEN-induced Group II rats show spleen with congestion and moderate degeneration of red and white pulp. Post-treated Group-III rats show spleen with mild congestion. Pre-treated Group IV rats show spleen with normal architecture and Group V rats show spleen with normal histological structure compared to the control Group I rats.

Heart

Figure 5 depicted the photomicrographs of heart sections stained with eosin and haematoxylin (40X) from experimental and control rats of EEEA. Group I control rats show cardiac myocytes with normal striated muscle, homogenous sarcoplasm (indicates arrow mark). DEN-induced Group II rats show heart with broken cardiac myocytes and irregular striated muscles (indicates arrow mark). EEEA post-treated Group III rats show heart with mild distraction of cardiac myocytes, intra muscular wall and centrally placed plump oval nuclei (indicates arrow mark). 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 as comparatively to Group I rats (indicates arrow mark).

Brain

Figure 6 depicted the photomicrographs of brain sections stained with eosin and haematoxylin (40X) from experimental and control rats of EEEA. Group I control rats show brain tissue with glial cells (indicates arrow mark). DEN-induced Group II rats show brain tissue with reactive gliosis, perivascular oedema and congestion (indicates arrow mark).
Mark). EEEA post-treated Group III rats show brain tissue with proliferation of neuroglial tissue, mild oedema and prominent vessels. Pre-treated Group IV rats show normal brain tissue. Silymarin-treated Group V rats show brain tissue with glial cells (indicates arrow mark).
Conclusion

The present finding culminates that EEEA has the chemopreventive potential in DEN-induced hepatic carcinoma which might be attributed 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 possible of EEEA. This proven anticancer activity of EEEA is may be due to enriched therapeutic phytochemical constituents such as phenols, flavonoids and terpenoids. Supportive studies are desired to depict their mechanisms of action which is accountable for the inhibition of hepatic carcinoma. Overall, the experimental studies recommend that ethanolic extract of Enhalus acoroides possesses potential chemopreventive activity against DEN-induced hepatoma in Wistar albino rats.

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Author Contribution The corresponding author PA designed the protocol, assistance in animal euthanasia and supervised the research work. MJ executed the experiment — efficacy of EEEA in hepatocellular carcinoma using Wistar albino rats and euthanasia of the animals. RV: assistance in animal euthanasia. BNP helped in writing of the manuscript. All authors read and approved the final manuscript.

Data Availability If required will share the data.

Code Availability Not applicable.

Declarations

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

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.

Conflict of Interest The authors declare no competing interests.

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