Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 5, Suppl 1, 2013

Research Article

ETHANOLIC EXTRACT OF *CARUM CARVI* (EECC) PREVENTS N-NITROSODIETHYLAMINE INDUCED PHENOBARBITAL PROMOTED HEPATOCARCINOGENESIS BY MODULATING ANTIOXIDANT ENZYMES

GOWTHAM KUMAR SUBBARAJ[1,3], LANGESWARAN KULANTHAIVEL[2], REVATHY RAJENDRAN[3] RAMAKRISHNAN VEERABATHIRAN[1]

[1]Department of Genetics, Faculty of Allied Health Sciences, Chettinad Academy of Research and Education, Kelambakkam, Chennai 603103, [2]Department of Industrial Biotechnology, Bharath University, Tambaram, Chennai 600073, [3]Department of Pharmacology and Environmental Toxicology, University of Madras, Taramani Campus, Chennai 600113. Email: gowtham_phd@yahoo.com

Received: 04 Dec 2012, Revised and Accepted: 26 Jan 2013

ABSTRACT

Introduction: Hepatocellular carcinoma (HCC) is one of the most fatal hepatic cancers and its occurrence has been increasing steadily over the past decades. In recent years, natural agents such as fruits, vegetables have drawn considerable attention in scientific community for their therapeutic efficacy against various ailments such as cancer prevention and therapy.

Materials and methods: Liver cancer was induced in experimental rats by administration of single intraperitoneal (ip) injection of N-nitrosodiethylamine (NDEA) at a dose of 200 mg/kg body weight in saline. Two weeks after administration of NDEA, phenobarbital (PB) at a concentration of 0.05% was incorporated into rat chow for upto 14 successive weeks to promote the liver cancer. After the liver cancer was initiated in Group II and III, ethanolic fruit extract of *Carum carvi (EECC)* was treated at the dose of 10 mg/kg /bw/orally to Group III and Group IV animals for up to 28 days.

Results: After the experimental episode, levels of enzymic and non-enzymic antioxidants, levels of pro-apoptotic expression of Bcl-2 and the histopathological investigation of control and treated groups were analyzed. From the results we found that EECC was able to prevent the cancer progression by modulating the antioxidant system and also regulatory role in the proteins of anti-apoptotic flow against the NDEA induced oxidative stress mediated ailments.

Conclusion: The ethanolic fruit extract of *Carum carvi (EECC)* could be as safe and more efficient in the liver cancer treatment.

Keywords: Ethanolic extract of *Carum carvi,* N-nitrosodiethylamine, Phenobarbital, Antioxidant, Bcl2 protein

INTRODUCTION

N-Nitrosodiethylamine (NDEA) is an N-nitroso alkyl compound depicts as an effective hepatotoxin to experimental animals, producing toxicity after repeated administration. NDEA became metabolically active by the action of cytochrome p450 enzymes to produce reactive electrophiles, which increase oxidative stress level leading to cytotoxicity, mutagenecity and carcinogenicity. Oxidative stress is considered as critical mechanism contributing to NDEA induced hepatotoxicity. An enhancement of NDEA-initiated liver carcinogenesis by Phenobarbital (PB) in male wistar rats was previously reported by Weisburger *et al*., [1]. In NDEA-initiated male wistar rats, PB increased the development of hepatic tumors and the mortality rate through β catenin mutation was reported by Awuah *et al*., [2].

Natural dietary substance that could modulate lipid peroxidation, TCA enzymes,membrane bound ATPase and antioxidant systems thereby alleviates liver carcinogenesis [3; 4; 5]. *Carum carvi* Linn, commonly known as caraway (Umbelliferae) have been reported to possess hypolipidic activity [6].The main constituents of *Carum carvi* are the volatile oils including carvone (40–60%), limonene, carveol, dihydrocarveol and thymol in addition to glycosides and flavanoids [7]. Kamaleeswari *et al*., [8] have reported that *Carum carvi* has antioxidant activity in rat colon carcinogenesis. Therefore, the present investigation was undertaken to evaluate the modulatory effect of ethanolic fruit extract of *Carum carvi (EECC)* against N-nitrosodiethylamine induced liver carcinogenesis in male wistar rats with reference to antioxidant and pro-apoptotic activity.

MATERIALS AND METHODS

Plant material and preparation of extract

The whole fruit of *Carum carvi* was collected from Chennai, Tamil nadu, India and was identified and authenticated by a botanist and a voucher specimen has been kept in the laboratory for future reference. The whole material was dried under shade, powdered and extracted with boiling water for 30 minutes. The filtrate was evaporated under vacuum below 70^{o}C in a vacuum drier and the yield of ethanolic fruit extract of *Carum carvi (EECC)* was used to treat the experimental rats.

Animals

Male adult albino rats of Wister strain weighing between 180 ± 30 g were procured from Central Animal House Facility, Dr.ALMPGIBMS, University of Madras, Chennai, India. The animals were kept in polypropylene cages and received standard diet and water *ad libitum*. Guidelines of experiments on animals, as mentioned by the Ministry of Social Justice and Empowerment of India were followed (IAEC: 07/013/08 Phase II).

Tumor induction

Group II and III animals received a single intraperitoneal (ip) injection of N-Nitrosodiethylamine (NDEA) at a dose of 200 mg/kg body weight in saline to induce liver cancer. Two weeks after administration of NDEA, Phenobarbital at a concentration of 0.05% was incorporated into rat chow for up to 14 successive weeks to promote liver cancer.

Experimental design

The male wistar rats were divided into four groups, comprising of six animals in each groups and were given 1ml of 1 % methylcellulose (as a vehicle to the ethanolic extract of *Carum carvi (EECC)* for 28 days: Group I animals served as control. Group II and III animals received a single intraperitoneal (ip) injection of N-nitrosodiethylamine (NDEA) at a dose of 200 mg/kg body weight in saline to induce liver cancer. Two weeks after administration of NDEA, phenobarbital (PB) at a concentration of 0.05% was incorporated into rat chow for upto 14 successive weeks to promote the liver cancer. Group III Liver cancer bearing animals were treated with 10 mg of *EECC* orally/kg body weight for 28 days. Group IV animals received 10 mg of *EECC* orally/kg body weight for 28 days.

Collection of samples

At the end of the experimental period, all the animals were anesthetized with diethyl ether, which were sacrificed by cervical decapitation. Blood was collected and the serum was separated. The liver tissue was dissected out and washed 2 to 3 times with saline and stored in formalin for histopathological investigation.

Western blotting analysis

Tumors were disaggregated by treatment with an enzyme mixture containing 2.0 g/l collagenase, 2.0 g/l DNase for 90 min at 37°C. The resulting cell suspensions were filtered through a 30 µm nylon mesh. Centrifuged cells were washed in phosphate-buffered saline and boiled in Laemmli lysis buffer for 5 min. Liver tissue proteins (50 µg /lane) were separated on 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane (PVDF). The membrane was probed with Bcl-2 specific antibody (1:2000 dilutions) to determine their levels which were then visualized under Gel Doc (GeNei) system using β actin as an internal control.

Statistical analysis

Data are presented as the mean ± standard deviation (SD). One way analysis of variance (ANOVA) followed by Tukey's multiple comparison method was used to compare the means of different groups of by using SPSS 10.0 student's versions. Comparisons were made between group II and III with group I and IV for animal studies. P<0.05 was considerable statistically significant in all cases.

RESULTS

Table 1, 2 and Fig 1, 2, and 3 shows the activities of SOD, CAT, GPx, GSH, Vitamin C and Vitamin E in serum and liver of experimental animals. NDEA induced cancer bearing animals shows a significant decrease of enzymic antioxidants (p<*0.05*) and non-enzymic antioxidants (Glutathione, Vitamin C and Vitamin E p<0.05) in serum. In *Ethanolic extract of Carum carvi (EECC)* treated group III animals, these antioxidants were significantly increased (SOD, CAT, GPx, GSH, Vitamin C, Vitamin E p<0.05).There was no significant differences were observed in group IV compared with group I animals.

Table: 1 The levels of enzymic antioxidants enzyme in liver of control and experimental animals

Parameters	Group I	Group II	Group III	Group IV
Super Oxide dismutase (Units/mg protein / min)	12.51±0.96	7.75±0.62 $a^{\#}$	8.85±0.74 $b^{\#}$	12.58±0.54 c^{NS}
Catalase (µ mol of H_2O_2 consumed / mg protein / min)	54.33±4.41	40.57±3.21 $a^{\#}$	47.32±3.77 $b^{\#}$	54.61±4.32 c^{NS}
Glutathione peroxidase (µ g of GSH utilized / mg protein / min)	9.69±0.73	6.83±0.58 $a^{\#}$	7.77±0.65 $b^{\#}$	9.58±0.74 c^{NS}

Each Value represents mean ± SD of Six animals. a – Compared with Group I, b – Compared with Group II,

c – Compared with Group I *p <0.001, $^{@}$p<0.01, $^{\#}$p<0.05, ^{NS} – Not Significant

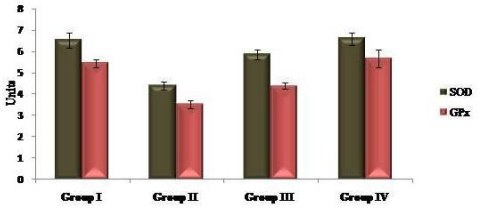
Table: 2 The levels of non-enzymic antioxidants enzymes in liver of control and experimental animals

Parameters	Group I	Group II	Group III	Group IV
Glutathione (mg of GSH/mg protein / min)	11.63±0.99	8.21±0.64 $a^{\#}$	10.83±0.73 $b^{\#}$	11.38±0.85 c^{NS}
Vitamin C (mg/g of wet tissue)	3.54±0.25	2.04±0.16 $a^{\#}$	2.96±0.24 $b^{\#}$	3.17±0.30 c^{NS}
Vitamin E (mg /g of wet tissue)	5.0±0.41	3.50±0.26 $a^{\#}$	4.12±0.24 $b^{\#}$	5.17±0.42 c^{NS}

Each Value represents mean ± SD of Six animals. a – Compared with Group I, b – Compared with Group II,

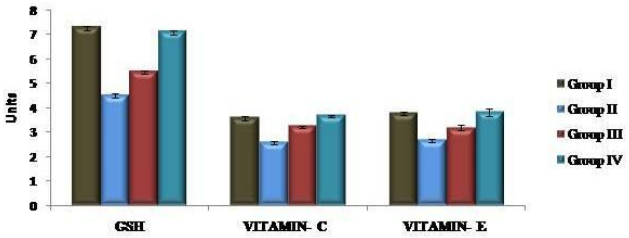
c – Compared with Group I *p <0.001, $^{@}$p<0.01, $^{\#}$p<0.05, ^{NS} – Not Significant

Fig 1.Effect of *Carum carvi* on enzymic antioxidant levels in serum of control and experimental animals



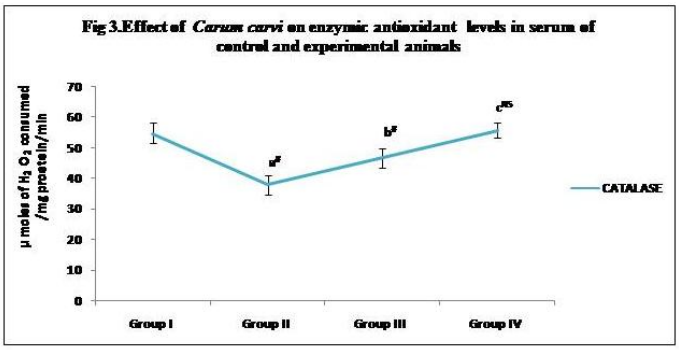
Units: SOD-50% inhibition of pyrogallol auto-oxidation/mg protein/min; GPX- µM of Glutathione oxidized/mg protein/min
Each Value represents mean ± SD of Six animals;a – Compared with Group I; b – Compared with Group II; c – Compared with Group I
***p <0.001, @p<0.01, $p<0.05, NS – Not Significant.**

Fig 2.Effect of *Carum carvi* on non-enzymic antioxidant levels in serum of control and experimental animals



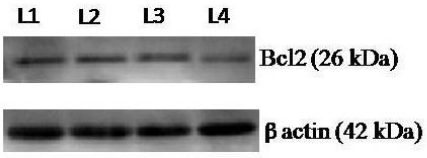
Units -GSH-µg/mg protein.; Vit-C-mg/mg protein; Vit -E- mg/mg protein. Each Value represents mean ± SD of Six animals
a – Compared with Group I; b – Compared with Group II; c – Compared with Group I;*p <0.001, @p<0.01, $p<0.05, NS – Not Significant.

Fig 3.Effect of *Carum carvi* on enzymic antioxidant levels in serum of control and experimental animals



Each Value represents mean ± SD of Six animals;a – Compared with Group I; b – Compared with Group II; c – Compared with Group I
***p <0.001, @p<0.01, $p<0.05, NS – Not Significant.**

Fig 4.Effect of *Carum carvi* on Bcl2 anti-apoptotic protein expression



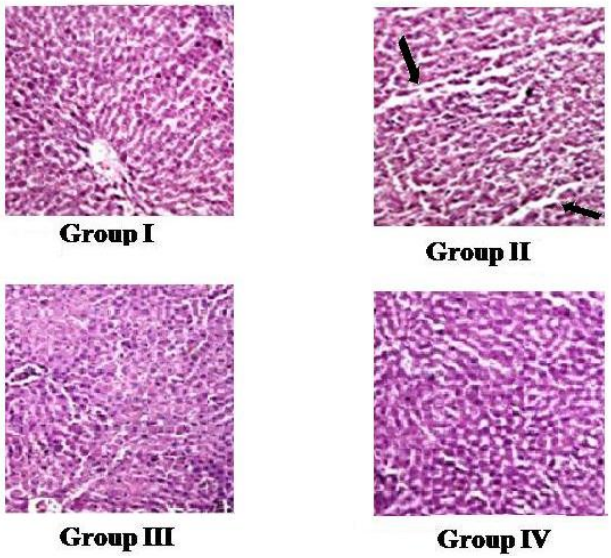
L1-Control; L2-NDEA; L3-NDEA+ Carum carvi ;L4- Carum carvi

Western blot analysis of Bcl-2 protein expression in liver of control and experimental animals.

Figure 4 showed the expression of the Bcl-2 protein in control and experimental animals. The expression of Bcl-2 protein was found to be significantly increased in group II liver cancer bearing animals when compared to group I control animals. Conversely, the expression of Bcl-2 protein was significantly decreased in group III ethanolic extract of *Carum carvi (EECC)* treated animals, when compared to group II cancer-bearing animals. However, there was no significant change in the expression of Bcl-2 protein was observed in group IV *ethanolic extract of Carum carvi (EECC)* alone treated animals when compared to group I control animals.

Figure 5 depict the histopathological observation of experimental rats. Group I animals' revealed normal architecture and cells with granulated cytoplasm and small uniform nuclei. Group II animals revealed loss of architecture, showed a tendency to spread by intra-hepatic veins, both hepatic and portal with significant tumor thrombi within portal vessels. Group III animals showed almost uniform appearance of hepatocytes with regular vesicular nuclei. Group IV animals exhibited normal architecture compared to group I animals indicating the non-toxic nature of the tested extract, *EECC*.

Fig 5 Histopathological examination of control and experimental rats



DISCUSSION

The anti-oxidative defense system may scavenge Reactive Oxygen Species (ROS) that play a significant role in the initiation of lipid peroxidation. This defense system works through enzymatic and non-enzymatic components. GPx and SOD are key enzymes in the body to eliminate free radicals. SOD can change the highly toxic superoxide anions ($O2^-$) to O_2 and H_2O_2, then H_2O_2 and O_2^- react while the iron chelating compounds exist, and produce OH^- which has strong activity; in the interim, GSH-Px can further catalyzed the reduction of GSH and H_2O_2, oxidize H_2O_2 to H_2O and prevent the production of highly toxic OH [9].

CAT causes direct breakdown of hydrogen peroxide to oxygen and water [10]. GR is the key enzyme in the conversion of oxidized glutathione (GSSG) back to the reduced form (GSH) [11]. GSH scavenge the electrophilic moieties formed by toxic chemicals and conjugate them to less toxic products [12]. Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages in liver.

In the present study there was increase manyfold in the levels of hydroxyl free radicals in NDEA induced animals that was neutralized to near normalcy in ethanolic extract of *Carum carvi (EECC)* treated animals, thus indicating prevention of increased ROS production [13;14]. SOD is said to act as the primary defense mechanism against superoxide radical generated as a by-product of oxidative phosphorylation [15]. Further, CAT or GPx converts H_2O_2 to H_2O. The activities of antioxidant enzymes depletion in can be owed to an enhanced radical production during N-nitrosodiethylamine metabolism. In the present investigation, an increase in MDA was presumably associated with increased free radicals, consistent with the observation that these free radicals reduced the activity of the endogenous antioxidant enzyme SOD [16]. Glutathione is essential to maintain the normal reduced state of cells and to counteract all the deleterious effects of oxidative stress. GSH is said to be involved in many cellular processes including the detoxification of endogenous and exogenous compounds. NDEA, an electrophilic carcinogen may interact with the large nucleophilic pool of GSH thereby reducing the macromolecule and carcinogen interaction [17]. In ethanolic extract of *Carum carvi (EECC)* treated rats, there was a significantly higher level of GSH both in liver and serum when compared to NDEA induced animals consistent with the idea of attenuation of DNA carcinogen interaction and thereby averting a favorable ambiance for carcinogenesis. Decreases in the activities of SOD, CAT, GPx, GSH, Vit C and Vit E are seen in tumor cells. The protection offered by ethanolic extract of *Carum carvi (EECC)* to the enzymatic antioxidant system may be explained by the increase in the level of these antioxidants probably due to the direct reaction of *Ethanolic extract of Carum carvi (EECC)* with ROS. Ethanolic extract of *Carum carvi (EECC)* may also protect the membrane and antioxidants from ROS. Additionally the compound could act by upregulation of endogenous antioxidant defenses. The endogenous antioxidant system may counteract ROS and reduce oxidative stress with the help of enzymic antioxidants [18].Oxidative stress leads to excessive liver damage caused by N-nitrsodiethylamine depleted the levels of non-enzymic antioxidants like GSH, vitamin-C and vitamin-E was observed. Non-enzymic antioxidants like vitamin-C and E act synergistically to scavenge the free radicals formed in the biological system. Chaudiere [19] has reported that the GSH acts synergistically with vitamin-E in inhibiting oxidative stress and acts against lipid peroxidation. Vitamin-C also traps and detoxifies free radicals in combination with vitamin-E and glutathione [20]. It plays an important task by regenerating the reduced form of vitamin-E and preventing the formation of excessive free radicals [21]. The decreased levels of these antioxidant vitamins and GSH observed during N-nitrsodiethylamine administration might be because of the excessive utilization of these vitamins in scavenging the free radicals formed during the metabolism of N-nitrsodiethylamine. Ethanolic extract of *Carum carvi (EECC)* treatment effectively restored the decreased levels of these nonenzymic antioxidants caused by N-nitrsodiethylamine. This shows that *EECC* maintains the levels of antioxidant vitamins by maintaining GSH homeostasis, thereby protecting the cells from further oxidative stress.

Bcl-2 proteins exist in the mitochondrial outer membrane and have a role in the regulation of mitochondrial permeability transition pore opening and release the apoptogenic proteins from mitochondria in to the cytosol which results to apoptosis. The increased level of Bcl-2 expression in cancer cell survival by inhibiting apoptosis was reported by Khalil and Booles [22].On the other hand, ethanolic extract of *Carum carvi (EECC)* treatment significantly decreased the expression anti-apoptotic protein Bcl-2

and the observed result clearly confirms that *EECC* has significant apoptotic nature. On the other hand expression of Bcl-2 did not show any difference in ethanolic extract of *Carum carvi (EECC)* alone treated animals when compared with control which ultimately suggests that it has no toxic effects in normal liver tissue.

Histopathological examination reveals the livers from control rats revealed normal architectures. Liver from rats treated with NDEA alone showed clear signs of severe hepatic injury manifested as areas with periportal with diffuse ballooning degeneration, intra-acinar lymphoplasmacytic and polymorphonuclear infiltrates with adjacent hepatocytes exhibiting feathery degeneration and regenerative cellular changes, proliferation of vascular channels are some of the regenerative cellular changes noted in most of the sections, multinucleated giant cells are seen, within some of the granulomas. Livers from group III rats treated with ethanolic extract of *Carum carvi (EECC)* showed a reduced amount of neoplastic cells, near normal architecture, and significant improvement in liver histopathology. There were no such pathological observation was revealed in group IV compared with group I animals.

CONCLUSSION

From this investigation, it is concluded that ethanolic extract of *Carum carvi (EECC)* suppressed the free radical processes by scavenging hydroxyl radicals. The efficacy of *Ethanolic extract of Carum carvi (EECC)* to modulate the levels of LPO and significantly increase the endogenous antioxidant defense mechanisms in NDEA induced hepatocellular carcinogenesis. Therefore we suggest that ethanolic extract of *Carum carvi (EECC)* may be developed as an effective chemotherapeutic agent. Further studies are underway to elucidate the molecular mechanisms involved to prove *EECC* efficacy as a potential antioxidant agent.

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