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Research Article
Ethanolic Extract of *Canthium coromandelicum* Leaves Exhibits Antioxidant, Anti-inflammatory and Apoptotic Activity in DEN-induced Hepatocellular Carcinoma

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Abstract

Background and Objective: *Canthium coromandelicum* is a traditional medicinal plant used in various regions of India for treating many diseases. However, its anticancer effects on hepatocellular carcinoma (HCC) have not yet been studied. The aim of this study was to investigate the chemopreventive action and the possible mechanisms of ethanolic extract of *Canthium coromandelicum* leaves (CEE) against diethylnitrosamine (DEN)-induced liver cancer in rats. Antioxidant, anti-inflammatory and apoptotic properties of CEE were studied by analyzing various proteins. **Materials and Methods:** Rats pretreated with leaves of CEE intragastrically at the dose of (500 mg kg^{-1} b.wt.) for 1 week before the administration of DEN and continued till the end of the experiment (i.e., 16 weeks). Analysis of serum HC-related markers, oxidative stress markers, inflammatory markers, DNA-fragmentation assay, protein and gene analysis of apopotic markers were done. **Results:** *Canthium coromandelicum* restored the normal liver function by normalizing the serum markers like AST, ALT, ALP and αFP. The extract significantly reduced oxidative markers like MDA and total ROS and increased antioxidant markers like SOD, CAT, GSH and GPx. Proinflammatory mediators like TNF-α and iNOS protein levels were significantly reduced by *Canthium coromandelicum.* It also inhibited NF-κB activation by reducing phosphor-IκBα and increased cleavage of caspase-3 as well as DNA damage. **Conclusion:** The present study concludes that CEE exerts its hepatoprotective effect against HCC through induction of apoptosis via modulating oxidative damage and suppressing inflammatory response.

Key words: *Canthium coromandelicum*, hepatocellular carcinoma, TNF-α, iNOS, NF-κB, caspase-3

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common solid tumors and the third leading cause of cancer-related deaths worldwide[1]. Its prognosis is yet to be studied in detail, with a 5 year survival rate[2] of <5%. The HCC carcinogenesis is a multistep process involving different genetic alterations that ultimately lead to malignant transformation of the hepatocytes[3].

The HCC has been proved to be associated with oxidative stress, defective apoptosis and increased cell proliferation. Cancer cells often show alterations in expression of tumor suppressor genes, DNA repair genes, genes regulating the cell cycle and genes involved in apoptosis[3]. It has also been shown that inflammation-associated cancer actually promotes tumor growth and progression[4]. Several proinflammatory gene products [i.e., tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)] play a critical role in apoptosis, proliferation, angiogenesis, invasion and metastasis. Their expression is mainly regulated by nuclear factor kappa-B, which is constitutively active in most of the tumors and induced by chemotherapeutic agents and carcinogens[5]. However, improper up regulation of iNOS and proinflammatory cytokines has been associated with the pathophysiology of certain cancers.

In hepatocarcinogenesis in both animal models and humans, ROS has been reported to play a major role in tumor initiation and survival due to the presence of a variety of agents, by mediating cellular signal transduction pathways. These signaling pathways are involved in the transmission of inter or intracellular information and are critical for tumor cell survival and cell fate[6].

Diethylnitrosamine (DEN) is one of the most important environmental carcinogens, present in tobacco-smoke, cosmetics, gasoline and various processed food such as milk and meat products. The DEN is also commonly used to induce lesions in rats that mimic different types of benign and malignant tumors in human[7]. The DEN became metabolically active by the action of cytochrome P_{450} enzymes to produce reactive electrophiles, which increase hepatocarcinogenesis level leading to cytotoxicity, mutagenecity and carcinogenicity. It has been shown that on primary metabolic activation, DEN produces the promutagenic adducts, O^6-ethyl deoxyguanosine and O^4 and O^6-ethyl deoxy thymidine that can produce DNA chain damage, depurination or binding to DNA and often generating a miscoding gene sequence, paving way to initiation of liver carcinogenesis through free radical mechanisms. The DEN is producing a reproducible tumor after repeated administration and also it serves as a standard model to study the beneficial effects of many drugs and treatments on hepatocarcinogenesis[8]. It is the carcinogen among nitrosamines and primarily induces tumors of liver. The presence of nitrosamines and their precursors in human environment, together with the possibility of their endogenous formation in human body from ingested secondary amines and nitrites, have led to the suggestions of their potential involvement in HCC. It is now widely used as a standard experimental model for HCC. Chemoprevention is one of the strategies by which we can revert or delay the response of carcinogen.

Chemotherapy for cancer is associated with side effects such as myelosuppression and its consequent risks of infection, anemia and bleeding[9]. For patients with advanced cancer, traditional herbal medicine is frequently prescribed in most of the countries like Taiwan, either in conjunction with or as an alternative to mainstream medicine. Recently, there has been a lot of interest in exploring the chemopreventive properties of natural herbs and plants.

Canthium coromandelicum (Burm. f.) Alston (Syn. *C. parviflorum*) belonging to Family: Rubiaceae is a bushy thorny suffruticose herb, native of India found mainly in coast of coromandelicum region of India. A few preclinical pharmacological evaluations have been carried out in various parts of this plant revealed from the literature through scientific investigation. *Canthium coromandelicum* leaves have been reported to exhibit significant antimicrobial and anti-HIV activity[10], hypocholesterolaemic activity[11], oral hypoglycemic activity[12], wound healing activity[13] and antioxidant properties[14]. Its major chemical constituents include kaempferol 3-O-β-D-glucopyranoside, squalene, phytol, β-sitosterol and n-hexadecanoic acid[14-16]. This plant has been used traditionally in East Asia particularly in India to treat various diseases like Intestinal worms in children, antioxidant and diuretics, scabies and the ring worm infections, leucorrhoea, headache, fever, diabetes, snake bite, indigestion, nausea, dysuria, impotence, GI disorders like gastric ulcer, diarrhea and constipation[17-19]. Based on its pharmacological evaluations, it is aimed to study the hepatoprotective effect of ethanolic extract of *C. coromandelicum* leaves by examining its apoptotic, antioxidant and anti-inflammatory effects on DEN-induced HCC rat model.

MATERIALS AND METHODS

Preparation of *Canthium coromandelicum* leaves ethanolic extract (CEE): *Canthium coromandelicum* leaves used for this study were collected from in Thanjavur, Tamilnadu, India The leaves were identified by Botanical Survey of India,

Coimbatore and the voucher samples are kept in the BSI herbarium for reference (BSI/SRC/5/23/2011-12/Tech-542). A voucher specimen has been deposited in the herbarium of the department .

The leaves (300 g) were extracted with 70% ethanol using "Soxhlet apparatus" for 48 h. The liquid extract was filtered through sieve and the filtrate was concentrated up to two parts on a rotary vacuum evaporator. After elimination of alcohol under reduced pressure, concentrated liquid was obtained and it was dried to obtain dry powder.

Dosage fixation: Different doses of *C. coromandelicum* leaves extract (CEE) (50, 100, 250, 500 mg and 750 mg kg^{-1} b.wt.) were given for 4 weeks. The effective dose of CEE was assessed based on the contents of liver and kidney lipid peroxidation (oxidative damage marker). Supplementations of *C. coromandelicum* extract at doses of 250, 500 and 750 mg kg^{-1} b.wt., for 4 weeks were found to be effective in rats. Among these doses, the minimal effective dose 500 mg was fixed as therapeutic dosage for the subsequent studies.

Animals: Male wistar albino rats, weighing 150-180 g were used for this experiment. Animals were acclimatized under standard laboratory conditions at 25±2°C and normal photoperiod (12 h light:dark cycle). The animals were fed with standard rat chow and water ad libitum. The food was withdrawn 18-24 h before animal sacrifice. Experimental hepatocarcinogenesis was induced by using DEN (Sigma, USA). All animal experiments were performed in the laboratory according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (IAEC).

Experimental design: The experimental animals were divided into four groups, each group containing six animals, analyzed for a total experimental period of 16 weeks as follows:

- **Group I (GpI):** Normal control rats fed with standard rat chow and pure drinking water
- **Group II (GpII):** Rats were orally given CEE (500 mg kg^{-1} b.wt.) in the form of aqueous suspension daily once a day for 16 weeks
- **Group III (GpIII):** Rats were induced with DEN (0.01%) alone in drinking water for 16 weeks
- **Group IV (Gp IV):** Rats pretreated with CEE intragastrically at the dose of (500 mg kg^{-1} b.wt.) for 1 week before the administration of DEN and continued till the end of the experiment (i.e., 16 weeks)

At the end of 16 weeks, experimental rats (n = 6 per group) were sacrificed. Body weights and liver weights of the animals were recorded in all groups.

Collection of blood and preparation of serum sample: At the end of the experimental period, the animals were anaesthetized using chloroform vapour prior to dissection. Blood was collected by cardiac puncture into serum separator tubes. The blood was allowed to clot by standing at room temperature for 30 min and then refrigerated for another 30 min. The resultant clear part was centrifuged at 3000 rpm for 10 min and then the serum (supernatant) was isolated and stored at refrigerated until required for analysis.

Tissue collection: The liver was sectioned and fixed in phosphate-buffered 10% formaldehyde for histological analysis. For protein and gene expression studies, the liver were immediately immersed in protease inhibitor cocktail and RNA-later solution (Qiagen) and finally stored at -20°C until further use.

Serum markers of liver damage and cancer: Functional state of the liver was determined by spectrophotometrically estimating biochemical parameters such as aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP). The assay was performed using Bio Merieux Reagent Kit according to the manufacturer's instructions.

Alpha-fetoprotein (αFP) secreted by the fetal liver was used as marker for hepatic carcinoma. It was determined in serum by enzyme immunoassay method using a commercial kit (Calbiotech Inc., CA, USA) according to the manufacturer's instructions.

Lipid peroxidation and antioxidant enzyme assay: Liver was weighed and homogenized using a teflon homogenizer. Tissue homogenate was prepared in 0.1 M tris-HCl buffer (pH 7.4) and used for the estimation of lipid peroxidation from MDA content[20] and antioxidant enzymes activities such as superoxide dismutase (SOD)[21], catalase (CAT)[22], glutathione peroxidase (GPx), glutathione reduced content and GSH[23]. All analyses were performed spectrophotometrically.

Total Reactive Oxygen Species (ROS) in liver homogenate were assayed spectro fluorometrically. Tissue homogenate was mixed with CM-H_2DCFDA for 15 min. Fluorescence of the H2DCFDA was read at 480 nm excitation/520 nm emission in the Hitachi F2000 Spectrofluorimeter. Total protein content of liver tissues was determined according to the Lowry method.

Enzyme-linked immunosorbent assay: The TNF-α and iNOS in tissue homogenate were measured using TNF-α rat ELISA Kit (catalogue No. ab46070, Abcam, UK) and iNOS Rat ELISA Kit (Novus, KA 1634) according to manufacturer's instructions.

DNA fragmentation assay: Apoptotic changes in the liver were analyzed by agarose gel electrophoresis. The DNA content in hepatic tissues was determined. Hepatic tissues were homogenized and the homogenate was suspended in ice-cold trichloroacetic acid (TCA). After centrifugation, pellet was extracted with ethanol. Levels of DNA were determined by treating the nucleic acid extract with diphenylamine reagent and reading the intensity of blue color at 600 nm. Liver samples were homogenized in 700 µL hypotonic lysis buffer and centrifuged for 15 min at 11,000 rpm. Supernatant containing small DNA fragments was treated with equal volumes of absolute isopropyl alcohol and NaCl to precipitate DNA. Extracted DNA was electrophoresed on 1% agarose gels containing 0.71 µg mL^{-1} ethidium bromide. Then, gels were examined using UV transillumination[24].

Western blot analysis: Liver tissues were washed with PBS and homogenated using 200 µL ice-cold RIPA lysis buffer supplemented with protease inhibitor cocktail and protein concentration was measured using BCA protein assay kit. Equal amount of protein extract (50 µg) was loaded, separated by 12% SDS-PAGE and blotted onto nitrocellulose membrane. Then, membranes were incubated with rabbit polyclonal anticaspase-3 and antiphospho IκBα (Santa Cruz Biotechnology, CA, USA) antibody overnight at 4°C. After washing, the membranes were incubated with anti-rabbit or anti-mouse IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, CA, USA) for 2 h at room temperature. The membrane was incubated with standard ECL (enhanced chemiluminescence) reaction (RPN2106, Amersham/Pharmacia Biotech, Uppsala, Sweden) as a substrate and dried. The black bands developed were visualized, photographed and then subjected to densitometric tracing using ImageJ software (http://rsb.info.nih.gov/ij/)[25].

Real-time PCR analysis: Total RNA was extracted from the liver of control and experimental rats using Trizol (Invitrogen, USA). Reverse transcription was carried out using Prime Script TM RT Reagent Kit (TakaRa, Japan) according to the manufacturer's instructions. The SYBR Green PCR Mastermix (Takara, Japan) was used for quantitative RT-PCR and 10 µL reaction mixture was added to Axygen 384-well reaction plates sealed with optical adhesive covers (Axygen). The cDNA levels of caspase-3 and NF-kB-p65 were normalized to β-actin. Primers sequences used for real-time PCR analyses were as follows:

- **Caspase-3:** Forward 5'tggttcatccagtcgctttg3' and reverse 5'cattctgttgccacctttcg3'
- **NF-κB-p65:** Forward 5'gcgtacacattctggggagt3' and reverse 5'accgaagcaggagctatcaa3'
- **β-actin:** Forward 5'gcagaaggagattactgccct3' and reverse 5'gctgatccacatctgctggaa3'
- ABI PRISM 7900HT (Applied Biosystems, USA) was used for quantitative RT-PCR for each sample in triplicate. The PCR program was as follows: 95°C for 30 sec then 40 cycles were performed (5 sec at 95°C and 31 sec at 60°C) and then 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec

Statistical analysis: Data were analyzed using SPSS version 16.0. All values were expressed as Mean±SD. Statistically significant differences between mean values were determined by one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. Real-time PCR values were analyzed by comparative C_T method. The $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

Morphometric analysis: Macroscopic appearance of the liver cells of control group 1 animals shows normal morphology (Fig. 1a). The DEN alone administered group 3 animals showing enlargement and several grayish white nodules and foci on the peripheral surface of the liver (Fig. 1c). Liver cell shows normal morphology in CEE alone treated group 2 animals (Fig. 1b). Most of the foci and nodules disappeared in the liver from DEN and CEE treated group (group 4) of rats showing the effect of chemoprevention (Fig. 1d).

Table 1 presents the changes in the whole body weight and organs weight in control, cancer induced and drug

Table 1: Effect of *Canthium coromandelicum* on body and liver weight in control and experimental rats

Parameters	Group I	Group II	Group III	Group IV
Body weight				
Initial weight (g)	164.00±10.21	169.00±12.8	162.00±12.9	163.00±12.5
Final weight (g)	261.00±19.52	262.00±20	198.00±15.8[a$]	252.00±19.84[b$]
Liver weight (g)	7.81±0.60	8.28±0.58	14.17±1.18[a$]	8.48±0.70[b$]

Each value is expressed as Mean±SD for six rats in each group, [a]As compared with group I, [b]As compared with group III, [$]$p<0.001$

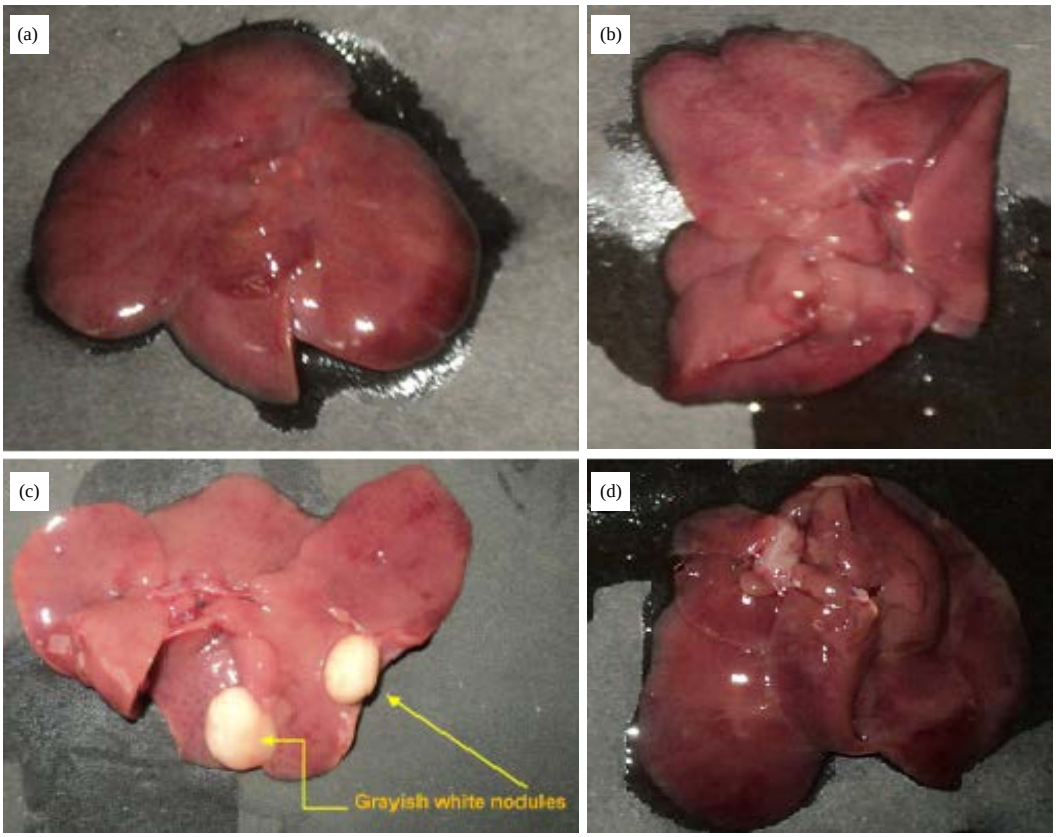

Fig. 1(a-d): Morphometric analysis of liver, (a) Normal control rat liver, (b) CEE alone treated rat liver, (c) DEN alone treated rat liver and (d) DEN and CEE treated rat liver

Table 2: Effect of *Canthium coromandelicum* on serum markers

Parameters	Group I	Group II	Group III	Group IV
αFP	0.69±0.03	0.71±0.19	3.14±0.80[a$]	1.70±0.03[b$]
AST	47.12±3.76	51.23±4.09	130.65±5.45[a$]	49.65±2.10[b$]
ALT	36.54±0.92	41.82±0.34	131.56±0.52[a$]	42.01±0.36[b$]
ALP	8.81±0.90	10.78±0.88	26.17±2.18[a$]	11.48±1.07[b$]

Values are expressed as Mean±SEM of six rats per group. Level of αFP is expressed as ng mL^{-1}, activity is expressed as IU L^{-1} for ALT, AST and ALP, Significance was determined by one-way analysis of variance, $Denotes p<0.001, [a]Comparison of group III with I and II, [b]Comparison of group IV with III

treated rats. The body weight was significantly decreased in hepatocellular carcinoma bearing rats when compared to control. The organ weights as liver were significantly increased in hepatocellular carcinoma bearing rats when compared to control. Oral administration of CEE significantly recouped the body weight and organ weights as compared with group 3 rats.

Serum liver markers: Serum ALT, AST and ALP were measured as biochemical markers of liver function. Serum αFP level was measured as an indicator of cancer incidence. Table 2 shows that animals treated with CEE alone showed non-significant changes in serum ALT, AST, ALP and αFP levels, while animals treated with DEN (cancer group) showed significantly ($p<0.001$) higher serum levels of ALT, AST, ALP and αFP levels, when compared to their counterparts in the normal control group. Rats treated with CEE (CEE-DEN group) showed significantly ($p<0.001$) reduced serum ALT, AST, ALP and αFP levels compared to rats treated only with DEN, which shows its property of restoring the normal liver function.

Oxidative markers and antioxidant status: Hepatic MDA and total ROS were measured as oxidative stress markers. Hepatic SOD GSH, GPx and CAT levels were measured to determine antioxidant status. Table 3 shows that, animals treated with CEE alone showed non significant changes in MDA, total ROS, SOD, GSH, GPx and CAT, while animals treated with DEN (cancer group) showed significantly ($p<0.001$) higher levels of MDA and total ROS levels and lower levels of SOD, GSH, GPx and CAT ($p<0.001$), when compared to their counterparts in the normal control group. Rats treated with CEE (CEE-DEN group) showed significantly ($p<0.001$) reduced oxidative stress markers MDA and total ROS and increased

Table 3: Effect of *Canthium coromandelicum* on the hepatic antioxidants parameters in rats

Parameters	Group I	Group II	Group III	Group IV
Total ROS	0.74±0.02	0.78±0.02	2.14±0.04$^{a\$}$	0.87±0.02$^{b\$}$
MDA	0.28±0.06	0.32±0.04	1.13±0.12$^{a\$}$	0.39±0.06$^{b\$}$
SOD	5.87±0.02	5.79±0.03	4.35±0.17$^{a\$}$	5.76±0.04$^{b\$}$
CAT	113.70±0.24	114.00±0.23	98.25±0.32$^{a\$}$	112.60±0.73$^{b\$}$
GSH	4.65±0.37	5.75±0.46	1.65±0.13$^{a\$}$	5.89±0.47$^{b\$}$
GPx	7.02±0.56	6.87±0.54	3.86±0.30$^{a\$}$	6.89±0.55$_{b\$}$

Values are expressed as Mean±SEM of six rats per group. Concentration is expressed as nmol mg^{-1} protein for total ROS and MDA. Activity is expressed as U mg^{-1} protein for CAT, SOD and as µg mg^{-1} protein for GSH and GPx. Significance was determined by one-way analysis of variance, $^{\$}$Denotes $p<0.001$, ^{a}Comparison of group III with I and II, ^{b}Comparison of group IV with III

Table 4: Effect of *Canthium coromandelicum* on hepatic levels of the pro-inflammatory mediator TNF-α and iNOS enzymatic activity

Parameters	Group I	Group II	Group III	Group IV
TNF-α	7.93±0.62	7.74±0.71	17.39±2.52$^{a\$}$	8.18±0.64$^{b\$}$
iNOS	4.80±0.30	4.90±0.36	12.95±1.31$^{a\$}$	5.06±0.26$^{b\$}$

Hepatic levels of TNF-α and iNOS were expressed as pg mg^{-1} of protein. Values are given in Mean±Standard Deviation (SD), n = 6. Significant differences between groups are analyzed by one-way analysis of variance (ANOVA), where, $^{\$}p<0.0001$, ^{a}Comparison of group III with I and II, ^{b}Comparison of group IV with III

($p<0.001$) antioxidant markers SOD, GSH, GPx and CAT levels compared to rats treated only with DEN. Reduction in oxidative stress markers and increase in antioxidant levels demonstrate antioxidant property of CEE.

Level of proinflammatory cytokine (TNF-α): Concentration of the proinflammatory cytokine TNF-α in the liver tissue was determined by ELISA according to the manufacturer's instructions. Table 4 shows that administration of DEN significantly ($p<0.001$) increased the levels of TNF-α in rats of the cancer group relative to those of the normal control group; this increase was significantly ($p<0.001$) reversed in the rats of the CEE-DEN group. Rats treated with CEE alone did not show any significant changes in TNF-α levels compared to the normal control group rats. Decrease in the proinflammatory cytokine levels by CEE in cancer group showed its anti-inflammatory activity.

iNOS enzymatic activity: The inhibitory effect of CEE on the activity of hepatic iNOS enzyme was also examined. Table 4 shows that DEN significantly ($p<0.001$) elevated the enzymatic activity of iNOS in the cancer group rats, while CEE did not elevate iNOS enzymatic activity, when compared to iNOS activity in normal control rats. More interestingly, iNOS enzyme activity in rats in the CEE-DEN group was significantly ($p<0.001$) reduced when compared to that in the cancer group rats. As iNOS is considered as an important inflammatory marker, decrease in its activity confirmed the anti-inflammatory property of CEE.

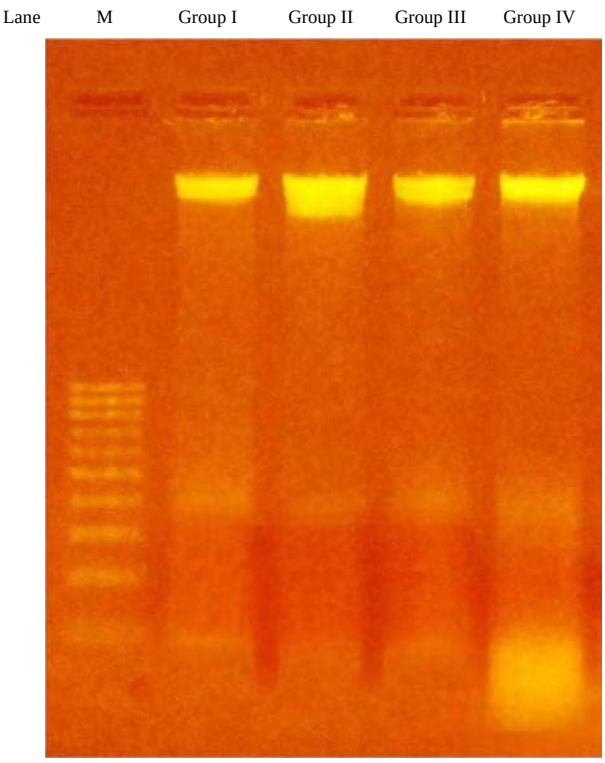

Fig. 2: Effects of *Canthium coromandelicum* on DNA fragmentation of hepatic tissue in DEN-induced cancer rats. Agarose gel electrophoresis pattern of DNA isolated from liver tissues of different groups, M: DNA marker

DNA fragmentation analysis: Figure 2 shows the agarose gel electrophoretic pattern of control, CEE alone, cancer and CEE-DEN groups. Progressive DNA fragmentation is a marker for the later stages of apoptosis. Fragments of DNA were not found in DEN-induced cancer rats, whereas, they were found in the CEE-DEN group. It indicates that CEE induces DNA fragmentation in genomic DNA of cancer group.

Expression of antiapoptotic protein phospho-IκBα and NF-κB-p65 mRNA: Figure 3 shows the protein and gene expression of antiapoptotic protein phospho-IκBα and NF-κB-p65 mRNA. Phospho-IκBα protein expression in the liver was analyzed by using Western blot (Fig. 3a). The densities of bands corresponding to the phospho-IκBα protein were normalized on the basis of β-actin and analyzed relative to that of the normal control group as shown in Fig. 3b. Administration of DEN significantly ($p<0.01$) upregulated liver phospho-IκBα protein expression in the cancer group rats. Phospho-IκBα protein expression in the liver of CEE-DEN rats was significantly ($p<0.001$) decreased when compared to that of cancer group rats.

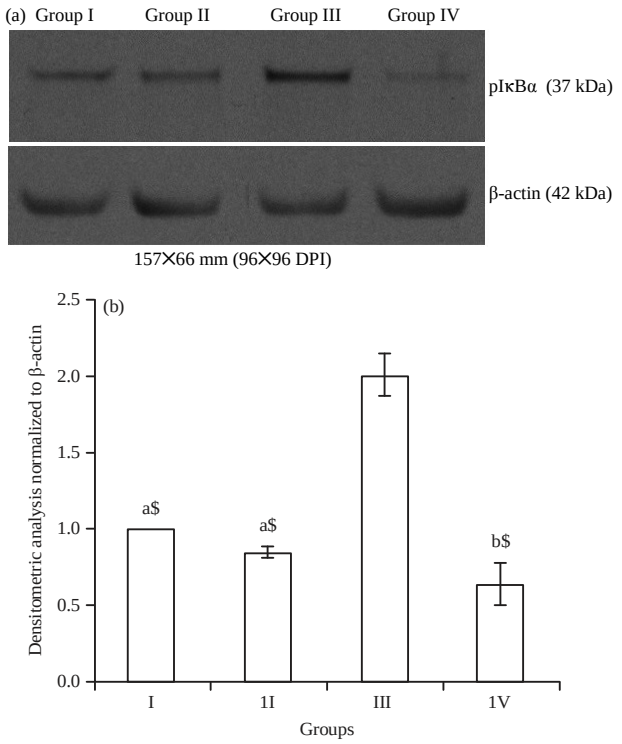

Fig. 3(a-b): Protein expression of anti-apoptotic protein phospho-IκBα after CEE treatment in DEN induced rat, (a) Representative Western blot image for the different groups and (b) Densitiometric analysis of the Western blot bands for phospho-IκBα normalized to b-actin. All values were expressed as Mean±SD where, $Denotes p<0.001, aComparison of group III with I and II, bDenotes comparison of group IV with III

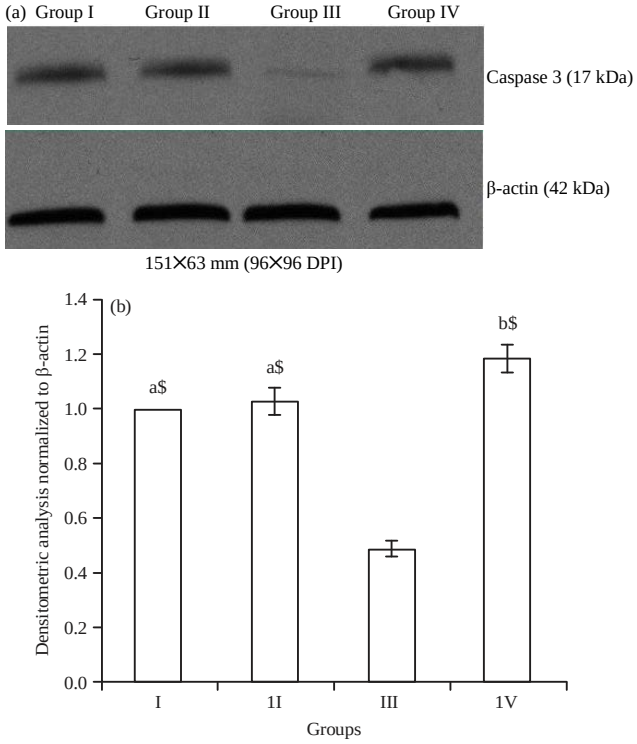

Fig. 4(a-b): Protein expression of apoptotic protein caspase 3 after CEE treatment in DEN induced rat, (a) Representative Western blot image for the different groups and (b) Densitiometric analysis of the Western blot bands for caspase 3 normalized to β-actin. All values were expressed as Mean±SD where, $Denotes p<0.001, aComparison of group III with I and II, bDenotes comparison of group IV with III

Gene expression of NF-κB-p65 in the liver was analyzed by real-time PCR: Comparative C_T method was used to quantify the NF-κB-p65 gene expression, which was normalized on the basis of β-actin and analyzed relative to that of the normal control. The DEN increased the NF-κB-p65 mRNA level 2.16 fold in cancer group rat when compared to normal control group. In CEE-DEN group, NF-κB-p65 mRNA expression was reduced 0.78 fold when compared to cancer group. Reduction in NF-κB-p65 mRNA transcripts by CEE showed its apoptotic and anti-inflammatory properties.

Protein and gene expression of apoptotic protein caspase-3: Figure 4 shows the protein and gene expression of apoptotic protein caspase-3. Caspase-3 protein expression in the liver was analyzed using Western blot (Fig. 4a). The densities of bands corresponding to the caspase-3 protein were normalized on the basis of β-actin and analyzed relative to that of the normal control group as shown in Fig. 4b. Administration of DEN significantly ($p<0.01$) down regulated liver cleaved caspase-3 protein expression in the cancer group rats. Expression of cleaved caspase 3 in the liver of CEE-DEN rats was significantly ($p<0.001$) increased when compared to that of cancer group rats.

Gene expression of caspase-3 in the liver was analyzed by real-time PCR (Fig. 5). Comparative C_T method was used to quantify active caspase-3 gene expression, which was normalized on the basis of β-actin and analyzed relative to that of the normal control. The DEN down regulated caspase-3 mRNA level 0.55 fold in the cancer group compared to normal control group. In CEE-DEN group, caspase-3 mRNA expression was increased 1.45 fold compared to cancer group. The CEE treatment increases active caspase-3 mRNA transcripts, which shows its apoptotic property.

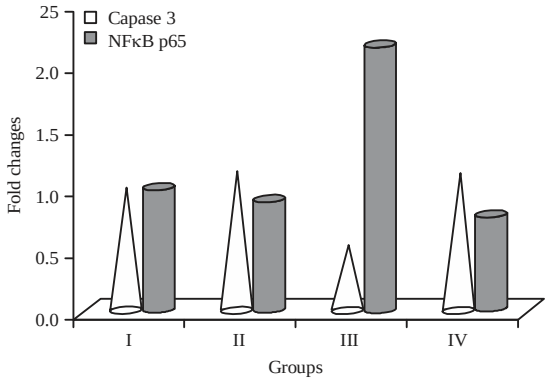

Fig. 5: Quantification of caspase 3 and NF-κB-p65 mRNA by real time PCR using comparative C_T method. The C_T values were normalized on the basis of β-actin and analyzed relative to that of the normal control. Gene expression were given as fold changes (n = 6)

The present study aims, for the first time to the best of our knowledge, to evaluate the hepatoprotective effect of *C. coromandelicum* leaves by examining its apoptotic, antioxidant and anti-inflammatory effects on DEN-induced HCC rat model. Hepatocarcinogesis is a multistep process associated with oxidative stress and unbalanced production and/or activation of proinflammatory mediators such as TNF-α and nitric oxide. These proinflammatory mediators cause increased cell proliferation and defective apoptosis in liver, which in turn leads to HCC. Increased ROS, chronic inflammation and defective apoptosis are associated with the pathological condition of HCC[6].

Antioxidant activity of CEE in DEN-induced rats: Oxidative stress plays a major role in all chronic diseases. In this study, we found increased oxidative stress markers and decreased antioxidant markers in DEN-treated rats. Increase in oxidative stress markers leads to liver damage, which causes release of liver markers such as AST, ALT, ALP and αFP. On the other hand, decrease in GP_X and SOD in the liver of DEN-treated rats might indirectly lead to an increase in oxidative DNA damage[26]. Administration of CEE counteracts DEN-induced oxidative stress by restoring the antioxidant levels of SOD, CAT, GSH and GPx in the liver and by diminishing the oxidative markers MDA and total ROS in the liver. This antioxidant property of CEE was also accompanied by a decrease in the levels of liver damage markers such as serum AST, ALT, ALP, αFP. Patro and Sasmal[14] reported that CCE has a free radical scavenging activity. This activity may be due to the presence of certain known phytoconstituents such as flavonoids, tannins, saponins and terpenoids in CEE[16]. Prevention of oxidative stress and hepatic toxicity by CEE might feature its recovery of hepatic damage through its antioxidant property.

Anti-inflammatory activity of CEE in DEN-induced rats: Chronic inflammation also plays a major role in hepatocarcinogenesis, which is associated with early changes in the development of cancer. A link between oxidative stress and chronic inflammation is found in most of the diseases. Ability of *Canthium parviflorum* extract to bring down thermal denaturation of protein is possibly a contributing factor for its anti-inflammatory activity. The anti-inflammatory activity of *Canthium parviflorum* extract found may be due to the presence of therapeutically active flavonoids[27].

In this study, we observed increased concentration of pro-inflammatory mediators like TNF-α and iNOS in DEN-treated rats. Increased expression of TNF-α and iNOS has been observed in several human tumor tissues and in chemically induced animal tumors. The iNOS is considered as a key enzyme involved in producing proinflammatory signals. It is over expressed in hepatocellular carcinoma[28]. The main proinflammatory mediator induced by iNOS is TNF-α. Its expression level in precancerous and tumor cells was associated with the progression of various malignant diseases[29]. The DEN induces inflammation via an oxidative-dependent manner involving ROS.

Treatment with CEE revealed its efficacy to protect against DEN-induced liver inflammation by decreasing the level of pro inflammatory mediators TNF-α and iNOS. These decreases are mainly due to the free radical scavenging property of CEE. Decreased serum TNF concentration during chemotherapy in various cancer tissues is well correlated with the extent of therapy responses, suggesting that serum TNF level could be an indicator of chemotherapy response and prognosis[29]. Moreover, inflammatory mediators TNF-α and iNOS have been repeatedly used as targets to radiosensitize tumor cells[6]. Altogether, reduction in TNF-α and iNOS levels by CEE suggests that its protection against carcinogenesis could be mediated by its anti-inflammatory activity.

Apoptotic activity of CEE in DEN-induced rats: Chronic inflammation produced by ROS leads to increased cell proliferation, which may be due to defect in the regulation of apoptosis and survival pathway. Progressive DNA fragmentation is a marker for the later stages of apoptosis. In the present study, DNA fragments are not found in cancer group rats, whereas, CEE induces fragmentation of genomic

DNA in cancer groups. Isolation of DNA from liver tissue mainly constitutes hepatocytes of the cellular population, which demonstrates that DNA fragmentation may be mainly due to hepatocyte apoptosis. This proves the cytotoxic effect of CEE on DEN-induced cancer.

The NF-κB is the main antiapoptotic protein as well as an important transcription factor for inflammation. It is normally present in the cytoplasm bound to an inhibitory protein, IkB and critically plays an important role in the regulation of cell cycle as well as influencing cell death pathways[30].

These results showed that there is an increased expression of phospho-IκBα protein and NFκB-p65 mRNA in DEN-treated rats. This may be due to the presence of ROS, inflammatory cytokines like TNF-α and interleukin-1[31]. The TRAF2, 5 and 6 are reported to activate NF-κB. Among these factors, TRAF6 is the major mediator of TNF receptor, IL-1 receptor and Toll-like receptor signaling. The TRAF6 activates TAK1-TAB2/3 complex and then activates the IKKα, β complex by phosphorylation[32]. Phosphorylation of IκB causes the release of NF-κB, which is then transported into the nucleus where it induces the transcription of a wide range of target genes that induce inflammatory (COX-2 and iNOS) and antiapoptotic responses[6]. All these factors lead to uncontrolled cell proliferation in HCC.

Administration of CEE to DEN-treated rats reversed DEN-induced upregulation of NF-κB-p65 mRNA. Similar result is also reported in protein expression studies, where CEE treatment caused a decrease in the phosphorylation of IkB. This decrease is due to the decrease in the levels of total ROS, TNF-α and iNOS by CEE. These results showed the anti-inflammatory as well as apoptotic effects of CEE.

In this study, we observed that the protein and gene expression of active caspase-3 was down regulated in DEN-induced cancer rats compared to normal control. This indicates that there is defective apoptosis. This defective apoptosis may be due to the increase in iNOS levels; NO inhibits apoptosis by nitrosylating essential components of the apoptosis mechanism such as caspases. The TNF-α increased the production of iNOS and nitric oxide, which causes partial inactivation[33] of caspase-3.

The CEE treatment results in upregulation of caspase-3 in DEN-induced rats. This may be due to decrease in iNOS levels, which in turn decreases nitrosylation of caspases and facilitates apoptosis. This study also demonstrates that CEE decreases TNF-α and NF-κB levels, which might lead to the activation of JNK through transcriptional factor GADD45β. The JNK directly activates Bid, causing cytochrome c release[34] and caspase-3 activation in cancer rats. The results of caspase-3 and NF-κB suggest that CEE's effect against hepato carcinogenesis could be mediated by its apoptotic activity.

CONCLUSION

In summary, data presented here show that CEE dramatically inhibited both nodular and FAH formation in livers of DEN-treated rats. This inhibition was associated with induced apoptosis by up regulating caspase-3 and down regulating NF-κB-p65, decreased oxidative stress and down regulation of inflammatory markers such as iNOS TNF-α. Thereby it is concluded that CEE exerts its hepato protective effect against HCC through induction of apoptosis via modulating oxidative damage and suppressing inflammatory response. This effect can be beneficial in clinical practice.

REFERENCES

1. Yang, J.D. and L.R. Roberts, 2010. Hepatocellular carcinoma: A global view. Nat. Rev. Gastroenterol. Hepatol., 7: 448-458.
2. Shariff, M.I.F., I.J. Cox, A.I. Gomaa, S.A. Khan, W. Gedroyc and S.D. Taylor-Robinson, 2009. Hepatocellular carcinoma: Current trends in worldwide epidemiology, risk factors, diagnosis and therapeutics. Expert Rev. Gastroenterol. Hepatol., 3: 353-367.
3. Moradpour, D. and H.E. Blum, 2005. Pathogenesis of hepatocellular carcinoma. Eur. J. Gastroenterol. Hepatol., 17: 477-483.
4. Balkwill, F., K.A. Charles and A. Mantovani, 2005. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. Cancer Cell, 7: 211-217.
5. Aggarwal, B.B., S. Shishodia, S.K. Sandur, M.K. Pandey and G. Sethi, 2006. Inflammation and cancer: How hot is the link? Biochem. Pharmacol., 72: 1605-1621.
6. Reuter, S., S.C. Gupta, M.M. Chaturvedi and B.B. Aggarwal, 2010. Oxidative stress, inflammation and cancer: How are they linked? Free Radical Biol. Med., 49: 1603-1616.
7. Park, D.H., J.W. Shin, S.K. Park, J.N. Seo, L. Li, J.J. Jang and M.J. Lee, 2009. Diethylnitrosamine (DEN) induces irreversible hepatocellular carcinogenesis through overexpression of G1/S-phase regulatory proteins in rat. Toxicol. Lett., 191: 321-326.
8. Ueno, S., D. Aoki, F. Kubo, K. Hiwatashi and K. Matsushita *et al*., 2005. Roxithromycin inhibits constitutive activation of nuclear factor κB by diminishing oxidative stress in a rat model of hepatocellular carcinoma. Clin. Cancer Res., 11: 5645-5650.

9. Ahles, T.A., A.J. Saykin, C.T. Furstenberg, B. Cole and L.A. Mott *et al*., 2002. Neuropsychologic impact of standard-dose systemic chemotherapy in long-term survivors of breast cancer and lymphoma. J. Clin. Oncol., 20: 485-493.
10. Chinnaiyan, S.K., M.R. Subramanian, S.V. Kumar, A.N. Chandu and K. Deivasigamani, 2013. Antimicrobial and anti-HIV activity of extracts of *Canthium coromandelicum* (Burm.f.) Alston leaves. J. Pharm. Res., 7: 588-594.
11. Bandara, W.V.R.T.D.G., S. Ekanayake, E.R. Jansz and K. Balasubramanium, 2009. *Canthium coromandelicum* leaf as a functional food. Vidyodaya J. Sci., 14: 105-109.
12. Widanagamage, R. and S. Ekanayake, 2009. Oral hypoglycaemic effect of *Canthium coromandelicum* leaf extract on wistar rats. Vidyodaya J. Sci., 14: 53-57.
13. Mohan, S.C., K. Sasikala and T. Anand, 2014. Antimicrobial and wound healing potential of *Canthium coromandelicum* leaf extract-a preliminary study. Res. J. Phytochem., 8: 35-41.
14. Patro, S.K. and D. Sasmal, 2015. *In vitro* antioxidant study and search for a novel bioactive compound from leave fractions of *Canthium coromandelicum* (Burm.F.) Alston. Int. J. Pharmaceut. Sci. Res., 6: 3841-3852.
15. Krishnan, K.R., F. James and A. Mohan, 2016. Isolation and characterization of n-hexadecanoic acid from *Canthium parviflorum* leaves. J. Chem. Pharmaceut. Res., 8: 614-617.
16. Sasikala, K. and S.C. Mohan, 2014. Total phenolic, flavanoid contents and GC-MS analysis of *Canthium coromandelicum* leaves extract. Int. J. Pharm. Pharmaceut. Sci., 6: 379-381.
17. Anitha, B., V.R. Mohan, T. Athiperumalsami and S. Sutha, 2008. Ethnomedicinal plants used by the Kanikkars of Tirunelveli District, Tamil Nadu, India to treat skin disease. Ethnobot. Leaflets, 12: 171-180.
18. Ayyanar, M., K. Sankarasivaraman and S. Ignacimuthu, 2008. Traditional herbal medicines used for the treatment of diabetes among two major tribal groups in South Tamil Nadu, India. Ethnobot. Leaflets, 12: 276-280.
19. Suresh, K., R. Kottaimuthu, T.S.J. Norman, R. Kumuthakalavalli and S.M. Simon, 2011. Ethnobotanical study of medicinal plants used by Malayali tribals in Kolli hills of Tamil Nadu, India. Int. J. Res. Ayurveda Pharm., 2: 502-508.
20. Uchiyama, M. and M. Mihara, 1978. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal. Biochem., 86: 271-278.
21. Nandi, A. and I.B. Chatterjee, 1988. Assay of superoxide dismutase activity in animal tissues. J. Biosci., 13: 305-315.
22. Aebi, H., 1984. Catalase *in vitro*. Methods Enzymol., 105: 121-126.
23. Habig, W.H., M.J. Pabst and W.B. Jakoby, 1974. Glutathione S-transferases: The first enzymatic step in mercapturic acid formation. J. Biol. Chem., 249: 7130-7139.
24. El-Nekeety, A.A., S.H. Abdel-Azeim, A.M. Hassan, N.S. Hassan, S.E. Aly and M.A. Abdel-Wahhab, 2014. Quercetin inhibits the cytotoxicity and oxidative stress in liver of rats fed aflatoxin-contaminated diet. Toxicol. Rep., 1: 319-329.
25. Cao, L., X.D. Zhou, M.A. Sens, S.H. Garrett and Y. Zheng *et al*., 2010. Keratin 6 expression correlates to areas of squamous differentiation in multiple independent isolates of As^{+3}-induced bladder cancer. J. Applied Toxicol., 30: 416-430.
26. El-Nekeety, A.A., S.R. Mohamed, A.S. Hathout, N.S. Hassan, S.E. Aly and M.A. Abdel-Wahhab, 2011. Antioxidant properties of *Thymus vulgaris* oil against aflatoxin-induce oxidative stress in male rats. Toxicon, 57: 984-991.
27. Karthik, K., P.B.R. Kumar, R.V. Priya, K.S. Kumar and R.S.B. Rathore, 2013. Evaluation of anti-inflammatory activity of *Canthium parviflorum* by *in-vitro* method. Indian J. Res. Pharm. Biotechnol., 1: 729-731.
28. Fathy, M. and T. Nikaido, 2013. *In vivo* modulation of iNOS pathway in hepatocellular carcinoma by *Nigella sativa*. Environ. Health Prev. Med., 18: 377-385.
29. Wang, X. and Y. Lin, 2008. Tumor necrosis factor and cancer, buddies or foes? Acta Pharmacologica Sinica, 29: 1275-1288.
30. Kundu, J.K. and Y.J. Surh, 2005. Breaking the relay in deregulated cellular signal transduction as a rationale for chemoprevention with anti-inflammatory phytochemicals. Mutat. Res./Fundam. Mol. Mech. Mutagen., 591: 123-146.
31. Hayden, M.S. and S. Ghosh, 2008. Shared principles in NF-κB signaling. Cell, 132: 344-362.
32. Inoue, J.I., T. Ishida, N. Tsukamoto, N. Kobayashi, A. Naito, S. Azuma and T. Yamamoto, 2000. Tumor necrosis factor Receptor-Associated Factor (TRAF) family: Adapter proteins that mediate cytokine signaling. Exp. Cell Res., 254: 14-24.
33. Dimmeler, S., J. Haendeler, M. Nehls and A.M. Zeiher, 1997. Suppression of apoptosis by nitric oxide via inhibition of Interleukin-1β-Converting Enzyme (ICE)-like and Cysteine Protease Protein (CPP)-32-like proteases. J. Exp. Med., 185: 601-607.
34. Madesh, M., B. Antonsson, S.M. Srinivasula, E.S. Alnemri and G. Hajnoczky, 2002. Rapid kinetics of tBid-induced cytochrome *c* and Smac/DIABLO release and mitochondrial depolarization. J. Biol. Chem., 277: 5651-5659.